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# Surface Layer Proteins of *Clostridium difficile* as Novel Vaccine Candidates

**A Thesis Submitted for the Degree of Doctor in Philosophy (Ph.D.)**

**By**

**Julie Bridget O'Brien**

**At**

**University of Dublin, Trinity College**

**2005**

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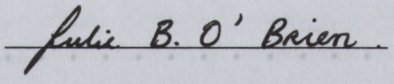
## DECLARATION

I hereby declare that this thesis has not been previously submitted for a degree at this or any other university. Except where otherwise stated the work described in this thesis is entirely my own. Part of the work described in Chapters IV and V was carried out jointly with Dr. Déirdre Ní Eidhin and Dr. Matthew McCabe and includes the unpublished and published work of others. Drs. Ní Eidhin and McCabe are duly acknowledged in the text where appropriate. I hereby give my permission to the library of Trinity College Dublin to lend or copy this thesis upon request for the purpose of study.



Julie B. O'Brien

# SUMMARY

*Clostridium difficile* is a major cause of antibiotic associated diarrhoea and pseudomembranous colitis in hospitalised patients. The major precipitating factor for *C. difficile*-associated disease (CDAD) is disruption of the normal gut flora by antibiotic therapy. *C. difficile* can then colonise the gut and produce the toxins, which mediate much of its pathogenesis. Currently, there is no *C. difficile* vaccine licensed for human use. A toxin-based vaccine is under development and has shown promise. However, this vaccine will not prevent colonisation and thus transmission is likely to remain a problem. *C. difficile* expresses two major surface layer proteins (SLPs), the low-molecular weight (MW) SLP and the high-MW SLP, which form a crystalline regular array on its outer surface. A single gene, *slpA*, encodes a precursor protein which is processed to remove a signal peptide and then undergoes a second cleavage to release the two mature SLPs. The low-MW SLP exhibits a high degree of inter-strain variability and can elicit a serum antibody response in infected individuals. The high-MW SLP is putatively involved in gut colonisation. The SLPs, therefore, represent good novel targets for vaccine development against *C. difficile*. This study was chiefly concerned with the generation of strong immune responses against the SLPs with a view to developing a vaccine against *C. difficile*.

The parts of the *slpA* gene, from a *C. difficile* Ribotype 1 clinical isolate, that encode for the low- and high-MW SLPs were cloned and expressed in *E. coli*. The low-MW SLP was purified to homogeneity and underwent crystallography analysis. Following trials, initial crystallisation conditions were identified; these are being refined with the intention of producing suitable crystals for structure solution by x-ray crystallography. Solving the three dimensional structure of this protein may help elucidate its role(s) in the pathogenicity of *C. difficile*. Highly purified native SLPs in the quantities required for *in vivo* studies were produced using a combination of urea extraction and anion exchange chromatography.

Passively administered rabbit anti-SLP serum delayed progression of CDAD in a lethal hamster challenge model of infection. A potential mechanism of action of the antiserum was shown to be through enhanced phagocytosis of *C. difficile*. This indicates that anti-SLP antibodies play a protective, albeit incomplete, role in an *in vivo* model of *C. difficile* infection. The mechanism of action of the anti-SLP serum is most likely multifactorial. The prolonged survival of anti-SLP treated hamsters might be explained through an increase in phagocytosis of *C. difficile* at damaged mucosal surfaces. However, as shown

*in vitro,* the anti-SLP serum might also have inhibited colonisation by agglutinating *C. difficile in vivo* and therefore inhibiting binding of the bacterium to the epithelium. The prolonged survival in the anti-SLP treated group most likely reflected exposure to lower toxin concentrations from a lower bacterial load achieved by increased phagocytosis.

Active immunisation of hamsters with the SLPs in combination with aluminium hydroxide adjuvant resulted in the generation of high-titre serum IgG anti-SLP antibodies. However, these antibodies were not protective against CDAD. Active immunisation with the SLPs in combination with the mucosal adjuvants cholera toxin (CT) and Ribi and a combination of CT/Ribi resulted in low serum IgG anti-SLP responses. Low levels of protection against CDAD were observed with Ribi and CT/Ribi adjuvants, but no correlation between antibody titre and protection was evident. The immune response to the SLPs in BALB/c mice was examined in parallel. Low serum IgG and IgA and mucosal IgA anti-SLP titres were generated in mice using CT, CT/Ribi and two chitosan adjuvants.

The SLPs were shown to be Th1 inducing proteins, characterised by IL-2 and IFN-$\gamma$, and a lack of IL-4 and IL-5, production from SLP re-stimulated murine splenic cells. The SLPs were shown to have immunomodulatory properties on murine dendritic cells (DCs) *in vitro*. Specifically, they induced secretion of both pro-inflammatory cytokines IL-12p70 and TNF-$\alpha$ and anti-inflammatory cytokines IL-10 and TGF-$\beta$. This profile of cytokine secretion is representative of a Th1 type immune response. CT blocked the production of SLP-induced IL-12p70 and TNF-$\alpha$ and concomitantly greatly enhanced the production of SLP-induced IL-10 and TGF-$\beta$. Ribi adjuvant also induced a Th1 type immune response, which was modulated in a similar fashion to the SLPs when combined with CT.

In conclusion, passively administered anti-SLP serum delayed progression of CDAD in a hamster model of infection. It proved difficult to induce strong active immune responses against the SLPs in either hamsters or mice. Low levels of protection from CDAD were observed in SLP immunised hamsters but this was not correlated to antibody response. The SLPs were shown to be Th1 inducing proteins in mice and to have immunomodulatory properties on murine DCs *in vitro*. CT in combination with either the SLPs or Ribi has the potential to be used to modulate immune responses *in vivo*, particularly in Th1 dysregulated inflammatory and autoimmune diseases.

## DEDICATION

**This thesis is dedicated to my parents,**

**Teddy and Rita O'Brien.**

But man, proud man,
Dress'd in a little brief authority,
Most ignorant of what he's most assur'd –
His glassy essence – like an angry ape
Plays such fantastic tricks before high heaven
As makes the angels weep; who, with our spleens,
Would all themselves laugh mortal.

William Shakespeare, Measure for Measure, act II, scene II.

"Veldurat sá er varar"

"He causes who warns"

Njáls Saga, 13th century Iceland.

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# ABBREVIATIONS

| | |
|---|---|
| AAD | Antibiotic associated diarrhoea |
| $Al(OH)_3$ | Aluminium hydroxide |
| $AlPO_4$ | Aluminium phosphate |
| ANOVA | Analysis of variance |
| APS | Ammonium persulphate |
| BHI | Brain heart infusion |
| BSA | Bovine serum albumin |
| $CaCl_2$ | Calcium chloride |
| CDAD | *Clostridium difficile*-associated diarrhoea |
| CFA | Complete Freund's adjuvant |
| CFSE | 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester |
| CFU | Colony forming units |
| CT | Cholera toxin |
| CTL | Cytotoxic T lymphocytes |
| DC | Dendritic cell |
| DMSO | Dimethylsulphoxide |
| ECL | Enhanced chemiluminescence |
| EDTA | Ethylene diamine tetra-acetic acid |
| ELISA | Enzyme linked immunosorbent assay |
| FAB | Fastidious anaerobe broth |
| FCS | Foetal calf serum |
| FHA | Filamentous hemagglutinin |
| FSC | Forward scatter |
| GFP | Green fluorescent protein |
| GM-CSF | Granulocyte-macrophage colony stimulating factor |
| HBSS | Hanks buffered salts solution |
| HEPES | N-(2-Hydroxyethyl)piperazine-N'-2-ethanesulfonic acid |
| HLA | Human leukocyte antigen |
| HRP | Horseradish peroxidase |
| $H_2SO_4$ | Sulphuric acid |
| IFA | Incomplete Freund's adjuvant |
| IFN-$\gamma$ | Interferon gamma |
| IgA | Immunoglobulin A |
| IgG | Immunoglobulin G |

| | |
|---|---|
| IgM | Immunoglobulin M |
| IgY | Immunoglobulin Y |
| IL | Interleukin |
| i.n. | Intranasal |
| i.p. | Intraperitoneal |
| kDa | Kilodalton |
| LAL | Limulus amoebocyte lysate |
| LB | Luria-Bertani media |
| LPS | Lipopolysaccharide |
| LT | *E. coli* heat-labile toxin |
| MAP | Mitogen activated protein |
| $MgCl_2$ | Magnesium chloride |
| MHC | Major histocompatibility complex |
| MOI | Multiplicity of infection |
| MPL | Monophosphoryl lipid A |
| MW | Molecular weight |
| MYD88 | Myeloid differentiation factor 88 |
| NaCl | Sodium chloride |
| NaOH | Sodium hydroxide |
| NK | Natural killer |
| OPD | 1,2-phenylenediamine dihydrochloride |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| p*I* | Isoelectric point |
| PMA | Phorbol 12-myristate 13-acetate |
| PMC | Pseudomembranous colitis |
| PMN | Polymorphonuclear leukocyte |
| PRR | Pathogen recognition receptor |
| PVDF | Polyvinylidene difluoride |
| RAS | Ribi adjuvant system |
| REA | Restriction endonuclease analysis |
| rEK | Recombinant enterokinase |
| RFLP | Restriction fragment length polymorphism |
| RNA | Ribonucleic acid |

| | |
|---|---|
| SCC | Side scatter |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| SEM | Standard error of the mean |
| S-IgA | Secretory IgA |
| S-layer | Surface layer |
| SLP | Surface layer protein |
| TAE | Tris-acetate EDTA |
| TBE | Tris-boric EDTA |
| TBS | Tris buffered saline |
| TE | Tris-EDTA |
| TEMED | N,N,N',N'-tetramethlyenediamine |
| TDM | Trehalose dicorynomycolate |
| TGF-$\beta$ | Transforming growth factor beta |
| Th1 | T-helper 1 |
| Th2 | T-helper 2 |
| TLR | Toll like receptor |
| TMB | Tetramethylbenzidine |
| TMC | N-trimethyl chitosan chloride |
| TNF | Tumour necrosis factor |
| Tr | T-regulatory |
| Tris | Tris (hydroxymethyl) aminomethane |

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# CHAPTER I

# GENERAL INTRODUCTION

# CHAPTER I

## 1.1 HISTORICAL BACKGROUND OF *CLOSTRIDIUM DIFFICILE* DISEASE

*Clostridium difficile* is today recognised as a common cause of nosocomial diarrhoea and the most frequent cause of pseudomembranous colitis (PMC) in hospitalised patients (Poutanen and Simor, 2004, Surawicz and McFarland, 1999). PMC lesions of the intestine were first described in 1893 by Finney, as a postoperative complication of gastrojejunostomy for an obstructive peptic ulcer. Following surgery, this patient developed diarrhoea, which became bloody, and the patient subsequently died. On autopsy, the patient was found to have what Finney described as "diphtheritic colitis". In 1935, Hall and O'Toole first isolated *C. difficile* from the meconium and faeces of newborn infants. They termed this new organism *Bacillus difficilis* because it grew slowly in culture and was difficult to isolate. The organism was shown to produce a lethal toxin in experimental animals, but since it was commonly found in the stools of healthy neonates it was classified as a commensal. The conclusion was that either the organism did not produce the toxin *in vivo* or that humans were not susceptible to the effects of the toxin. The link between *Bacillus difficilis* and PMC was not made until four decades later and in the intervening years the organism was assigned to the genus *Clostridium* (Brazier, 1998a).

PMC was relatively rare until the 1950s after which time it became a common complication of antibiotic treatment following the introduction of penicillin, tetracycline and chloramphenicol (Tabaqchali and Jumaa, 1995). There was a further resurgence of interest in PMC when clindamycin was introduced in the 1960s for anaerobic infections. Clindamycin is a semi-synthetic antibiotic derived from lincomycin by the addition of chloride (Weingarten-Arams and Adam, 2002). Tedesco *et al.* (1974) described PMC lesions in patients receiving clindamycin and found that 21% of patients treated with this antibiotic developed diarrhoea and 10% developed PMC. In 1977 Larson *et al.* reported that a cytotoxin detected in the stools of five of six patients with histologically proven PMC had a cytotoxic effect on tissue culture cells. In 1978, 85 years since Finney first described PMC lesions, *C. difficile* was definitively identified as the primary cause of PMC and antibiotic-associated colitis and diarrhoea (Bartlett *et al.*, 1978, Larson *et al.*, 1978). It is now clear that *C. difficile* is responsible for virtually all cases of PMC and for up to 20% of cases of antibiotic-associated diarrhoea (AAD) without colitis (Kelly *et al.*, 1994, Surawicz and McFarland, 1999). The spectrum of disease caused by *C. difficile* has become known as *C. difficile*-associated disease (CDAD) and this anaerobic pathogen

continues to infect up to 20% of individuals admitted to hospital (Kelly and LaMont, 1998).

## 1.2 *C. DIFFICILE* INFECTION

### 1.2.1 Epidemiology of *C. difficile* infection

*C. difficile* is widely distributed in the environment and has been isolated from soil, river and lake water, raw vegetables, farm and pet animal faeces, and general surfaces in the home, hospitals and veterinary clinics (Brazier, 1998b). During the first month of life up to two thirds of infants become colonised with *C. difficile*, usually reflecting acquisition from the hospital environment. Because the neonatal intestine is immature and does not have the protective normal flora established in adults it is susceptible to *C. difficile* colonisation and overgrowth (McFarland *et al.*, 2000). For reasons that remain unclear, most colonised neonates remain asymptomatic, even when toxin production can be demonstrated (Borriello and Wilcox, 1998, Kelly and LaMont, 1998). One hypothesis put forward for the resistance of neonates to the *C. difficile* toxins is the immaturity of their enterocyte membrane toxin receptors (Kelly *et al.*, 1994). Maturation of the receptors may include microbial degradation of glycoproteins and exposure of internal motifs. It is thought that this may be age-dependent and may parallel the development of a normal gut flora (Borriello and Wilcox, 1998). In addition, the neutralising effect of maternal antibodies may play a role. As childhood progresses, carriage rates decline to adult levels and both sporadic and outbreak CDAD begins to appear (McFarland *et al.*, 2000).

Reported carriage rates of *C. difficile* in the colonic flora of healthy adults range from less than 3% to as high as 15% (Beaugerie and Petit, 2004, Poutanen and Simor, 2004, Tabaqchali and Jumaa, 1995). These differences most likely reflect variations in the sensitivity of methods of culture and in the selection of subjects. Asymptomatic carriage among healthy adults following antibiotic treatment has been reported to be as high as 46% (Tabaqchali and Jumaa, 1995). *C. difficile* infection occurs primarily among hospitalised patients, causing as many as three million cases of diarrhoea and colitis per year in the U.S.A. (Kelly and LaMont, 1998). Hospitals and long-term care facilities have been reported as having an infection rate of 25-60 per 100,000 occupied bed days (Mylonakis *et al.*, 2001). Among hospital inpatients, the prevalence of *C. difficile* culture positivity ranges from 7% to 11% (Poutanen and Simor, 2004). Among patients with AAD, *C. difficile* infection is detected in less than 10% of cases in the community, but up to 60% to 70% of cases in hospitalised patients (Beaugerie and Petit, 2004, Oldfield, 2004). If

pseudomembranes are present, *C. difficile* is found to be responsible for nearly 100% of cases (Oldfield, 2004). Rates of colonisation and infection increase rapidly beyond the age of 65, such that in England and Wales *C. difficile* is the predominant enteric pathogen among people in this age group (Djuretic *et al.*, 1999).

In industrialised countries, the mean rate of antibiotic exposure is approximately one course per inhabitant per year (Beaugerie *et al.*, 2003) and the use of antibiotics continues to increase (Oldfield, 2004). Furthermore, the trend towards shorter hospital stays results in an increasing number of patients returning home on antibiotic therapy (Djuretic *et al.*, 1999). Given these factors, a high incidence of community-acquired *C. difficile* infection might also be expected. However, the incidence of community-acquired CDAD appears to be substantially lower than rates observed in hospitals, with an estimated 7 to 18 cases per 100,000 (Beaugerie *et al.*, 2003, Mylonakis *et al.*, 2001, Poutanen and Simor, 2004). Recently, a prospective study in France found a *C. difficile* infection rate during antimicrobial therapy in the community of 2700 per 100,000 exposures to antibiotics (Beaugerie *et al.*, 2003). This is the first study to demonstrate such a high rate of *C. difficile* infection during antimicrobial therapy in the community.

Reports of hospital outbreaks of *C. difficile* are common in the literature. The National *C. difficile* Standards Group, U.K. (Report to the Department of Health, February 2003) defined an outbreak as "the occurrence of two or more related cases over a defined period, agreed locally, taking account of the background rate". These outbreaks are often prolonged and difficult to control and create a serious financial burden on healthcare resources. The estimated number of cases of CDAD in the U.S.A. exceeds 250,000 per year (Voth and Ballard, 2005) and a recent prospective study estimated the cost of managing CDAD in the U.S.A. at over US $1.1 billion (Kyne *et al.*, 2002). A study on the secular trends of hospital-acquired CDAD in the U.S.A. over the period 1987 to 2001 documented an increase in the number of cases of *C. difficile* infection in the ICUs of large general hospitals. Hospital wide rates also increased in smaller general hospitals over the same period (Archibald *et al.*, 2004). In 1991/1992 an outbreak in Manchester, U.K., which lasted for six months, involved 175 patients and contributed to 17 deaths (Djuretic *et al.*, 1999). It was estimated that this single outbreak cost in excess of St£100,000. A prospective case-control study by Wilcox *et al.* (1996) estimated the cost of a single case of CDAD in the U.K. at St£4000, implying that the annual cost in the U.K. of CDAD, based on the number of toxin positive laboratory reports, was in excess of St£48 m. Data

collected by the Communicable Disease Surveillance Centre, Northern Ireland, documents an almost continuous increase in *C. difficile* toxin positive laboratory reports in Northern Ireland over the period 1992 to 2003.

The Health Protection Surveillance Centre, Ireland, does not collect data on the incidence of *C. difficile* infection, however anecdotal evidence and the literature indicate high rates of infection in Irish hospitals. From January to June 1995, two simultaneous outbreaks of CDAD, each caused by a single common strain, were documented in St. James's Hospital, Dublin, Ireland (Kyne *et al.*, 1998). In this outbreak, 139 patients were identified and 9 deaths occurred. Additionally, a large increase in the number of toxin positive laboratory reports in the Munster region of Ireland was documented over the period 1992 to 2002, with the number of reports rising from negligible in 1992 to greater than 350 in 2002. Following the introduction of a strict antibiotic policy, which reduced the use the intravenous cephalosporins, there was a significant reduction in the number of patients found to have *C. difficile* toxin-positive diarrhoea. The relative risk of developing CDAD with the old antibiotic policy compared to the new was 3.24 (O'Connor *et al.*, 2004).

In the U.K. *C. difficile* strains have been allocated to 117 distinct ribotypes. In total, 54 different ribotypes have been identified from hospital patients, with 16 of these accounting for 90% of isolates. Strains belonging to Ribotype 1 (corresponding to Serogroup G) account for over half of all hospital isolates in England and Wales (Brazier, 1998b). Furthermore, Stubbs *et al.* (1999) found that 55% of U.K. hospital isolates belonged to Ribotype 1. However, this ribotype was responsible for only 7.5% of infections in the community, indicating that Ribotype 1 has become endemic in hospital environments, possibly due to selection by the particular antibiotics in use in U.K. hospitals. There is evidence that subgroups exist within Ribotype 1 and a recent report from the U.K. identified eight subgroups within this strain (Northey *et al.*, 2005, Fawley *et al.*, 2003).

Although no national studies have been carried out to identify the most prevalent strains in Irish hospitals, a prospective study was carried out in St. James's Hospital, Dublin, Ireland, over a sixteen-month period in 1998-1999 (Dr. Rachael Doyle, MD thesis, Trinity College Dublin 2004, Immune Response to *Clostridium difficile*-Associated Disease, call number TX-1-359). In this study, Ribotype 1 was the most common strain isolated, accounting for approximately 48% of cases. This correlates well with the U.K. findings of Brazier (1998b) and Stubbs *et al.* (1999). Ribotype 12 (corresponding to Serogroup C) was also prevalent

in St. James's hospital, accounting for approximately 12% of cases. Ribotype 17, a toxin B only strain, accounted for approximately 7% of cases. In contrast, Brazier (1998b) found Ribotype 12 to account for only 2.6% of hospital isolates in England and Wales. **Table 1.1** summarises the ribotypes and corresponding serogroups isolated during this study.

During the course of this thesis, data on the number of toxin positive individuals were collected from the Central Pathology Laboratory (CPL) in St. James's hospital. The number of individuals with toxin positive laboratory reports in the hospital decreased substantially over the period 2002–2004, with the majority of this decrease observed in 2004 **(Figure 1.1)**. A particular downward trend is evident in the second half of 2004. A potential explanation for this decrease is a reduction in the use of cephalosporins, as well as major infection control efforts in the hospital (Mary Kelleher, surveillance scientist, St. James's Hospital, personal communication). As expected, *C. difficile* infection was most common in individuals in the 70-80 and 80-90 age brackets. No seasonal trends were evident in the data **(Figure 1.2)**. *C. difficile* infection was not distributed equally between wards throughout the hospital, with some locations having many more individuals with toxin positive reports than others **(Figure 1.3).**

The most recent reports of *C. difficile* outbreaks come from Canada, where a major outbreak occurred in 2003/2004 in Quebec (Pépin *et al.*, 2004). Pépin *et al.* (2004) studied the patterns of CDAD over the period 1991 to 2003. The annual incidence of CDAD in Quebec increased from 35.6 per 100,000 of the population in 1991 to 156.3 per 100,000 of the population in 2003. Furthermore, in this study a rate of 866.5 per 100,000 among adults of 65 years of age or more was recorded in 2003, representing a major epidemic among the elderly. Of particular concern was a dramatic increase in the case-fatality rate of CDAD over the study period, suggesting that a more virulent and more transmissible *C. difficile* strain might be involved.

### 1.2.2 Pathogenesis of CDAD

The pathogenesis of CDAD involves the disruption of host defences mediated by the indigenous colonic microflora. Healthy adults carry at least 500 recognised species in the colon, over 90% of them anaerobes (Kelly *et al.*, 1994). They form a stable ecosystem together with the intestinal mucosa and it is this complex population that has an inhibitory effect on incoming, non-indigenous species. This phenomenon has been termed "colonisation resistance" (Bergogne-Bérézin, 2000, Borriello, 1998). If the normal colonic

| Ribotype | Sero-group | Sequence looks like | Comment |
|---|---|---|---|
| **1** | **G** | **Identical to UK Type 1** | **Most common hospital type in UK and SJH** |
| 2 | A2 | New sequence | Several isolates |
| 5 | Unknown | 3 nt difference, similar to UK Type 16, one Belgian Group A1, one Belgian Group A10 sequence | Several isolates, also found in UK |
| 54 | A1 | | Infrequent |
| 10 | D | Similar to 10 in UK, Identical to D in Belgium, Paris | Non-toxigenic |
| **12** | **C** | **Identical to Group C sequences from Belgium, Italy, Switzerland 1 nt different to 46/92** | **Most common in Europe, 2nd most in SJH** |
| 46 | Unknown | Identical to one another | Rare |
| 92 | Unknown | | Several isolates |
| **17** | **F** | **Identical sequences from UK, Belgium Japan** | **Fairly common, toxin B only** |
| 14 | H | 1 nt difference Belgian Group H sequence v similar | Rare, commoner in UK |
| 66 | A9 | | Non-toxigenic |
| 78 | Unknown | Similar to group A?, French strain | Rare, weak toxigenicity, also found in UK |
| 31 | K | Similar to French and Belgian Group K sequences | Non-toxigenic |
| 94 | Unknown | | Several isolates |

**Table 1.1 Summary of *C. difficile* ribotypes isolated from patients in St. James's hospital, Dublin, Ireland, with corresponding serogroups and *slpA* sequences.** The most prevalent Ribotypes, 1 (48%), 12 (12%) and 17 (7%) are highlighted in bold. The information contained in Table 1.1. is from Dr. Déirdre Ní Eidhin, personal communication, and Dr. Rachael Doyle, MD thesis 2004, Trinity College Dublin, call number TX-1-359.

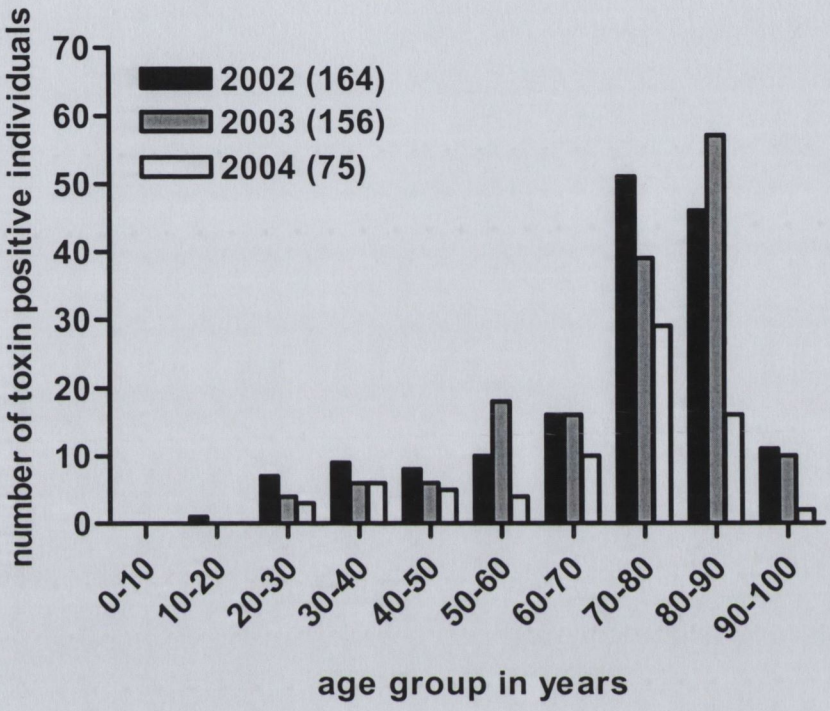



**Figure 1.1** ***C. difficile*** **toxin positive individuals per age category, 2002-2004, St. James's Hospital, Dublin.** The number of *C. difficile* toxin positive reports for 2002-2004 inclusive, were collected from the Central Pathology Laboratory, St. James's Hospital, Dublin. One toxin positive report per individual was included. Totals for each year are represented in brackets after the legends.

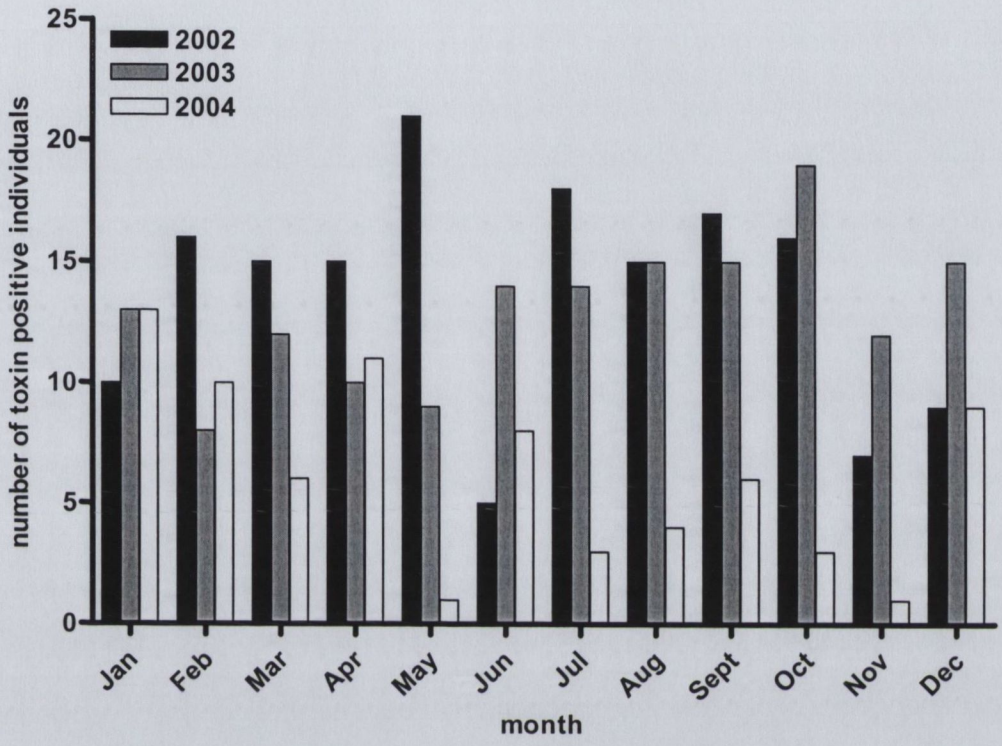
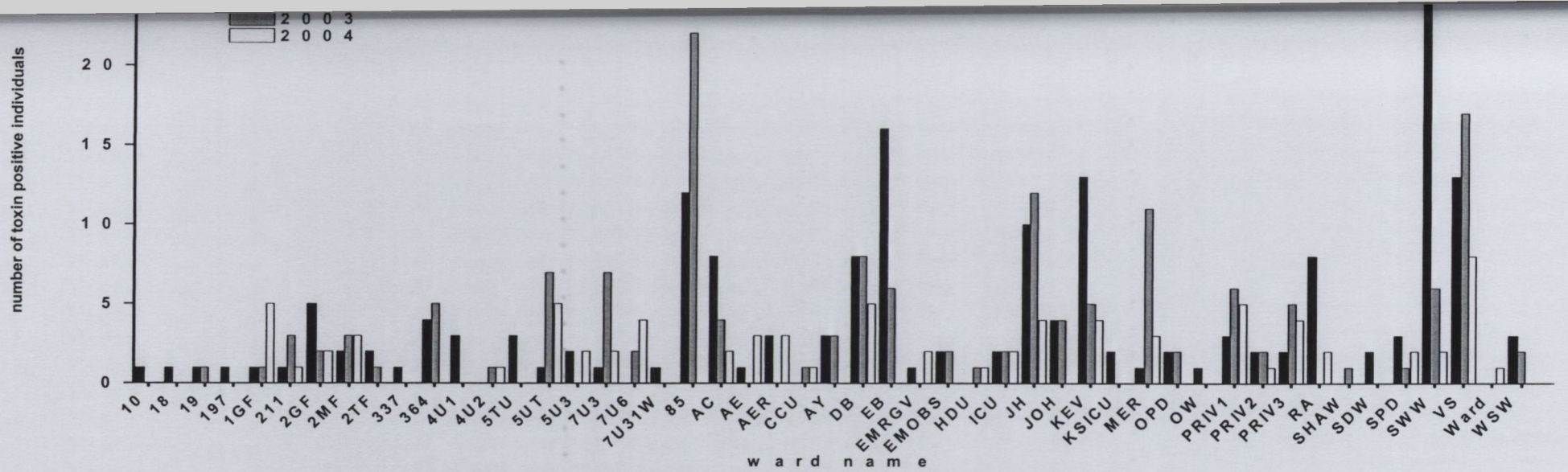



**Figure 1.2** ***C. difficile*** **toxin positive individuals per month, 2002-2004, St. James's Hospital, Dublin.** The number of *C. difficile* toxin positive reports for 2002-2004 inclusive, were collected from the Central Pathology Laboratory, St. James's Hospital, Dublin, Ireland. One toxin positive report per individual was included.




| Key to Ward Names | | | |
|---|---|---|---|
| 10 = Patients not registered with St. James's hospital | 4U2 = Hospital 4, unit 2 (rehabilitation) | DB = Denis Burkitt (haematology/bone marrow transplant) | OW = Observation Ward |
| 18 = Cherry Orchard Hospital, Dublin | 5TU/TU = Hospital 5, transition unit | EB = Edward Hallaran Bennett ward (GIT / emergency surgery) | PRIV1 = Private 1 (oncology/haematology/medical) |
| 19 = St. Patrick's Hospital, Dublin | 5U3 = Hospital 5, unit 3 (infectious diseases) | EMRGV = Emergency Virtual ward | PRIV2 = Private 2 (mixed general surgery with various specialities) |
| 197 = Orthopaedic Hospital Clontarf, Dublin | 7U3 = Hospital 7, unit 3 (general medicine) | EMOBS = Emergency Medicine Observation | PRIV3 = Private 3 (medical, surgical, oncology, cardiology) |
| 1GF = Hospital 1, ground floor | 7U6 = Hospital 7, unit 6 (general medicine) | HDU = High Dependency Unit | RA = Robert Adam's ward (cardiac/thoracic) |
| 211 = Peamount Hospital, Dublin | 7U31W = Hospital 7, unit 3 | ICU = Intensive Care Unit | SHAW = Keith Shaw Unit (cardiac surgery) |
| 2GF = Hospital 2, ground floor (rehabilitation) | 85 = Cavan General Hospital | JH = John Houston (respiratory/ medical) | SDW = Day Surgery Ward (day surgery procedures) |
| 2MF = Hospital 2, middle floor (rehabilitation) | AC = Abraham Colles ward, (orthopaedics/trauma) | JOH = John's ward (ear, nose and throat) | SPD = Sir Patrick Dun's ward (vascular & general surgery) |
| 2TF = Hospital 2, top floor (care of the elderly) | AE = Accident and Emergency | KEV = St. Kevin's ward (acute medical admissions) | SWW = William Wild ward (acute medical admissions) |
| 337 = unknown | AER = Accident and Emergency Recovery | KSICU = Keith Shaw Intensive Care Unit (cardiothoracic) | VS = Victor Synge ward (endocrinology / rheumatology) |
| 364 = General Practice | CCU = Coronary Care Unit | MER = Mary Mercers ward (acute medicine for the elderly) | WSW = Walter Stevenson Unit (oncology and haematology) |
| 4U1 = Hospital 4, unit 1 (care of the elderly) | AY = Anne Young Ward (reconstructive plastic surgery, oro-maxillo facial surgery) | OPD = Outpatients Department | |

**Fig. 1.3** ***C. difficile*** **toxin positive individuals per ward, 2002-2004, St. James's Hospital, Dublin.**

flora is disrupted, colonisation resistance is lost and *C. difficile* can colonise and subsequently cause disease. Antibiotic therapy is the key factor that alters the colonic flora and allows *C. difficile* infection to occur (Pothoulakis, 1996). Almost all antibiotics have been implicated in CDAD, including oral, intravenous, intramuscular and topical antibiotics. Broad-spectrum antibiotics with activity against enteric bacteria are the most frequently implicated agents (Kelly *et al.*, 1994). Clindamycin is notorious for its ability to induce CDAD, however in practice, ampicillin, amoxycillin, cephalosporins, quinolones, erythromycin, tetracyclines, trimethoprim, chlormaphenicol and sulphonamides are the most common culprits, reflecting their widespread use (Farthing, 1996, Kelly *et al.*, 1994, Tabaqchali and Jumaa, 1995). For third generation cephalosporins, an almost perfect correlation between increased use and increased incidence of CDAD has been recognised (Oldfield, 2004). Even vancomycin and metronidazole, which have been very successful at treating *C. difficile* infection, have occasionally been associated with CDAD (Pothoulakis, 1996). The relative risk of an antibiotic inducing CDAD depends on how frequently it is used and for how long (Tabaqchali and Jumaa, 1995). However, even brief exposure to a single dose for surgical prophylaxis can precipitate diarrhoea (O'Connor *et al.*, 2004). Following antibiotic therapy, *C. difficile* colonisation occurs via the oral-faecal route. Most ingested vegetative cells are killed in the stomach, with only 1% of the inoculum passing into the small bowel. *C. difficile* spores, however, are acid resistant and readily pass through the stomach. It is thought they germinate in the small bowel upon exposure to bile acids (Poutanen and Simor, 2004). In order to cause disease, *C. difficile* has to establish itself in the colon and produce the toxins that cause mucosal damage, inflammation and fluid secretion (Tabaqchali and Jumaa, 1995) **(Figure 1.4)**.

### 1.2.2.1 Toxins A and B

CDAD is a toxin mediated disease and when established in the colon, pathogenic strains of *C. difficile* produce two well-characterised toxins, toxin A and toxin B. Toxins A and B have molecular masses of 308 kDa and 270 kDa, respectively, and are the main known virulence factors of the organism (Voth and Ballard, 2005). Toxins A and B are intracellularly acting cytotoxins which get access to their cells via receptor mediated endocytosis (von Eichel-Striber *et al.*, 1996, Just and Gerhard, 2004). The toxins bind to specific receptors on the luminal aspect of the colonic epithelium and are then transported into the cytoplasm. Toxin A binds to the trisaccharide sequence Gal$\alpha$1-3Gal$\beta$1-4GlcNAc and this or a similar carbohydrate structure may act as the toxin A receptor. Conversely, little is known about the toxin B receptor, except that it is different to that of toxin A (Just

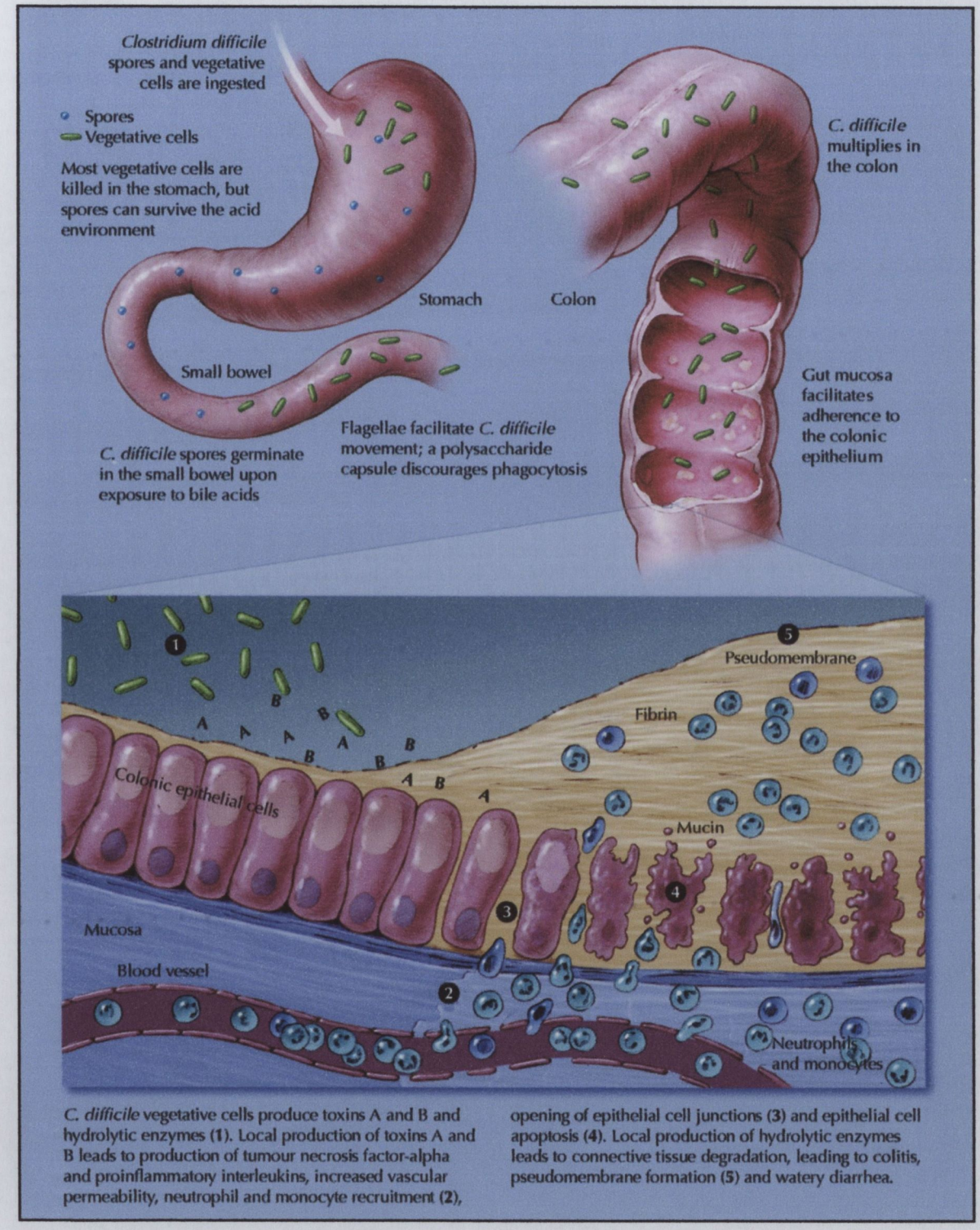



**Fig. 1.4 Pathogenesis of *C. difficile*-associated diarrhoea in adults.** Taken from Poutenan and Simor, CMAJ, July 6, 2004; 171 (1).

and Gerhard, 2004, Kelly, 1996). Toxin B has been shown to intoxicate a broad range of cell types, indicating that the receptor for this toxin is ubiquitous (Voth and Ballard, 2005). Once they have gained entry into the cell, both toxins catalyse a specific alteration of GTPases, a family of small GTP-binding proteins important in actin polymerisation, cytoskeletal architecture and cell movement. Specifically, toxins A and B catalyse the addition of a glucose moiety to a threonine residue on RhoA, Rac and Cdc42 (Kelly, 1996, Voth and Ballard, 2005). This modification inactivates these proteins, leading to the depolymerisation of actin filaments, disruption of the cytoskeleton, cell rounding and cell death. Thus, mucosal barrier function is severely compromised. The toxins also induce the production of proinflammatory cytokines, especially IL-1, IL-6, IL-8, tumour necrosis factor alpha (TNF-$\alpha$) and interferon gamma (IFN-$\gamma$), from immune cells of the mucosa, which contribute to the associated inflammatory response and pseudomembrane formation. Release of chemoattractants causes invasion of neutrophils, which are characteristic of PMC (Just and Gerhard, 2004, Poutanen and Simor, 2004). A severe inflammatory reaction in the lamina propria and the formation of microulcerations of the colonic mucosa, which are covered by an inflammatory pseudomembrane, are a typical feature of CDAD (Kelly and LaMont, 1998). Furthermore, the toxins activate the enteric nervous system through an unknown sequence of steps, causing secretory diarrhoea and amplifying inflammation (Just and Gerhard, 2004, Mylonakis *et al.*, 2001). Although highly homologous and encoded contiguously, the two toxins have very different characteristics (Bongaerts and Lyerly, 1994). Toxin A is thought to play a more critical role than toxin B in the pathogenesis of CDAD, because only it is associated with extensive tissue damage and fluid accumulation in experimental animal models. Toxin B has no evident direct enterotoxic activity and is thought to play a role only after the gastrointestinal wall has been damaged by toxin A (Just and Gerhard, 2004). However, a recent report suggested that both toxins were comparable in human disease or that even toxin B is of more relevance (Savidge *et al.*, 2003). Interestingly, as toxin A-negative/toxin B-positive virulent *C. difficile* strains have been described, it is now clear that toxin A is not essential for virulence (Borriello *et al.*, 1992, Johnson *et al.*, 2001, Poutanen and Simor, 2004).

### 1.2.3 Clinical presentation of *C. difficile* infection

A wide spectrum of clinical presentation is observed in *C. difficile* infection, ranging from asymptomatic carriage to life-threatening colitis. The majority of hospital inpatients infected with *C. difficile* are asymptomatic (Kelly and LaMont, 1998). These asymptomatic carriers are an important hidden reservoir of *C. difficile*, particularly in medical facilities.

They shed the organism, contaminate the environment and perpetuate the chain of infection (Kelly *et al.*, 1994). Symptoms of *C. difficile* infection usually begin either during or shortly after the beginning of antibiotic therapy, but are occasionally delayed for several weeks or even longer (McFarland, 2005, Poutanen and Simor, 2004).

Mild to moderate CDAD is usually accompanied by lower abdominal cramping pain but no systemic symptoms or physical findings. The results of sigmoidoscopy are frequently normal in patients with mild disease and are therefore of little diagnostic help (Kelly *et al.*, 1994). Moderate to severe CDAD usually presents with profuse diarrhea, abdominal distention and pain, and occult bleeding. Common systemic manifestations include fever, nausea, anorexia, malaise and dehydration (Mylonakis *et al.*, 2001, Kelly *et al.*, 1994). PMC is the manifestation of full-blown *C. difficile* infection and is accompanied by similar but often more severe symptoms than those of patients with colitis without pseudomembranes (Kelly and LaMont, 1998). PMC is well defined histologically and shows focal ulceration of the colonic mucosa, covered by an exudate of inflammatory cells, fibrin and necrotic debris, the so-called "summit" or "volcano" lesion (Kelly and LaMont, 1998) and is almost invariably restricted to the colon and rectum (Collier *et al.*, 1996). The pseudomembranes form in multiple, friable yellow-white plaques that vary in size from 2 mm to about 2 cm in diameter, and are attached to the mucosal surface. These pseudomembranes are easily visualised at sigmoidoscopy or colonoscopy (Collier *et al.*, 1996). Fulminant PMC develops in 1% to 3% of patients with CDAD, with ileus, toxic megacolon, perforation and death (Mylonakis *et al.*, 2001). Although diarrhoea may be present, severely ill patients may have little or no diarrhoea as a result of toxic megacolon and loss of colonic muscular tone (Tabaqchali and Jumaa, 1995). Mortality associated with toxic megacolon is high, ranging from 24% to 38% (Poutanen and Simor, 2004). Fortunately, the most severe forms of *C. difficile* infection are also the least common.

### 1.2.4 Laboratory diagnosis of *C. difficile* infection

The laboratory diagnosis of *C. difficile* infection is generally based on the detection of toxin A or toxin B. A tissue culture cytotoxicity assay detecting the presence of toxin B in stool filtrates is considered to be the gold standard for diagnosis because of its high specificity (99-100%). ELISAs have been developed for the detection of toxin A or both toxin A and B from stool filtrates. Test kits able to detect both toxins are more sensitive because they are also able to identify disease caused by toxin A-negative/toxin B-positive strains of *C. difficile*. Stool culture for detection of the organism is generally less useful

because of the potential for asymptomatic carriage of *C. difficile* strains that are non-toxigenic. However, stool culture does have the advantage of allowing strain typing for investigation of an outbreak (Poutenan and Simor, 2004, Tabaqchali and Jumaa, 1995).

### 1.2.5 Recurrent *C. difficile* infection

Recurrence of CDAD following completion of initial successful treatment is common, with reports of relapse rates varying from 5% to 40% (Kelly and LaMont, 1998, Poutanen and Simor, 2004, Tabaqchali and Jumaa, 1995). Relapse is manifested by the appearance of symptoms usually within a week of stopping antimicrobial therapy. Patients who relapse once are at a greater risk of further relapses, with the relapse rate in patients who have suffered two or more previous relapses reported to be 45% to 65% (Kelly and LaMont, 1998, McFarland *et al.*, 2002). There has been much debate about the distinction between relapse and re-infection. Almost half of recurrences have been shown to be due to re-infection rather than relapse, suggesting that exposure to *C. difficile* from other patients or the environment is a major source of recurrent symptoms (Poutanen and Simor, 2004). True relapses arise after antibiotic therapy is discontinued and endogenous spores, which have not been destroyed by the antibiotics, germinate in the susceptible colon (McFarland, 2005, Poutanen and Simor, 2004). Kyne *et al.* (2001) showed that independent risk factors for recurrent CDAD include age greater than 65 years, increased severity of underlying illness and exposure to additional antibiotics after treatment. Controlling for these factors, this study also showed that a low serum antibody response to toxin A during an initial episode of CDAD is associated with an increased risk of recurrence. Relapse due to antibiotic resistance is not thought to be common, given the high intraluminal concentrations of antibiotics that can be achieved and the relatively rare occurrence of antimicrobial resistance *in vitro* (Poutanen and Simor, 2004).

### 1.2.6 Host risk factors

Antibiotic use is the most important risk factor for CDAD but other important host risk factors exist. The basis for the extreme variability in clinical presentation of *C. difficile* infection is not entirely clear, but host factors appear to be as important as bacterial virulence factors (Kelly and LaMont, 1998). Increasing age has been a consistently noted risk factor, with a more than ten-fold increased risk for those in the age group 60–90 years (Mylonakis *et al.*, 2001, Oldfield, 2004). It is possible that this may be due to age-related changes in faecal flora, resulting in poorer colonisation resistance, and/or decreased humoral immunity (Kyne *et al.*, 1999). Species such as bifidobacteria, which are

considered as being protective, are thought to decline in numbers with age, whereas clostridia and enterobacterial populations, which are viewed as being detrimental to health, increase with age (Hopkins and Macfarlane, 2002). The risk of diarrhoea is also related to the virulence of the infecting *C. difficile* strain and to the immune response to the organism's toxins. The majority of clinical isolates are toxin producers and many asymptomatic carriers have high toxin levels in their stools (Kelly, 1996). The ability of the host's immune system to produce protective antibodies against the toxins plays an important role in reducing the severity of disease and preventing recurrent episodes (Giannasca and Warny, 2004, Kyne *et al.*, 2001, McFarland, 2005). Gastrointestinal surgery and the use of nasogastric tubes, stool softeners, gastrointestinal stimulants, antiperistaltic drugs, antacids and enemas have been associated with an increased risk of CDAD (Poutanen and Simor, 2004). Antineoplastic and immunosuppressive drugs have also been implicated in CDAD and colitis (Mylonakis *et al.*, 2001, Pothoulakis, 1996). Underlying disease severity has recently been identified as a major risk factor for CDAD (Kyne *et al.*, 2002). Furthermore, the risk of acquiring CDAD is related to the length of hospital stay and rates of *C. difficile* colonisation of inpatients have been reported to be as high as 50% to 55% for patients hospitalised for greater than four weeks, suggesting acquisition from personnel, patients, or the environment (Archibald *et al.*, 2004, Bergogne-Bérézin, 2000). Debility and cognitive impairment at the onset of symptoms are associated with prolonged symptoms and more severe disease (Kyne *et al.*, 1999). Not everyone colonised with *C. difficile* experiences CDAD. In fact, studies have shown that colonisation with *C. difficile* protect against the development of symptomatic disease (Kyne *et al.*, 2000).

## 1.3 CLASSIFICATION AND TYPING

*C. difficile* belongs to the genus *Clostridium*, which comprises a group of Gram positive, anaerobic or aero-tolerant bacilli. The genus *Clostridium* contains thirty-five species that are considered pathogenic **(Figure 1.5)**. *C. difficile* is widely distributed in the soil and intestinal tracts of animals. Its vegetative cells are capable of forming spores, which confer resistance to heating, drying and chemical agents, and permit the organism to survive harsh environments. Strain typing is important for outbreak investigation and epidemiology of *C. difficile* and numerous typing methods exist. These are broadly divided into two categories, namely phenotypic and genotypic methods.

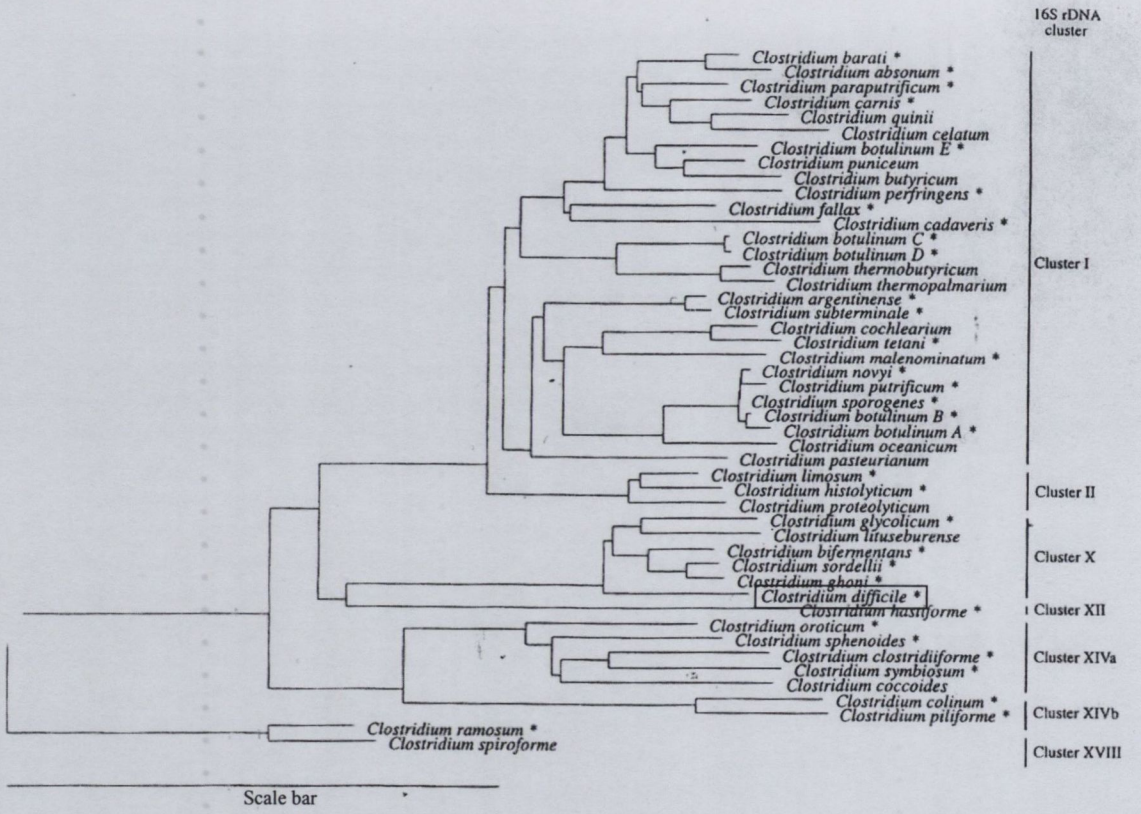



**Fig. 1.5** **Phylogenetic dendrogram of the pathogenic *Clostridium* species*.** Clusters as defined by Collins *et al.*, 1994. The scale bar represents 10 nucleotide substitutions per 100 nucleotides. The position of *C. difficile* is highlighted with a text box. Adapted from original in "The Clostridia, Molecular Biology and Pathogenesis", Erko Stackebrandt and Fred A. Rainey, Academic Press, 1997.

Historically, phenotypic typing methods were used and included SDS-PAGE of soluble cellular proteins, immunoblotting and serotyping. Nakamura *et al.* (1981) were the first to apply serogrouping, by using antisera raised against *C. difficile* to differentiate serovars amongst isolates from healthy carriers. Delmeé *et al.* (1985) further developed this method, by carrying out serogrouping using antibodies raised in laboratory animals to cell surface antigens. Other early phenotypic methods included antibiograms (Burdon, 1982), where distinct patterns of resistance to antibiotics in isolates from cases were compared with those of isolates in the rest of the hospital. This method was superseded by that of Wust *et al.* (1982), who applied plasmid analysis, PAGE, immunoelectrophoresis of extracellular antigens and antibiograms to sixteen isolates from related cases of CDAD. Using this combination of methods, twelve of the sixteen isolates analysed were indistinguishable, strongly suggesting that cross-infection had taken place.

Other phenotypic methods used included a combination of bacteriocin and bacteriophage (Sell *et al.*, 1983), immunochemical fingerprinting of EDTA-treated cell extracts, (Poxton *et al.*, 1984) and PAGE of [$^{35}$S] methionine-labelled proteins (radio-PAGE) (Tabaqchali *et al.*, 1984). Tabaqchali's radio-PAGE scheme had a typeability rate of 98% compared to Delmeé's serogrouping scheme, which had a typeability rate of 99%. The latter study gave rise to a serotying scheme that was able to recognise nineteen distinct serogroups and is frequently used as the standard by which other typing methods are compared. Toma *et al.* (1988) further developed serogrouping and found that typing by this method correlated well with that by PAGE. A correlation between serogrouping and toxicity was also found in this study. Heard *et al.* (1986) combined PAGE and serotyping using Tabaqchali's nine radio-PAGE types to immunise rabbits to confirm that certain protein bands detected on the gel were immunogenic and strain specific. A multiple typing approach was described by Mahony *et al.* (1991), which used bacteriocin, bacteriophage and plasmid analysis. However, this combination of methods could only type 84% of the isolates analysed.

A number of studies have compared the types recognised by one method with those recognised by another. Mulligan *et al.* (1988) found good correlation between the types recognised by plasmid profiling, serotyping, PAGE of cell surface antigens and immunoblotting. PAGE of cell surface antigens has been compared with serogrouping and generally correlated well with the results of serogrouping (Ogunsola *et al.*, 1995). Putative *C. difficile* outbreaks have been investigated by whole cell analysis using Pyrolysis Mass

Spectrometry (PyMS) (Cartmill *et al.*, 1992, Magee *et al.*, 1993). This is now regarded mainly as a 'fingerprinting' method and is useful to determine if a single strain is responsible for an outbreak. A typing method based on the molecular masses of the two major surface layer proteins (SLPs) of *C. difficile*, called 'S-typing', has recently been described (McCoubrey *et al.*, 2003, McCoubrey and Poxton, 2001). This method takes advantage of the high degree of variation of the molecular masses of the two major *C. difficile* SLPs. Each isolate is designated a four digit 'type number' consisting of the molecular mass, in kDa, of the heavier SLP followed by that of the lighter SLP. Due to the low levels of cross-reactivity between the SLPs from different strains, immunoblotting can further discriminate between isolates with SLPs of similar molecular mass (McCoubrey and Poxton, 2001).

In more recent times, a number of genotypic typing methods have been applied to *C. difficile*. Plasmid analysis has been investigated as a typing method, but has proved largely unsuccessful, mainly due to the fact that only 30–60% of *C. difficile* strains harbour plasmids (Jumaa *et al.*, 1996). Analysis of chromosomal DNA of *C. difficile* using whole cell DNA restriction endonuclease analysis (REA) has been investigated and has used a number of restriction enzymes including *Hind*III and *Cfo*I (Kuijper *et al.*, 1987). This method resulted in complex digestion products of greater than fifty bands, hence making comparisons difficult. REA is highly discriminatory and reproducible but is technically demanding, labour intensive and expensive to set up. Restriction fragment length polymorphism (RFLP), involving initial restriction endonuclease digestion followed by gel electrophoresis, has been used to type *C. difficile* but is labour intensive and offers little advantage over REA as a typing method. Pulsed field gel electrophoresis also allows analysis of the whole chromosome following digestion with rare cutting enzymes such as *Sma*I, *Ksp*I, *Sac*II or *Nru*I. Up to ten RFLPs are produced by this method per strain, consequently making analysis and comparisons relatively simple (Brazier, 1998b).

PCR-based typing methods have also been used to type *C. difficile*. Random or specific PCR primers can be used in these methods, with arbitrarily primed PCR and random amplified polymorphic DNA both using oligonucleotide primers of random sequence. Even minor changes in this method, such as changing the supplier of *Taq* polymerase, use of a different thermocycler or the amount of DNA used, can profoundly affect the resulting amplicons (Brazier, 1998b, Brazier, 2001). PCR ribotyping uses specific primers complementary to sites within the RNA operon and was first applied to *C. difficile* by

Gürtler (1993). Gürtler was able to distinguish 14 different PCR ribotypes among the 24 isolates analysed, indicating that PCR ribotyping offered good discrimination. Cartwright *et al.* (1995) and O'Neill *et al.* (1996) further developed PCR ribotyping, such that nowadays it is now routinely used to type *C. difficile*. The U.K. Anaerobe Reference Unit in Cardiff uses PCR ribotying routinely and has a library consisting of 117 distinct ribotypes (Brazier, 2001, Stubbs *et al.*, 1999). Karjalainn *et al.* (2002) investigated the usefulness of S-layer gene sequencing for subtyping of strains. This study found that the sequence of the variable region of the *slpA* gene was identical within a given serogroup but different between serogroups, suggesting that *slpA* gene sequencing may be an alternative to serogrouping.

## 1.4 PREVENTION OF *C. DIFFICILE* INFECTION

Primary prevention of *C. difficile* infection involves ensuring that patients do not become susceptible to *C. difficile* infection through disruption of their normal gut flora. Therefore, reducing the inappropriate use of broad-spectrum antibiotics can reduce the risk of CDAD (Worsley, 1998). There are many studies demonstrating that restrictive antibiotic policies are associated with reduced rates of CDAD (Climo *et al.*, 1998, Ludlam *et al.*, 1999, McNulty *et al.*, 1997, Pear *et al.*, 1994).

Transmission of *C. difficile* requires the organism or its spores to reach the patients gastrointestinal tract, either by ingestion or by direct inoculation into the bowel by contaminated equipment. *C. difficile* spores may survive for months or years and environmental contamination with spores has been demonstrated in 34% to 58% of sites in hospital wards (Fawley and Wilcox, 2001). Contamination of endoscopes has been noted to be 67% after use in patients with CDAD (Oldfield, 2004). Outbreaks in hospitals suggest, that, generally, transmission is either via staff hands or by direct contact with infected patients or contaminated surfaces (Worsley, 1998). One of the key interventions shown to be effective against the spread of *C. difficile* is hand washing (Jones and MacGowan, 1998). Therefore, good hand hygiene by health care workers is essential to limit the spread of the organism. Contamination of health care workers' hands leads to environmental contamination with *C. difficile* and it has been demonstrated that healthcare worker hand contamination is proportional to the level of environmental contamination (Samore *et al.*, 1996). Environmental contamination has been linked with the spread of *C. difficile* via contaminated commodes, baby baths and electronic thermometer (Worsley, 1998). Thus, environmental cleaning of floors, furniture and equipment is essential to

control the spread of *C. difficile*. In cases of outbreaks, disposable equipment should be used where possible to reduce the spread of infection via contaminated surfaces and hands. Symptomatic patients should be isolated to reduce the dissemination of the organism (Wilcox, 1998a).

## 1.5 TREATMENT OF *C. DIFFICILE* INFECTION

### 1.5.1 Antibiotics

The first approach in the treatment of CDAD is the cessation of the precipitating antibiotic, and 15% to 25% of patients respond to this approach (Poutanen and Simor, 2004, Wilcox, 1998b). This conservative approach allows for reconstitution of the normal colonic flora and markedly reduces the risk of relapse (Kelly and LaMont, 1998). However, sometimes it is not possible to stop antibiotic therapy if it is being given for a serious infection (Tabaqchali and Jumaa, 1995). If this is the case, the antibiotic should be changed to one shown to have a lower risk for CDAD (Oldfield, 2004). If diarrhoea persists following withdrawal of the offending antibiotic, antimicrobial therapy directed against *C. difficile* is indicated. Two specific treatments of *C. difficile* infection are currently recommended, namely oral vancomycin and oral metronidazole for seven to ten days (Wilcox, 1998b). Metronizadole is considered the initial treatment of choice for moderately severe CDAD (Kelly and LaMont, 1998, Poutanen and Simor, 2004). There is evidence to suggest that vancomycin results in shorter mean duration of symptoms than metronidazole, however, whether this difference is significant in terms of clinical benefit is not established (Wilcox, 1998b). Metronidazole has consistently achieved cure rates of 95%, with relapse rates of 5% to 15% (Oldfield, 2004). Given the considerably higher cost of vancomycin over metronidazole, and concern about selection for vancomycin-resistant enterococci, vancomycin is reserved for patients who cannot tolerate metronidazole, who do not response to metronizadole, or who are severely immunocompromised and have severe AAD or PMC (Poutanen and Simor, 2004, Tabaqchali and Jumaa, 1995). Prolonged courses of vancomycin, pulsed vancomycin and a mixture of vancomycin and metronizadole have been used to treat recurrent CDAD (Tabaqchali and Jumaa, 1995). It has also been common practice to switch from metronidazole to vancomycin (or vice versa) in patients with symptomatic recurrences (Wilcox, 1998b).

Alternative antibiotic therapies for CDAD include oral teicoplanin, bacitracin, rifampicin or fusidic acid, although these agents have not been studied in as much detail as metronidazole or vancomycin (McFarland, 2005, Oldfield, 2004, Poutanen and Simor,

2004, Surawicz and McFarland, 1999). Linezolid, the first of a new class of antimicrobial drugs, the oxazolidinones, has been shown to have *in vitro* activity against *C. difficile* isolates which are resistant to metronidazole or have reduced susceptibility to vancomycin (Peláez *et al.*, 2002). Surgical intervention may be necessary in patients who deteriorate despite aggressive antimicrobial therapy and in patients with suspected colonic perforation. The overall mortality rate for surgical intervention is high, approximately 35%, reflecting the poor clinical condition of such patients at the time of surgery (Kelly and LaMont, 1998, Surawicz and McFarland, 1999).

### 1.5.2 Immunisation against *C. difficile* infection

Vaccines based on the *C. difficile* toxins are being explored as a means of protecting high-risk individuals against CDAD. The aim of these vaccines is to produce neutralising antibodies against toxins A and B, and hence prevent symptomatic infection. As early as 1979, it was demonstrated that passively administered *Clostridium sordellii* anti-toxin, shown to neutralise cytotoxicity of the *C. difficile* toxins, could prevent CDAD in hamsters, strongly indicating that antibodies against the *C. difficile* toxins would also protect against CDAD (Allo *et al.*, 1979). Hamsters vaccinated parenterally with formalin-inactivated toxins A and B in culture filtrate, but not individual toxins in culture filtrate, were protected from lethal CDAD in the hamster model (Fernie *et al.*, 1983, Libby *et al.*, 1982). Kim *et al.* (1987) found that toxins A and B combined and toxin A alone, but not toxin B alone, similarly protected hamsters from CDAD, while culture filtrates from non-toxigenic strains of *C. difficile* did not confer protection. Colostrum from cows vaccinated with the *C. difficile* toxins was shown to prevent diarrhoea and death in hamsters when administered before and during clindamycin-induced CDAD (Lyerly *et al.*, 1991). From these early studies the essential role of toxin-specific immunity in protection from CDAD was clear.

Torres *et al.* (1995) evaluated various adjuvants and routes of delivery of a toxoid vaccine in hamsters. A sequential combination of intranasal and intraperitoneal immunisation with vaccine plus cholera toxin (CT) and Ribi adjuvants, respectively, provided complete protection from death and diarrhoea, suggesting that induction of both systemic and mucosal immunity was necessary for optimal protection. Torres *et al* (1995) also assessed the efficacy of a rectally administered inactivated whole cell vaccine to protect against CDAD, however this offered no protection from death and minimal protection from diarrhoea. Giannasca *et al.* (1999) furthered this work and tested various clinically

compatible immunisation regimens in hamsters and found that a combination of rectal immunisation with *E. coli* heat labile toxin (LT) and intramuscular immunisation with alum provided 100% protection against diarrhoea and death. Surprisingly, it was found that intramuscular immunisation with toxins only conferred the same high levels of protection from diarrhoea and death. No detectable anti-toxin antibodies were found in saliva or faeces, suggesting that serum antibodies were the principal effector molecules. However, IgA anti-toxin antibodies have been shown to be more important than the IgG fraction in neutralising the cytotoxic and enterotoxic properties of toxin A (Johnson *et al.*, 1995). In order to define the immunogenic domains of the toxins required for protection, Kink and Williams (1998) created multiple recombinant peptides that spanned the entire toxin proteins. Avian antibodies raised against these polypeptides were used for passive immunisation of hamsters. Antibodies to the cell binding domains of both toxins were most effective in eliciting toxin neutralising antibodies, and antibodies to both toxins were required to completely prevent diarrhoea and death.

In humans, the ability to mount an effective immune response to the toxins following exposure to *C. difficile* appears to affect the course of disease progression. Following symptomatic infection, many individuals develop serum antibodies to the toxins, including toxin neutralising IgA, and this response is associated with protection from subsequent infection (Johnson *et al.*, 1995). Furthermore, in a prospective analysis of hospital patients receiving antibiotics, a serum IgG response against toxin A has been correlated with asymptomatic carriage of *C. difficile* in humans (Kyne *et al.*, 2001). Kotloff *et al.* (2001) studied the safety and immunogenicity of an intramuscularly administered *C. difficile* toxoid A and B vaccine in healthy adult humans. This vaccine was found to be both safe and immunogenic, leading to a vigorous serum antibody response to both toxins in over 90% of subjects. In a recent preliminary study, anti-toxin whey protein concentrate has been shown to protect against CDAD in hamsters and humans, without relapse after discontinuation of treatment (van Dissel *et al.*, 2005).

### 1.5.3 Novel therapies

Several novel non-antibiotic, non-vaccine based therapies for CDAD are currently under investigation. Probiotics are thought to enhance re-establishment of the normal gut flora following disruption by antibiotics, to stimulate the immune response, to elicit production of enzymes that destroy pathogenic toxins and/or to block attachment sites in the colon (McFarland, 2005, Surawicz, 2004). Several probiotics have been used to treat CDAD

including *Lactobacillus GC*, *L. acidophilus*, *L. bulgaricus*, non-toxic strains of *C. difficile* and the non-pathogenic yeast *Saccharomyces boulardii* (Bergogne-Bérézin, 2000, Kelly and LaMont, 1998, Surawicz, 2004). Furthermore, it has been demonstrated that a combination of vancomycin and *S. bouliardii* results in a significant decrease in recurrent CDAD compared to vancomycin treatment alone (Surawicz *et al.*, 2000). It has been suggested that the protective effects of *S. bouliardii* may be due to the proteolytic digestion of the *C. difficile* toxins by serine proteases produced by this yeast (Bergogne-Bérézin, 2000). *S. cerevesiae* has also been used successfully to treat patients with refractory CDAD (Mylonakis *et al.*, 2001). *L. GG* and *L. casei* have been shown to reduce the number of relapses in humans (Bergogne-Bérézin, 2000). Sambol *et al.* (2002) demonstrated that colonisation with non-toxigenic strains of *C. difficile* could prevent CDAD in 87% to 97% of hamsters challenged with toxigenic strains. A mixture of normal stool flora administered in normal saline or donor stool administered by nasogastric tube has also been used to restore the normal gut flora. Although not aesthetically appealing, high success rates with difficult cases have been achieved using stool transplantation (Oldfield, 2004). Faecal enemas and rectal instillates of microbes have also been used with promising results (Pothoulakis, 1996, Surawicz, 2004). It has been suggested that *Bacteroides* species may be of particular importance in competing with *C. difficile* and in one study these bacteria were used for the treatment of recurrent CDAD (Freeman *et al.*, 2003, Tvede and Rask-Masden, 1989).

Passive oral immunotherapy may be achieved by administering intravenous pooled normal human immunoglobulin, which has a high titre of antibody against *C. difficile* toxins (Kelly and LaMont, 1998). Intravenous immunoglobulin has been used successfully in patients not responding to metronidazole or vancomycin (Oldfield, 2004, Surawicz and McFarland, 1999). Other non-antibiotic approaches have included the bile-salt-binding resin cholestyramine, which is thought to bind the *C. difficile* toxins, although this has not been shown *in vivo* (McFarland *et al.*, 2002). It should be noted that cholestyramine also binds vancomycin (Oldfield, 2004). Tolevamer (GT160-246), a sodium salt of styrene sulfonate polymer that has toxin-binding properties, is under development for the treatment of CDAD. This compound has already been shown to protect against CDAD in the hamster model of disease (Braunlin *et al.*, 2004, Kurtz *et al.*, 2001).

## 1.6 MODELS OF *C. DIFFICILE* INFECTION

### 1.6.1 Golden Syrian hamster

The gold standard animal model of *C. difficile* infection is the Golden Syrian hamster (*Mesocricetus auratus*). It has long been recognised that a lethal enterocolitis can be induced in hamsters by the administration of clindamycin, and as such the hamster is a natural model of *C. difficile* infection with some laboratory colonies experiencing outbreaks of CDAD (Giannasca and Warny, 2004). This model was first described by Small (1968) and further investigated by Bartlett *et al.* (1977) and Chang *et al.* (1978). These early studies confirmed that administration of lincomycin or clindamycin could induce lethal enterocolitis in hamsters. This phenomenon presented a valuable model for studying CDAD and has been used by many investigators since this time.

In this model, hamsters are predisposed to *C. difficile* infection by clearing their gut flora with clindamycin and are subsequently challenged with *C. difficile*. Some of the earlier models of infection did not include challenge with *C. difficile* (Fernie *et al.*, 1983, Libby *et al.*, 1982), but presumably relied on the proliferation of endogenous or exogenous *C. difficile* spores to induce disease following the administration of clindamycin. Various routes of administration of clindamycin have been used, including intragastric (Giannasca *et al.*, 1999, Lyerly *et al.*, 1991, Torres *et al.*, 1995) and subcutaneous (Fernie *et al.*, 1983, Libby *et al.*, 1982). Sambol *et al.* (2002) successfully modified the hamster model to reflect transmission of *C. difficile* via spores in the environment. The inoculum was produced by culturing *C. difficile* for 5–7 days to allow sporulation. The harvested cells were heat shocked to kill any remaining vegetative cells and the resultant spores were stored at -70°C until use. Following administration of clindamycin and inoculation with toxigenic *C. difficile*, disease develops within 24 to 48 hours, with diarrhoea, visible as a "wet tail", ruffled fur, lethargy and death occurring within 2–3 days (Giannasca and Warny, 2004) **(Figure 1.6, A and B)**. Not all toxigenic *C. difficile* strains are equally virulent in the hamster model. Borriello *et al.* (1987) demonstrated that toxigenic *C. difficile* can be differentiated into two categories – those that cause death in all hamsters within two days of infection and those that kill some hamsters five days or more after infection.

Gross pathological examination of *C. difficile* infected hamsters shows enlarged haemorrhagic caecum and severe haemorrhagic necrosis of the caecum and colon (Giannasca *et al.*, 1999, Libby *et al.*, 1982) **(Figure 1.6, C and D)**. On histological




**Fig. 1.6 Gross pathology of hamsters infected with *C. difficile*.** (A) Hunched, inactive, ruffled fur. (B) The "wet tail" typically seen in *C. difficile* infected hamsters. (C) and (D) Grossly enlarged and haemorrhagic caecum and colon. Images are representative of the gross pathology observed in the hamster model of *C. difficile* infection used in this thesis.

examination of the caecum and colon the crypts are elongated and their luminal halves infiltrated by polymorphs. The epithelial surface becomes irregular and thrown up into "tufts" of disorganised epithelial cells. There is haemorrhage, focal necrosis of the surface epithelium with underlying congestion, leukocyte infiltration of the lamina propria and focal aggregates of neutrophils (Borriello *et al.*, 1987, Libby et al., 1982). Petechial haemorrhage is sometimes evident. Less virulent strains result in mildly hyperplastic crypts, marked thickening of the mucosa, superficial inflammation and disorganisation of the epithelium (Borriello *et al.*, 1987).

Most of the important clinical observations regarding CDAD were initially made in the hamster model, including detection of the aetiological agent, discovery of the implicated antibiotics, development of the diagnostic test, and treatment with oral vancomycin (Bartlett, 2004). Although the hamster model parallels human infection in its susceptibility to *C. difficile* following antibiotic therapy, it results in a more severe, lethal disease than in humans. The hamster model, therefore, is a stringent test of any protective effects of vaccines and immunotherapies against CDAD.

### 1.6.2 Other models

Several other models have been investigated as potential *C. difficile* infection models, including hares, guinea pigs, prairie dogs, monkeys and mice (Corthier *et al.*, 1991). BALB/c mice have been used for active immunisation studies with the toxins, although the mice were not challenged with *C. difficile* (Qamar *et al.*, 2001). Gnotobiotic mice have been challenged with *C. difficile*, but with conflicting results with regard to mortality rates. Onderdonk *et al.* (1980) demonstrated that gnotobiotic mice challenged with *C. difficile* had polymorhonuclear cell infiltration of the lamina propria, diarrhoea and high caecal cytotoxin concentrations. However, the mortality rate of the mice was only 2%. Conversely, Corthier *et al.* (1991) infected C3He/J gnotobiotic mice by orogastric administration of *C. difficile*. All mice developed a disease characterised by intense caecal abrasion and a severe inflammatory response and all died within two days. Prairie dogs have also been proposed as a model of CDAD. Muller *et al.* (1987) observed that cefoxitin induces *C. difficile* caecitis in these animals, with significant weight loss, diarrhoea and PMC in all animals. However, the animals tolerated the disease well and it was not lethal in the majority of animals. Recently, an *in vitro* triple stage chemostat model of the human gut has been described (Freeman *et al.*, 2003). The model consists of three vessels, V1, V2 and V3, operating in a weir cascade system and emptying into a waste vessel. The vessels

contain faecal slurry derived from faeces from healthy elderly volunteers. The model is temperature controlled at 37°C and the vessels are controlled to pH 5.5, 6.2 and 6.8, respectively, reflecting increasing alkalinity of the human intestine from proximal to distal. Freeman and colleagues investigated the effect of the antibiotic cefotaxime and a metabolite of cefotaxime on *C. difficile* and components of the normal faecal flora in this model.

## 1.7 SURFACE LAYER PROTEINS

### 1.7.1 Crystalline bacterial cell surface layers

Surface layers (S-layers) have been identified on organisms of nearly every taxonomic group of Bacteria and are an almost universal feature of Archaea (Sleytr *et al.*, 2001). These two-dimensional proteinaceous cell wall structures cover the outer surface of the cell **(Figure 1.7)**, and are generally composed of a single protein or glycoprotein species with molecular weight ranging from 40 kDa to 170 kDa. Most S-layer proteins are acidic with a large proportion of hydrophobic amino acids and contain little or no sulphur-containing amino acids. Lysine is the most predominant amino acid while the arginine, histidine and methionine content is generally low (Pum *et al.*, 2000, Sleytr *et al.*, 1993). According to sequence analysis, the isoelectric point of most S-layers is in the weakly acidic pH range (Pum *et al.*, 2000). S-layers exhibit one of three 2-D lattice types: oblique (p1 or p2), square (p4) or hexagonal (p3 or p6) symmetry **(Figure 1.8)**, with center-to-center spacings of the subunits being in the range 2.5 to 35 nm (Sleytr *et al.*, 1999, Sára and Sleytr, 2000). The lattice subunits are symmetric monomers (p1), dimers (p2), trimers (p3), tetramers (p4) or hexamers (p6) (Šmarda *et al.*, 2002). High resolution and scanning force microscopy have shown that S-layers are very porous structures, with pores of identical size (range 2 to 8 nm) and morphology occupying up to 70% of their surface area. Most S-layers are 5 to 26 nm thick and have a smooth outer surface and a corrugated inner surface (Sára and Sleytr, 2000). Since S-layers are found in Gram positive and Gram negative bacteria they can be associated with quite different cell envelope structures, such as the peptidoglycan, pseudomurein, or components of the outer membrane or plasma membrane. Commonly, the subunits are linked together and are also linked to the underlying cell envelope layers by a combination of non-covalent forces including hydrogen or ionic bonds, and hydrophobic or electrostatic interactions (Pum *et al.*, 2000, Sleytr *et al.*, 1993). Post-translational modifications of S-layer proteins include cleavage of N- or C-terminal fragments, glycosylation and phosphorylation of amino acid residues (Sleytr *et al.*, 1999).

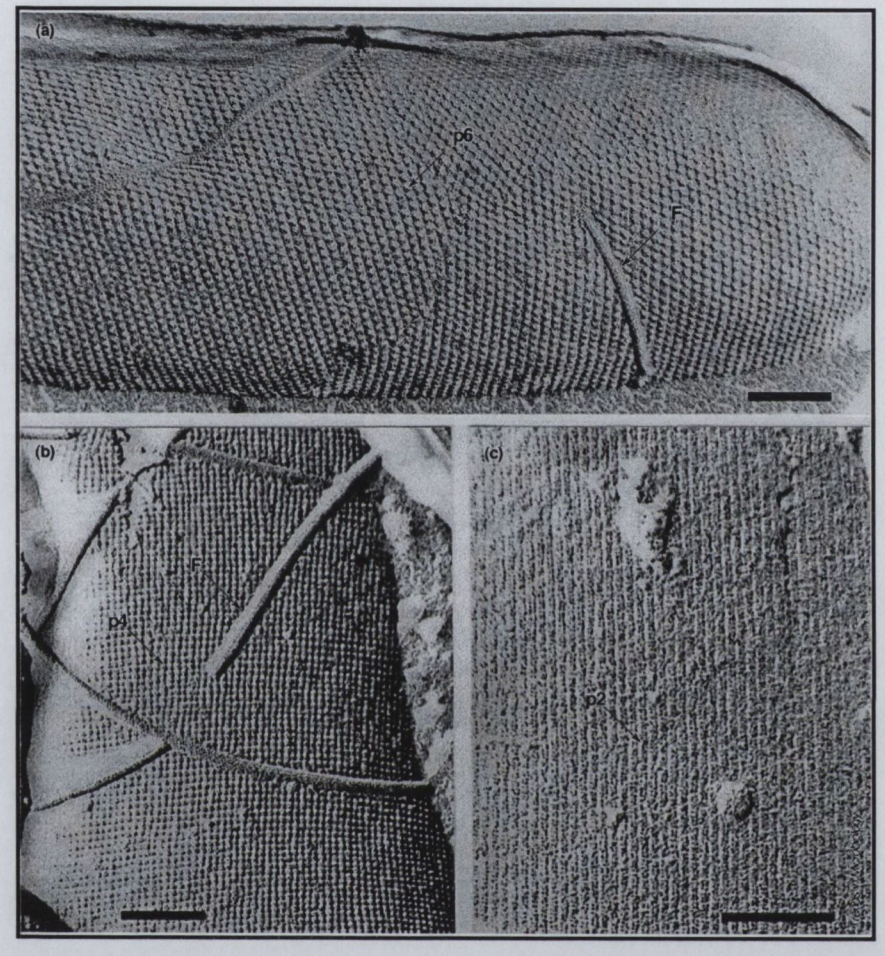



**Fig. 1.7 Electron micrographs of freeze-etched preparations of intact cells** (a) *Thermoanaerobacter thermohydrosulfuricus* showing a hexagonal (p6) surface lattice; (b) *Desulfotomaculum nigrificans* with square (p4) lattice; (c) *Lactobacillus helveticus* showing an oblique (p2) arrays of subunits. Modified from original of Sleytr and Beveridge, 1999.

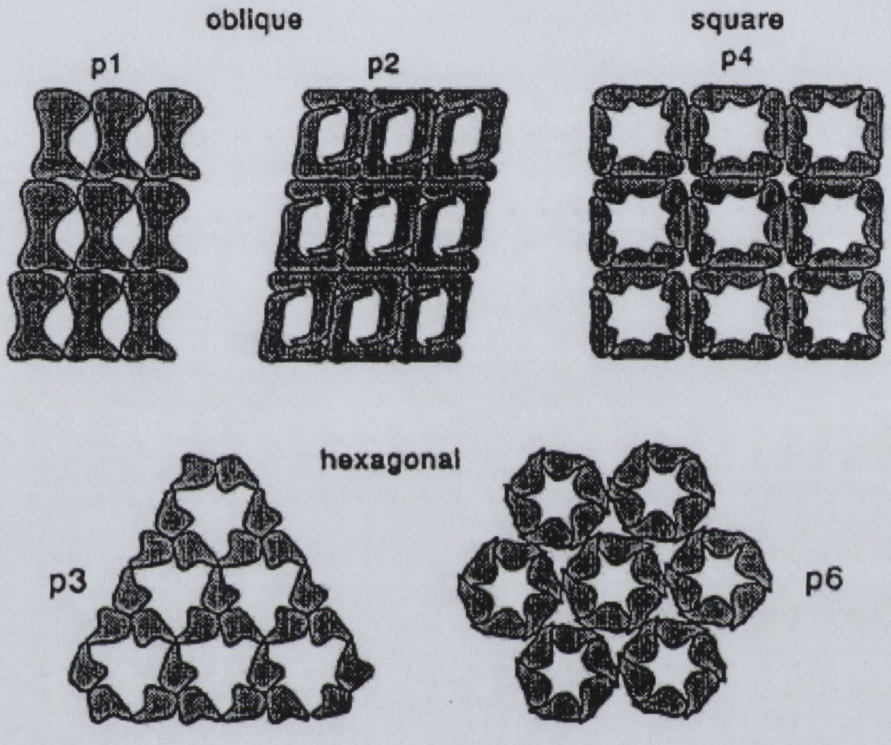



**Fig. 1.8** **Schematic illustration of S-layer lattice types.** The building blocks of each crystalline lattice are formed by its subunits, each of which is a symmetric array of identical monomers, dimers, trimers, tetramers or hexamers. Modified from original of Sleytr *et al.*, 1999.

S-layer proteins are often the most abundant species in cells, and can comprise up to 15% of the total cellular protein (Navarre and Schneewind, 1999). S-layers have the ability to completely cover the cell surface during all stages of bacterial growth and division by an entropy driven assembly process (Sára and Sleytr, 1996, Sleytr *et al.*, 1993). An intact S-layer on an average sized rod-shaped prokaryotic cell consists of approximately 1 x $10^5$ monomers. At least 500 copies of a single polypeptide species must be synthesised, translocated to the cell surface and incorporated into the existing lattice per second to maintain the complete S-layer on the surface of a cell growing with a generation time of 20 to 30 minutes. Presumably this structure evolved by selection in response to specific environmental and ecological pressures, and it is reasonable to assume that S-layers confer some advantage on bacteria or they would not produce this metabolically expensive structure. S-layers are often lost during prolonged cultivation under optimal laboratory conditions (Sára and Sleytr, 2000) and usually the S-layer deficient variant outgrows the S-layered parent (Sleytr *et al.*, 1999).

S-layers have been shown to function as protective coats, molecular sieves in the ultrafiltration range, and as a barrier, controlling the exchange of molecules between the cell and its environment, as well as structures involved in cell adhesion and surface recognition (Sleytr *et al.*, 1993). S-layers have also been shown to fulfill a shape-determining or shape-maintaining function. S-layers are essential for the virulence of some pathogens, including *Aeromonas salmonicida* and *Campylobacter fetus* (Grogono-Thomas *et al.*, 2003, Sára and Sleytr, 2000). The S-layer of *A. salmonicida* (termed A-layer) confers resistance to the bactericidal activity of complement systems, in both immune and non-immune serum. Serum resistance of *C. fetus* is due to the inability of the complement component C3b to bind to the S-layer surface and effective opsonisation occurs only after addition of antibodies to the A-layer (Sleytr *et al.*, 1999).

Due to their specific structural properties and ability to self-assemble *in vitro*, S-layers have many potential biotechnology applications. Since the pores of S-layers are identical in size and morphology, and have sharp molecular weight cut-offs in the 2 nm to 8 nm range, they can be used as isoporous ultrafiltration membranes. These membranes can be produced by depositing S-layer subunits, or cell wall fragments carrying S-layers, on microfiltration membranes, cross-linking the S-layer proteins with glutaraldehyde, and reducing Schiff bases with sodium borohydride (Sleytr *et al.*, 1999). Additionally, by exploiting the high density and defined positions of carboxy groups on the surface of S-

layers, they can be used for the immobilisation of functional molecules. A spectrum of functional macromolecules, including enzymes, antibodies, antigens and ligands have already been covalently bound to S-layer matrices (Pum *et al.*, 2000). S-layers can also be employed as supporting structures for functional lipid membranes, thereby increasing their stability. S-layer subunits can recrystallise on liposomes and be exploited as a matrix for the covalent attachment of molecules, giving rise to applications such as drug-delivery, drug-targeting systems or in gene therapy (Sleytr *et al.*, 1999).

### 1.7.2 S-layer of *C. difficile*

*C. difficile* expresses two major cell SLPs that form a crystalline S-layer on its surface (McCoubrey and Poxton, 2001). Kawata *et al.* (1984) first described the presence of this surface regular array by electron microscopy and negative staining, and described it as being composed of two squarely arranged proteins with apparent molecular masses of 32 kDa and 45 kDa. In this study, similar arrays were found on the cell walls of nine *C. difficile* strains. It was subsequently demonstrated that these proteins varied considerably between strains, both in molecular mass and immunogenic reactivity (Sharp and Poxton, 1988). Around the same time, Dailey and Schloemer (1988) reported the cloning and expression of two secreted antigens from *C. difficile* VPI 10463 in *E. coli*. These antigens had molecular masses of 32 kDa and 43 kDa, and probably corresponded to the two SLPs. Cerquetti *et al.* (2000) described the *C. difficile* S-layer as two superimposed and structurally different S-layers, each composed of a distinct protein. In contrast to the square symmetry first described by Kawata *et al.* (1984), Cerquetti *et al.* (2000) demonstrated that the outer S-layer is arranged in square symmetry and the inner in hexagonal symmetry.

A single gene, *slpA*, encodes a precursor protein, which is cleaved post-translationally to remove a signal peptide and to produce the two mature SLPs (Calabi *et al.*, 2001). The two mature peptides have different molecular masses to each other and are therefore termed the low- and the high-MW SLPs. The low-MW SLP is encoded by the 5' end of the *slpA* gene and the high-MW SLP by the 3' end (Calabi *et al.*, 2001, Karjalainen *et al.*, 2001). Generally, molecular masses are in the range 36-56 kDa (Cerquetti *et al.*, 2000), with the low-MW SLP having a molecular mass of 32-38 kDa and the high-MW SLP having a molecular mass of 42-48 kDa (Calabi *et al.*, 2001). Transcriptional analysis of *slpA* reveals that the gene is strongly transcribed during the whole growth phase (Savariau-Lacomme *et al.*, 2003). The presence of a typical signal sequence in the precursor indicates transport of the precursor protein across the plasma membrane by the general secretory pathway. SecA

is one of three subunits of the Sec translocase essential for this secretion. Interestingly, the *SecA* gene in *C. difficile* is situated directly downstream of the *slpA* gene (Calabi *et al.*, 2001, Mukherjee *et al.*, 2002). It may not be coincidental that a major secreted protein is coded for on a gene right next to a component of its possible translocase.

There is a high degree of variation in the molecular masses of the SLPs of different *C. difficile* strains and there is little antigenic cross-reactivity between SLPs of different strains unless they share identical SLPs (McCoubrey and Poxton, 2001). The high-MW SLP is relatively conserved between strains (Calabi *et al.*, 2001) and appears to be non-immunogenic, or at best weakly so. The high-MW SLP shows significant homology to N-acetylmuramoyl-L-alanine amidase and amidase enhancer protein of *Bacillus subtilis* (Calabi *et al.*, 2001, Karjalainen *et al.*, 2001, Mukherjee *et al.*, 2002). Recently, the sequences of the part of *slpA* encoding the high-MW SLP were found to be strictly identical within a given serogroup, but different between serogroups (Karjalainen *et al.*, 2002). The low-MW SLP is highly variable between strains and is the more immunogenic of the two (Calabi *et al.*, 2001, Karjalainen *et al.*, 2001, Poxton *et al.*, 1999). In early descriptions of the *C. difficile* S-layer, the component SLPs were not found to be glycosylated (Cerquetti *et al.*, 1992, Kawata *et al.*, 1984, Mastrantonio *et al.*, 1995). However, in recent times more sensitive assays have revealed that in certain strains, including a Ribotype 1 strain identical to that used in this study, both the low and high-MW SLPs are glycosylated (Calabi *et al.*, 2001, Cerquetti *et al.*, 2000, Mauri *et al.*, 1999). A third *C. difficile* SLP of approximately 70-75 kDa has been described and appears to be a common antigen conserved throughout different isolates (McCoubrey and Poxton, 2001, Poxton *et al.*, 1999).

Direct binding of *C. difficile* to Caco-2 and HT-29 colonic epithelial cell lines, as well as to primary intestinal epithelial cells, has been demonstrated (Cerquetti *et al.*, 2002, Drudy *et al.*, 2001). Due to their location at the outer surface of the cell, the SLPs have been proposed as adhesions and native forms of SLPs as well as a recombinant high-MW SLP are able to bind directly to human gastrointestinal tissue sections (Calabi *et al.*, 2002). Moreover, anti-sera against whole bacteria and the high-MW SLP can reduce binding of *C. difficile* to the intestinal epithelial cell lines HT-29 and Hep-2 respectively (Drudy *et al.*, 2001, Calabi *et al.*, 2002). However, the nature and precise cellular localisation of the ligand or ligands remains unclear. Cerquetti *et al.* (2002) demonstrated that *C. difficile* cells (strain C253) bound efficiently to fibronectin and collagen types I, III, IV and V, in a

dose dependent manner, and to fibrinogen and vitronectin, where binding was high regardless of the amount of protein. In contrast, Calabi *et al.* (2002) demonstrated binding of native SLPs and recombinant high-MW subunit to collagen I, thrombospondin and vitronectin (weakly). No binding of the SLPs was seen with collagen IV, fibronectin or laminin. This indicates that the binding of *C. difficile* to the gastrointestinal epithelium is multifactorial and other adhesions including the adhesin cwp66, flagella, the heat shock protein GroEL may be involved (Savariau-Lacomme et al., 2003, Waligora *et al.*, 2001). Furthermore, environmental factors have also been shown to modify *C. difficile* attachment to Vero cells (Waligora *et al.*, 1999).

### 1.7.3 Immune response of the host to *C. difficile* SLPs

A limited number of studies have investigated the immune response to the *C. difficile* SLPs in human serum samples. Pantosti *et al.* (1989) demonstrated that serum from 50% of patient's infected with *C. difficile* had IgG antibodies to the SLPs, and the low-MW SLP was the SLP most consistently recognised by individuals infected with *C. difficile.* However, no correlation between the development of serum IgG anti-SLP antibodies and clinical outcome was found in this study. Mulligan *et al.* (1993) compared serum IgA, IgM and polyvalent antibodies to dried whole *C. difficile* in asymptomatic carriers and symptomatic patients using ELISA. Significantly higher levels of IgA, IgM and polyvalent antibodies were detected in asymptomatic carriers compared to cases or controls. Given the fact that the immunological activity in the serum samples could be reduced by adsorption with *C. difficile* and not an unrelated organism, and since the SLPs of *C. difficile* cover the outermost surface of the bacterium, it is plausible that the serum antibodies detected were specific for the SLPs. This study indicated for the first time that an antibody response to the SLPs might play a role in the clinical outcome of patients infected with *C. difficile.* Most recently, Drudy *et al.* (2004) analysed serum IgM, IgG and IgA anti-SLP levels in *C. difficile* cases, carriers and controls. No differences in serum IgM, IgG or IgA levels between the groups were detected in this study. However, patients with recurrent CDAD had significantly lower serum IgM, but not IgG or IgA, anti-SLP levels, suggesting the importance of the immune response to the SLPs in the development of CDAD. All three studies observed a high degree of variability in the response to the SLPs and this may reflect the wide spectrum of disease associated with CDAD.

Phagocytosis of *C. difficile* is dependent on opsonisation of the bacterium. Antibodies against whole *C. difficile*, and to a much lesser extent, complement, can successfully

opsonise the bacterium. Resistance to phagocytosis in the absence of an opsonin may represent a mechansim evolved by the bacterium to persist in the gut despite the presence of many specialised phagocytic cells in this environment. Both toxigenic and non-toxigenic *C. difficile* are resistant to phagocytosis, indicating that the toxins are not the anti-phagocytic factor (Dailey *et al.*, 1987). It has also been shown that the ability of polymorphonuclear leukocytes (PMNs) from elderly and post-operative patients to phagocytose *C. difficile* is diminished, and that the defect is, in part, serum associated (Bassaris *et al.*, 1984). This defective PMN function may explain the increased incidence of CDAD in these two groups.

No more detailed studies exploring the host immune response to the *C. difficile* SLPs have been carried out. More in depth studies are necessary to elucidate the specific type of adaptive immune response elicited by these proteins. The role of innate immune cells, such as dendritic cells (DCs) and macrophages, in directing adaptive immunity has become clear in recent years. The SLPs may well have an important role in priming the adaptive immune response to *C. difficile* infection, and the cytokines elaborated by interaction of the SLPs with innate immune cells may be critical in directing adaptive immunity against this bacterium. Therefore, information regarding the role of the SLPs in the host immune response will allow for more rational vaccine design against this pathogen.

## 1.8 HOST IMMUNE RESPONSE TO INFECTION

### 1.8.1 Innate and adaptive immunity

The innate immune system provides the first line of defence against pathogens and includes mechanisms such as fever, mucosal secretions, chemical mediators and phagocytic cells, such as macrophages and neutrophils, which fight pathogens in an unspecific and non-intrusive way. The innate immune system defends against many common microorganisms and is essential for the control of common infections. However, this system cannot always eliminate infectious microorganisms. The lymphocytes of the adaptive immune system have evolved to provide a more versatile means of defence, which, in turn, provides increased protection against subsequent infection with the same pathogen (Janeway *et al.*, Immunobiology, 6$^{th}$ edition, 2004). The discovery that the innate immune system recognises microbial components directly through various pathogen recognition receptors (PRRs) expressed on innate immune cells has made it increasingly clear that the innate immune response determines the nature of the subsequent adaptive immune response (Pulendran, 2005). Acting initially through pattern recognition receptors,

such as Toll like receptors (TLRs) expressed on epithelial cells, macrophages and DCs, the innate immune system alerts the host to microbial infections via TLR binding of pathogen associated molecular patterns on the surfaces of microorganisms (Storni *et al.*, 2005). Once stimulated by the appropriate microbial ligand, TLRs activate the transcription factors NFκB and MAP kinase, leading to the production of important inflammatory cytokines of innate immunity such as IL-6, IL-12, IL-18, IFN-γ and TNF-α. Concurrently, TLR signaling induces expression of co-stimulatory molecules such as B7.1 (CD80) and B7.2 (CD86), which are essential for the induction of adaptive immune responses. These molecules, along with the surface presentation of microbial peptides on MHC molecules, activate $CD4^+$ T cells and initiate the adaptive immune response. The activation of antigen-specific T cells of an appropriate phenotype is central to effective immunisation. Cellular immunity, mediated by $CD4^+$ and $CD8^+$ T cells, plays a major role in protection against infectious pathogens. $CD4^+$ and $CD8^+$ T cells respond to antigens processed and presented by antigen presenting cells (APCs) by distinct mechanisms. $CD4^+$ T cells recognise exogenous antigen in association with MHC class II molecules, while $CD8^+$ cytotoxic T lymphocytes (CTLs) recognise endogenous antigen in association with MHC class I molecules (McNeela and Mills, 2001, Storni *et al.*, 2005). TLR-induced cytokines continue to direct the response by guiding the maturation of lymphocytes into antigen-specific effector and/or memory cells (Evans *et al.*, 2003). The end result of these events is antigen-specific acquired immunity.

### 1.8.2 T helper cell differentiation

#### 1.8.2.1 Th1 and Th2 cells

The $CD4^+$ T cell population may be divided into at least two subpopulations, namely T helper 1 (Th1) and T helper 2 (Th2) cells, on the basis of the cytokines they secrete and their function in the host immune response (Kidd, 2003) **(Figure 1.9)**. The role of effector Th cells is to control the whole immune response by secreting cytokines and by expressing surface molecules, such as CD40 ligand for activation of B cells, APCs and CTLs. The activation and differentiation of naïve $CD4^+$ T cells requires at least two separate signals. The first signal is delivered by the T cell receptor:CD3 complex after its interaction with antigen:MHC on APCs. The second signal is produced by a number of co-stimulatory molecules on the APC that interact with their ligands on T cells, for example CD28 and B7.1. The naïve T cells may pass through a transient, pre-activation state (T0) on their way to becoming Th1 or Th2 cells (Neurath *et al.*, 2002).

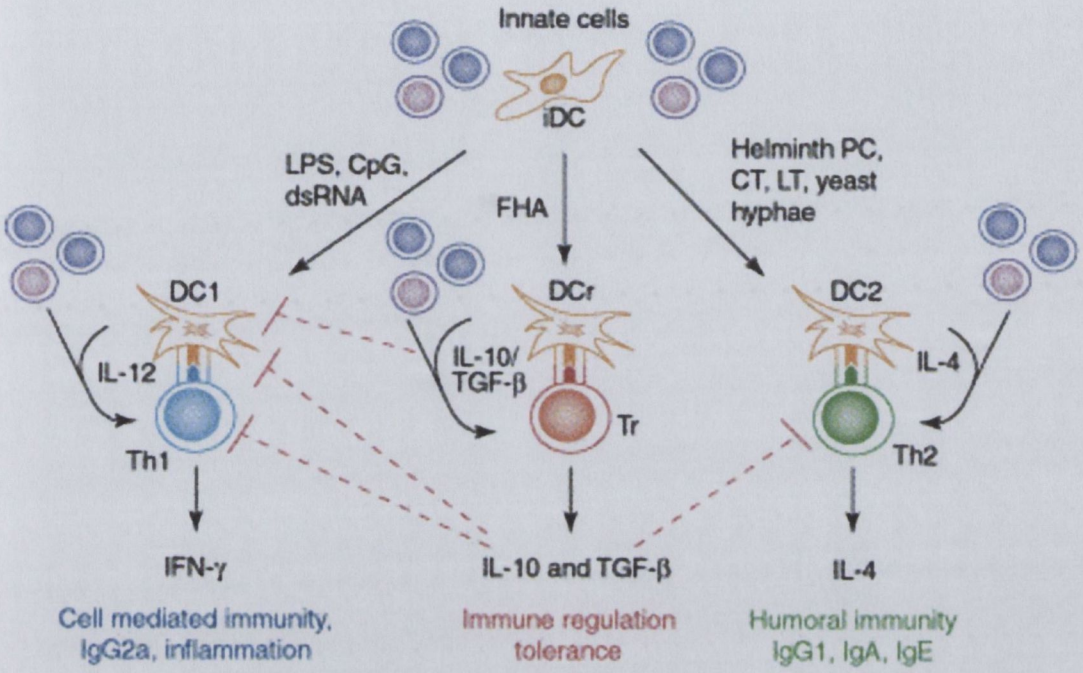



**Fig. 1.9 Proposed model for $CD4^+$ T-cell differentiation and function in immunity to infection.** Certain pathogen-derived immunomodulatory molecules bind to DCs or other innate cells, including macrophages, and stimulate maturation of immature DCs into DC1 or DC2, which direct the differentiation of Th1 and Th2 cells, respectively. Other pathogen-derived molecules can activate maturation of immature DCs into DCs that drive the differentiation of regulatory T cells, which suppress Th1, and in some cases, Th2 responses, by the release of anti-inflammatory cytokines or contact-dependent mechanisms, acting directly on the T cell or the APC. Figure taken from original of McGuirk and Mills, TRENDS in Immunology, Vol. 23, No. 9, September 2002.

Several different cytokines can influence the development of naïve Th cells into either Th1 or Th2 subtypes. Products of bacteria and viruses, such as LPS and bacterial DNA, activate cells of the innate immune system, including DCs and macrophages, to produce IL-12 or IL-18 and drive the differentiation of naïve precursor Th cells towards a Th1 subtype. Th1 cells secrete IFN-$\gamma$, TNF-$\beta$ and IL-2 and function in cell-mediated immunity, such as delayed type hypersensitivity, macrophage activation and inflammatory responses, for the protection against intracellular bacteria and viruses (Fujihashi *et al.*, 2000, Kidd, 2003, McNeela and Mills, 2001). However, Th1 cells can also provide helper function for specific IgG subclasses, particularly those involved in opsonisation and virus-neutralising IgG2a antibodies. Naïve Th cells are differentiated into Th2 cells by DCs matured in the presence of products from pathogens and allergens under the influence of IL-4. Induction of Th cells into Th2 cells is considered the default pathway, and in the absence of microbial products that stimulate the production of IL-12, antigens that lack immunomodulatory activity will induce Th2 cells. Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13 and provide effective help for B cell responses, in particular for IgG1, IgE and IgA synthesis (Fujihashi *et al.*, 2000, McNeela and Mills, 2001). With regard to IgA synthesis, IL-5 and IL-6 are of particular importance for inducing secretory IgA $(S\text{-}IgA)^+$ B cells to differentiate into IgA producing plasma cells (Fujihashi *et al.*, 2000). The two $CD4^+$ Th cell subsets can antagonise each other's actions, either by blocking polarised maturation of the opposite cell type or by blocking its receptor functions. For example, IFN-$\gamma$ secreted by Th1 cells can block the proliferation of Th2 cells, and high concentrations of IL-4 or IL-6 can block the generation of Th1 cells from naïve Th cells. Once one subset becomes dominant it is difficult to switch to another (McNeela and Mills, 2001, Kidd, 2003, Storni *et al.*, 2005).

Protective immunity against infectious pathogens is dependent on the differential induction of these Th1 or Th2 responses. The Th1/Th2 framework predicts that Th1 cells mediate immunity to intracellular pathogens, whereas, Th2 cells mediate immunity to extracellular pathogens (reviewed in Kidd, 2003). In fact, it has been shown for several intracellular pathogens that development of Th1 responses is required for protective immunity, while pathology may develop if Th2 cytokines are induced (McNeela and Mills, 2001). However, despite its dominance in the literature, the Th1/Th2 hypothesis has major inconsistencies. First, human cytokine patterns rarely fall exclusively into Th1 or Th2 patterns. Second, a further subpopulation of $CD4^+$ Th cells, termed regulatory T (Tr) cells, with immunosuppressive functions and cytokine profiles distinct from either Th1 or Th2,

has been described (McGuirk and Mills, 2002). Furthermore, Th cells are not the only, or even the dominant, source of Th1 and Th2 cytokines and there is evidence to suggest that DCs and other APCs have central importance in immunity (Kidd, 2003).

### 1.8.2.2 Regulatory T cells

Tr cells are a subset of $CD4^+$ T cells that function to control immune responses. Tr cells have been defined as "immunosuppressive T cells with cytokine profiles distinct from either Th1 or Th2 T cells" (McGuirk and Mills, 2002). Different populations of Tr cells have been described, including thymically derived $CD4^+CD25^+$ Tr cells and Tr1 cells induced in the periphery through exposure to antigen (Thompson and Powrie, 2004). Thymically derived Tr cells secrete high levels of immunosuppressive cytokines and are known to play a role in tolerance to self-antigens and in the prevention of autoimmune diseases. These Tr cells secrete IL-10 and/or TGF-$\beta$ but no IL-4, IL-5 or IL-2 and are poorly proliferative after stimulation *in vitro*. They have potent suppressive activity on normal responder T cell function by direct cell-to-cell contact or through the secretion of IL-10 or TGF-$\beta$ (Chen and Wahl, 2003, Jonuleit *et al.*, 2001). IL-10 producing Tr cells can act directly on activated Th1 cells and inhibit their antigen-specific proliferation and cytokine production in a cell contact dependent manner. The suppressive activity of these Tr cells is antigen non-specific and can be partially inhibited by the addition of exogenous IL-2 (Jonuleit *et al.*, 2001). In addition to naturally occurring Tr cells, recent studies suggest that IL-10 and TGF-$\beta$ secreting Tr1 cells can be induced against bacterial, viral and parasite antigens (McGuirk and Mills, 2002). Tr1 cells inhibit Th1 and Th2 responses via an IL-10 dependent pathway (Thompson and Powrie, 2004). Recently, pathogen derived molecules have been identified that stimulate IL-10 production from DCs by a TLR4 dependent mechanism, which in turn facilitates Tr1 cell development (Higgins *et al.*, 2003). This arm of the immune response might favour pathogen persistence and may have evolved as a host mechanism to limit pathology associated with microbial infection at mucosal sites.

The induction of Tr cells may not be desirable in immunisation, especially where Th1 or Th2 cells are known to mediate protection. However, Tr cells have considerable potential in the treatment of autoimmune and inflammatory diseases. Specifically, the ability to induce antigen-specific memory T cells that produce suppressive cytokines *in vivo* has opened up the possibility of developing vaccines against autoimmune diseases and

therapies to control graft rejection and graft versus host disease, Th1 mediated inflammation and allergy (Jonuleit *et al.*, 2001, Lavelle *et al.*, 2003).

### 1.8.3 Dendritic cells

DCs have been described as "natures adjuvant" because of their unique capacity to turn on naïve T cells (Audibert, 2003). DCs are critical in the function of the immune system, as they are the primary APC in the initiation of T cell responses **(Figure 1.9)**. For this reason, DCs are considered to be a major link between the innate and adaptive immune responses. DCs originate from the bone marrow, and their precursors migrate via the blood stream to almost every organ, where they reside in an immature state. DCs pass through several stages of maturation during their lifespan. Initially, immature DCs are characterised by a high rate of antigen uptake and low MHC class II and co-stimulatory molecule expression (Jonuleit *et al.*, 2001). Upon stimulation with bacterial products, cytokines, or CD40 ligation, DCs mature into potent APCs and migrate to the draining lymph nodes where they stimulate naïve T and B cells of the adaptive immune system. This maturation results in an increase in the expression of MHC molecules and co-stimulatory function, and a reciprocal decrease in the capacity to process protein antigens. In particular, DCs have been shown to up-regulate their expression of B7.1 and B7.2 molecules, which provide a co-stimulatory signal upon ligation to CD28 or CTLA-4 on T cells (McBride *et al.*, 2002, De Smedt *et al.*, 1997).

These mature DCs, now expressing high levels of co-stimulatory and MHC molecules, exit inflamed tissues and migrate to draining lymph nodes. Here they initiate the primary adaptive immune response by priming naïve T cells, thus determining their polarisation towards a Th1, Th2 or Tr subtype (Brossart *et al.*, 2000, Gagliardi *et al.*, 2000). It is generally accepted that by the time DCs arrive in the lymph nodes, they have lost the ability to capture new antigen. Impressively, a single mature DC is able to activate approximately 100 to 1000 T cells (Storni *et al.*, 2005). Furthermore, DCs can achieve cross priming, a phenomenon that consists of the capacity to present exogenous antigens on MHC class I molecules. Thus, DCs are the most potent APCs and are clearly central to the regulation, maturation and maintenance of a cellular immune response to antigens requiring cell-mediated immunity (Audibert, 2003, Storni *et al.*, 2005).

Functionally upstream to the polarisation of naïve T cells, the DCs themselves also become polarised **(Figure 1.9)**. DC polarisation is influenced by cytokines, chemokines and

eicosanoid prostaglandins such as PGE2. Naïve DCs exposed to high levels of IFN-γ can become DC1s, produce IL-12, and drive Th1 polarisation. Those exposed to relatively high levels of PGE2 are inhibited from producing high levels of IL-12 and polarise to become DC2, which drives Th2 differentiation. Those exposed to high IL-10 as well as PGE2 most likely become IL-12 inhibited and are also unable to stimulate naïve T cells. These may effectively be a DC3 subset that allows the emergence of immunosuppressive Tr populations (Kidd, 2003, McGuirk and Mills, 2002).

## 1.9 ROLE OF CYTOKINES IN THE IMMUNE RESPONSE

### 1.9.1 Brief introduction

Cytokines are proteins or peptides, some of which have sugar molecules attached. They are a large group of molecules and include the interferons, interleukins and various colony stimulating factors. They also include the TNFs and TGFs (Kidd, 2003). These soluble cytokines regulate many of the molecular and cellular events during an immune response, including the selective induction and differentiation of the different Th cell subsets (McNeela and Mills, 2001). Thus, the pattern and interaction of cytokines induced following infection or immunisation is critical in determining the type of Th response and therefore the outcome of infection or immunisation. Two pivotal cytokines that control Th1 and Th2 differentiation are IL-12 and IL-4, respectively. These two cytokines induce the generation of their own Th subset and simultaneously inhibit the development of the opposing subset (Kidd, 2003).

### 1.9.2 IL-12, IFN-γ and IL-4

IL-12, a 70 kDa heterodimeric cytokine composed of a p35 and a p40 chain, is important in mounting Th1 type immune responses. IL-12 can be produced by monocytes, macrophages and DCs in response to intracellular pathogens, bacteria and bacterial products, such as cell wall antigens or other smaller fragments of microorganisms. Ligation of CD40 and MHC class II molecules independently trigger IL-12 production by DCs. As IL-12 builds in concentration it begins to influence naïve T cells to become Th1 cells. Natural killer (NK) cells also respond to IL-12 and begin to release IFN-γ, which reinforces the APCs production of IL-12 and also helps drive the naïve T cell commitment process. As they attain maturity, Th1 cells also produce IFN-γ, which, together with the NK cells, stimulates the APC and naïve T cells to polarise into more Th1 cells, in a self-reinforcing "autocine" loop. Furthermore, IFN-γ secreted by Th1 cells can block the proliferation of Th2 cells (Kidd, 2003). IL-18 also modulates Th1 development. Although IL-18 alone cannot induce

Th1 cell differentiation, it strongly augments IL-12-dependent Th1 cell development, probably due to IL-18-induced up-regulation of IL-12R$\beta$2 chain expression on T cells and activation of the IFN-$\gamma$ promoter (Neurath *et al.*, 2002). The pro-inflammatory effects of IFN-$\gamma$ acting at sites of tissue inflammation are well established. The presence of IFN-$\gamma$ results in the activation of macrophages to release TNF-$\alpha$ and IL-1, and the up-regulation of pro-inflammatory chemokines to maintain inflammation. Tissue destruction in autoimmune diseases is associated with the presence of the pro-inflammatory cytokines IFN-$\gamma$, TNF-$\alpha$ and IL-1 (Hill and Sarvetnick, 2002). IFN-$\gamma$ has been implicated in the induction of the polymeric immunoglobulin receptor required for transport of S-IgA (McNeela and Mills, 2001).

$CD4^+$ T cells activated in the presence of IL-4, especially when IL-6 is also present, tend to differentiate into Th2 cells. Although several types of lymphocytes can secrete IL-4, the source of IL-4 that initiates the primary Th2 response is not yet entirely clear. Mast cells are potent producers of IL-4 after stimulation, and have been found to migrate to peripheral lymphoid organs, making them a possible candidate for an early source of IL-4 (Janeway *et al.*, Immunobiology, $6^{th}$ edition, 2004). IL-13 also appears to play an important role in Th2 development. While its function partially overlaps with IL-4, IL-13 can drive Th2 development and IgE synthesis in an IL-4 independent fashion.

### 1.9.3 IL-10 and TGF-$\beta$

High levels of immunosuppressive cytokines IL-10 and TGF-$\beta$ induce suppressor or regulatory responses. IL-10 is an immunosuppressive and anti-inflammatory cytokine that plays a major role in Th cell regulation by down-regulation of Th1 responses. The biological functions of IL-10 are mediated by its heterodimeric cell surface receptor, which activates a series of intracellular molecules, including STAT proteins (Corinti *et al.*, 2001). There is increasing evidence that IL-10 is a natural suppressor of inflammatory responses and can limit immunopathological damage due to excessive stimulation. Specifically, IL-10 deficient mice mount an exaggerated contact-hypersensitivity response, leading to irreversible tissue damage, whereas similar treatment of wild-type mice results in resolution of inflammation (De Smedt *et al.*, 1997). It has been demonstrated *in vitro* that IL-10 can inhibit the cytokine production and antigen-presenting function of DCs, monocytes and macrophages. Specifically, IL-10 inhibits IL-12 and TNF-$\alpha$ production from DCs generated from peripheral blood monocytes using granulocyte-macrophage-CSF and IL-4, and also down-regulates their capacity to active Th1 cells *in vivo* (Brossart *et al.*,

2000, De Smedt *et al.*, 1997, McBride *et al.*, 2002). Additionally, analysis of supernatants from IL-10 treated human DC cultures demonstrates an inhibited production of inflammatory cytokines such as IL-1$\beta$, IL-6 and TNF-$\alpha$, and a lack of IL-12 synthesis (Brossart *et al.*, 2000, Jonuleit *et al.*, 2001). Furthermore, addition of IL-10 to DCs during LPS activation has been shown to inhibit TNF-$\alpha$ and IL-12p70 production and to reduce IL-6 and IL-1$\beta$ production (McBride *et al.*, 2002). Changes in DC cell surface molecules, rather than inhibition of cytokine production, are thought to be the major mechanisms of IL-10 modulation of DCs, and therefore T cell function (McBride *et al.*, 2002). In particular, IL-10 has been shown to reduce DC expression of MHC class II molecules and several co-stimulatory and adhesion molecules and, in the human system, the DC-specific marker CD83. On exposure to IL-10, immature DCs show high levels of tyrosine phosphorylated STAT-3 and low STAT-1. In contrast, mature DCs lose sensitivity to the effects of IL-10, and display high basal phosphorylated STAT-3 and some STAT-1, that are only minimally increased by IL-10. This suggests an impaired early signal transduction defect to IL-10 in mature DCs (Corinti *et al*, 2001).

TGF-$\beta$ mediates its effects on cells through a heterodimeric complex consisting of type I (RI) and type II (RII) receptor components. These components are serine-threonine kinases that phosphorylate downstream signaling proteins once TGF-$\beta$ binds to TGF-$\beta$ RII, which in turn recruits and phosphorylates TGF-$\beta$ RI. The receptor complex then propagates the signal through the phosphorylation of cytosolic proteins, including the Smad proteins, Smad2 and Smad3. Once activated, Smad2 and Smad3 form a hetero-oligomeric complex and translocate to the nucleus where they modulate transcription of specific genes (Chen and Wahl, 2003).

TGF-$\beta$ affects a wide array of immune processes including T cell proliferation, T cell apoptosis, APC function and T cell differentiation. If present during primary stimulation of naïve $CD4^+$ T cells, TGF-$\beta$ can inhibit differentiation and expansion into either Th1 or Th2 effector cells by inhibiting key transcription factors required for polarisation. This results in T cells with severely impaired secondary responses, even if TGF-$\beta$ is not present during secondary stimulation (Lúdvíksson *et al.*, 2000). However, TGF-$\beta$ can also support expansion of differentiated $CD4^+$ T cells and can sometimes promote Th2 responses, perhaps by inhibition of Th1 responses (Hill and Sarvetnick, 2002). Some $CD4^+CD25^+$ Tr cells express surface membrane-bound TGF-$\beta$, although there are conflicting data on its functional role. Not only do $CD4^+CD25^+$ Tr cells express surface-bound latent TGF-$\beta$, but

these cells also bear TGF-$\beta$ in its active configuration on the cell surface. Recent studies suggest that autocrine production of TGF-$\beta$ by Tr cells is essential for their function (Chen and Wahl, 2003, Thompson and Powrie, 2004).

IL-10 and TGF-$\beta$ potently suppress inflammation *in vivo* and the generation of antigen-specific T cells that release IL-10 at the site of inflammation is promising for the treatment of autoimmunity and inflammatory disorders. It has been suggested that Tr cells that secrete high levels of IL-10 or TGF-$\beta$ may be prominent in the gut and in other mucosal tissues, where they play key roles in the maintenance of self-tolerance and the prevention of autoimmune diseases (McGuirk *et al.*, 2002).

## 1.10 IMMUNOLOGICAL MEMORY

The acquired immune response usually develops after infection is established and while it can help prevent infiltration of pathogens and assist in their elimination, it is also effective at preventing re-infection through the development of immunological memory. Immunological memory is the capacity of the immune system to respond more rapidly and effectively to previously encountered pathogens, and reflects the pre-existence of clonally expanded antigen-specific memory B and T cells. In immune individuals, secondary and subsequent responses are mediated solely by memory lymphocytes and not by naïve lymphocytes (Janeway *et al.*, Immunobiology, 6$^{th}$ edition, 2004). Protective immunological memory can persist for many years after the initial encounter with antigen and protects us from pathogens encountered on a daily basis. However, the most important and effective mechanism for maintaining immunity is periodic re-exposure to the pathogen. Such re-infections are usually asymptomatic or produce only mild symptoms and act as a natural booster to the immune system (Gourley *et al.*, 2004). It is this rapid and specific immunological memory response upon recall that is the basis of successful immunisation.

There are two arms of immunological memory, humoral immunity that includes pre-existing antibody, memory B cells and plasma cells, and cellular immunity that includes memory $CD4^+$ and $CD8^+$ T cells. Following the establishment of a B cell clone and clonal amplification of plasma cells producing specific antibodies, some of the stimulated B cells differentiate into antibody producing plasma cells. Others migrate into lymphoid tissues to form germinal centers, where B cells undergo hypermutation and affinity maturation. This differentiation process starts late in the primary immune response. During this process, the specificity of binding between antigen and antibody is refined through a process of

selection (Kurtz, 2004). Memory B cells divide slowly, if at all, and do not secrete antibody. However, upon re-encountering the same pathogen, the memory B cells start dividing at a high rate and differentiate into plasma cells producing specific antibody. Memory responses differ qualitatively from primary responses and the characteristics of antibodies produced in secondary and subsequent responses are distinct from those produced in the primary response to the same antigen (Storni *et al.*, 2005).

$CD4^+$ T cells can also differentiate into memory cells, which provide stronger stimulation to B cells than do naïve T helper cells. CTL memory leads to faster and more intense secondary CTL responses, which is crucial for protection against some viruses (Kurtz, 2004). After immunisation, the number of T cells reactive to a given antigen increases markedly as effector T cells are produced, and then falls back to persist at a level significantly above the initial frequency (Storni *et al.*, 2005). Two types of memory T cells have been identified. Central memory T cells, which display a lymph node-homing phenotype, are long-lived in the absence of antigen and are able to face systemic pathogenic infections. Effector memory T cells, which home to peripheral tissues, may be dependent on the presence of antigen, and produce IFN-$\gamma$ and perforin to protect against peripheral infection, and are relatively short-lived. Effector memory T cells probably develop from effector T cells (Storni *et al.*, 2005).

It is not yet clear whether memory cells stem from a common pool of precursor cells, or whether there is a subpopulation of memory cell precursors, which are separate from effector cell precursors, prior to contact with antigen (Kurtz, 2004). It is also unclear whether there is a need for memory cells to be periodically re-stimulated with antigen to maintain long-term memory. Such stimulation could occur through residual deposits of specific antigen, such as immune complexes retained for extended periods of time on follicular DCs, or through cross-reactive contact with environmental antigens. However, it has been shown that memory T cells can persist without a T cell receptor or in the absence of any MHC class II molecules, both of which would preclude antigenic stimulation. This "static" view of memory cells may not be correct, since there is also evidence for a rapid turnover of memory T cells (Kurtz, 2004, Stockinger *et al.*, 2004).

## 1.11 IMMUNISATION

### 1.11.1 Introduction

The principal goal of immunisation is the establishment of long-term, pathogen-specific, adaptive immunity. Transfer of immunity by antiserum or purified antibodies provides immediate protection against many pathogens and toxins produced by pathogens. This protection is temporary, lasting only as long as the transferred antibodies remain active in the recipient's body, and is therefore called passive immunity. Only active immunisation with antigen can provide lasting protection through the induction of immunological memory (Janeway *et al.*, Immunobiology, 6$^{th}$ edition, 2004).

Traditionally, vaccines have been designed using an empirical approach, based on killed or attenuated bacteria or viruses, with little knowledge of the protective antigens or immunological pathways leading to protection. Nevertheless, this approach was highly successful and killed and attenuated vaccines have eliminated or significantly reduced the incidence of many infectious diseases, including smallpox and poliomyelitis (Glück and Metcalfe, 2002, McNeela and Mills, 2001). Most newly developed vaccines are based on rationally selected target antigens and in some cases may be single molecules or even fragments thereof, derived from a pathogen, tumour cell, allergen or autoantigen (Moingeon, 2002). Recombinant DNA technology, genetic sequencing and tissue culture techniques have given rise to the development of modern vaccines, including recombinant protein, transgenic plant and DNA based formulations (Glück and Metcalfe, 2002).

Today, active vaccines against infectious diseases can be broadly classified into three general categories, namely, live vaccines, subunit or inactivated vaccines, and DNA vaccines. A live vaccine is an attenuated microorganism that can replicate in the host or infect cells in a fashion that elicits an immune response similar to that elicited by the natural infection. A subunit or inactivated vaccine is an immunogen that cannot replicate in the host and includes inactivated whole bacteria or viruses, native and recombinant proteins, fusion proteins and conjugate peptides (Ellis, 2001). DNA vaccines emerged from the observation that intramuscularly injected naked DNA resulted in the transfection of cells *in vivo* and expression of the encoded protein, leading to the induction of immune responses against that protein. DNA vaccines encoding antigens from a variety of viral, bacterial and parasitic pathogens have been found to elicit potent systemic immune responses and protection from infection in pre-clinical models (Ryan *et al.*, 2001). However, the early promise of DNA-based vaccines has not yet resulted in viable vaccines.

The arguments in favour of using DNA based vaccines are still strong and it may be that formulations will overcome the low response typically seen in man (Friede and Aguado, 2005). Viruses, including retroviruses, poxviruses and adenoviruses, have been used as immunisation vehicles to elicit antigen-specific immune responses. In such approaches, cDNA encoding an antigen is inserted into the viral vector. The resulting recombinant virus causes *in vivo* expression and subsequent presentation of the selected antigen to the immune system (Moingeon *et al.*, 2002).

The decision to develop a live, subunit or inactivated, or nucleic acid based vaccine should be based on knowledge of the pathogenesis, immunobiology and epidemiology of the given infection or disease, as well as technical feasibility.

### 1.11.2 Mucosal immunisation

The route by which an antigen is administered affects both the magnitude and type of immune response obtained. The commonest routes by which antigen is administered are subcutaneous injection, intramuscular injection, intravenous injection, and oral or nasal administration (Janeway *et al.*, Immunobiology, 6$^{th}$ edition, 2004) with almost all licensed vaccines given by intramuscular or subcutaneous injection (Ellis, 2001). However, mucosal routes of administration have attracted a lot of attention in recent years, both as a means of eliciting protective immunity against infectious diseases and as a possible approach to treat immunological disorders caused by aberrant immune responses associated with tissue-damaging inflammation.

The mucosal immune system is located in a variety of anatomical sites in which the external environment comes into contact with the host, including the gastrointestinal, oropharyngeal, genitourinary, respiratory and nasal mucosae (Freytag and Clements, 2005). The mucosal immune system is structurally and functionally divided up into sites for antigen uptake and processing at inductive sites, and effector sites engaging lymphocytes, granulocytes and mast cells. The key inductive sites for mucosal immunity are the nasopharyngeal-associated lymphoreticular tissue (NALT) and the gut-associated lymphoreticular tissue (GALT). Both possess APCs, T lymphocytes and IgA-committed B cells. Mucosal immunisation consists of either oral or nasal application of a vaccine formulation. In many cases nasal immunisation is more effective and requires smaller vaccine doses with less adjuvant. Most current protocols instill vaccine into each nostril and normal inhalation results in effective delivery to the NALT. The NALT is situated just

below the epithelial surface and contains aggregates of lymphoid follicles (B cell areas), inter-follicular areas (T cell areas), macrophages and DCs in a loose reticular network (Fujihashi *et al.*, 2002, Neurath *et al.*, 2002). The GALT is the largest lymphatic organ of the body (Tlaskalová-Hogenová *et al.*, 2004).

There are several important biological reasons for using a mucosal route of immunisation instead of a parenteral route. The first and most important is that the vast majority of pathogens infect via mucosal surfaces (Holmgren *et al.*, 2003). Traditional parenteral vaccine strategies do not prevent this initial interaction of the pathogen with the host mucosae, or in fact do not prevent infection itself, but rather resolve the infection before disease develops. The existence of a common mucosal immune system allows immunisation on one mucosal surface, for example the nasal mucosa, to induce secretion of antigen-specific antibodies at distant mucosal sites, for example the gastrointestinal mucosa. These mucosal antibodies, S-IgA in particular, can provide an early defense against invading pathogens by blocking attachment of bacteria and viruses and by neutralising bacterial toxins. Furthermore, mucosal immunisation has the advantage over conventional parenteral immunisation of stimulating both systemic and mucosal immune responses. While serum IgG may be required for prevention of systemic infection, it can also function at mucosal surfaces by transudation from the serum (Freytag and Clements, 2005, McNeela and Mills, 2001).

There are also important practical and logistical reasons for using mucosal immunisation. Most vaccines today are still delivered by injection, which carries with it the inherent risk of spread of infectious diseases due to contaminated syringes. Mucosal delivery systems, such as oral or nasal aerosols, would decrease this risk. Furthermore, a mucosal route of delivery would simplify the logistics of immunisation, resulting in increased stability and shelf life, lower cost, and the possibility of self-administration, therefore increasing compliance and vaccine coverage. The need for specially trained healthcare workers to administer vaccines would also be eliminated (Friede and Aguado, 2005, Freytag and Clements, 2005, Vajdy *et al.*, 2004).

In practice most mucosally administered vaccines are poorly immunogenic and are unable to induce protective immune responses. Therefore, mucosal vaccine strategies require the use of strong adjuvants. However, the major limiting factor for the development of mucosal vaccines is the availability of safe, effective adjuvants that function mucosally

(Freytag and Clements, 2005). Only a handful of vaccines currently approved for human used are administered mucosally, including the oral polio vaccine, oral killed whole-cell B subunit and live attenuated cholera vaccines, and an oral live attenuated typhoid vaccine (Holmgren *et al.*, 2003).

#### 1.11.2.1 Oral tolerance

A significant problem in mucosal immunisation is the risk of inducing peripheral systemic tolerance. This phenomenon, termed "oral tolerance", is characterised by the fact that animals which have been fed with or which have inhaled an antigen may become refractory or have diminished capability to develop an immune response when re-exposed to the same antigen introduced by parenteral injection (Holmgren *et al.*, 2003). This mechanism prevents the immune system from responding in a deleterious way to harmless antigens. Our mucosal surfaces are continuously exposed to a huge array of foreign antigens in the form of food, but these do not normally induce an adaptive immune response. In fact, oral tolerance to food antigens and to the commensal flora begins during the first few weeks of life (Tlaskalová-Hogenová *et al.*, 2004). This allows us to co-exist with our normal gut flora and to eat large amounts of foreign food proteins without inducing harmful systemic immune responses (Janeway *et al.*, Immunobiology, 6$^{th}$ edition, 2004, Holmgren *et al.*, 2003). It is thought two mechanisms are involved in the induction of oral tolerance: deletion and anergy associated with a high dose of antigen, and induction of active suppression mediated by Tr cells occurring with a low dose of antigen. The mechanism of suppression is caused by the induction immunosuppressive cytokines, mainly TGF-$\beta$ and IL-10, produced by the Tr cells (Tlaskalová-Hogenová *et al.*, 2004). Oral tolerance is specific for the ingested or inhaled antigen and does not influence the development of systemic immune responses against other antigens. Therefore, the induction of oral tolerance has become an attractive strategy for prevention and potential treatment of illnesses caused by immunopathological reactions against specific antigens encountered or expressed in preferentially non-mucosal tissues including both foreign antigens and auto-antigens (Holmgren *et al.*, 2003).

## 1.12 IMMUNE RESPONSES AND IMMUNISATION IN THE ELDERLY

*C. difficile* infection is most common in elderly individuals and therefore a vaccine against *C. difficile* would primarily be aimed at the elderly. In general, the elderly are more susceptible to infections due to age-associated decline of their immune function, termed immunesenescence (Castle, 2000). These age-related deficiencies in the immune system

appear to be related primarily to two phenomena: the atrophy of the thymus that occurs with age, restricting production of naïve T cells; and replicative senescence within the peripheral T cell pool (Lord *et al.*, 2001). Decreased *in vitro* T cell proliferation, delayed hypersensitivity reactions and decreases in naïve $CD4^+$ T cell function have all been documented with increasing age. Age-related decreases in $CD4^+$ T cell function, in particular, could potentially impact interactions with both $CD8^+$ T and B cells. This could result in diminished CTL activity and humoral responses, respectively, and ultimately result in reduced responses to both infections and vaccines (Eaton *et al.*, 2004). Production of IL-2, a key factor in cell-mediated responses declines with age in both humans and mice (Han and Meydani, 2000). A skewing of T cell responses during infection, towards a Th2 profile, resulting in lower production of IL-2 and IFN-$\gamma$, occurs in the elderly. Furthermore, there is a significant reduction in phagocytic ability in the elderly. Specifically, neutrophils from elderly donors can phagocytose bacteria, but with reduced efficiency. This would impact upon the efficiency of resolution of infections (Lord *et al.*, 2001). There is also a shift in the phenotype of circulating T cells with age, with a decrease in the number of naïve T cells and relative accumulation of memory T cells (Castle, 2000).

The increased incidence and severity of infectious diseases in elderly populations and a reduced ability to produce high affinity antibodies upon immunisation correlates with relatively weak and short-lived primary antibody responses in aged individuals. This can significantly affect the efficacy of vaccinations, which aim to produce high affinity neutralising antibodies (Eaton *et al.*, 2004). Response to vaccination, which requires intact cell mediated immunity to drive the humoral response, is diminished in many different elderly populations, as well as in laboratory animals (Castle, 2000, Lord *et al.*, 2001). With increasing age, the ability to produce protective antibodies in response to immunisation declines, resulting in reduced efficacy of vaccination (Eaton *et al.*, 2004). Successful immunisation of elderly individuals will have to overcome the decline in the immune system of aged individuals responsible for the poor immune responses on immunisation. Recently, immunisation of aged mice with protein antigens containing unmethylated immunostimulatory CpG motifs was shown to promote the successful development of immune responses that were qualitatively and quantitatively comparable to those induced in young animals immunised in a similar manner. New vaccines have been developed to induce a better immune response with fewer side effects for use in elderly individuals. For example, two phase I/II clinical trials of a new influenza vaccine developed using

recombinant IL-2 as the adjuvant show increased seroconversion when compared with current vaccines, both in young and elderly individuals (Tarazona *et al.*, 2002).

## 1.13 ADJUVANTS

### 1.13.1 Role of adjuvants in immunisation

New generation, rationally designed vaccines based on highly purified native proteins, recombinant proteins or naked DNA have considerably improved safety profiles, but are often poorly immunogenic (McNeela and Mills, 2001). This is because highly purified vaccine antigens are devoid of natural microbial components, once deemed irrelevant and potentially toxic, that are ligands for TLRs, which are required to elicit strong protective immune responses (Evans *et al.*, 2003). For example, diptheria-tetanus-pertussis vaccine contains two potent adjuvants from whole cell pertussis, LPS and pertussis toxin, whole cell typhoid and cholera vaccines contain LPS and CT, and bacille Calmette-Guérin (BCG) vaccine has strong non-specific immunostimulatory properties (Gupta and Siber, 1995). Therefore, highly purified vaccines require potent adjuvants to provide signals for the activation of innate immune cells, such as DCs and macrophages, which in turn activate specific subsets of antigen-specific T cells. Any material that helps the antigens or increases the humoral and/or cellular immune response is referred to as an adjuvant, which comes from the latin word, adjuvare, meaning "to help" (Gupta and Siber, 1995). Adjuvants encompass a highly heterogeneous group of substances capable of increasing or modulating humoral and/or cellular immune responses. Adjuvants may be used not only to augment the strength of the immune response, but also to modify the Th1/Th2 balance and induce anamestic responses (De Becker *et al.*, 2000).

There are four major ways in which adjuvants exert their activity. First, physical presentation of the antigen in the adjuvant:antigen formulation such that conformational epitopes are more effectively maintained and presented to appropriate effector immune cells. This maximises the amount of conformationally relevant (i.e. neutralising) antibody, influences the affinity of the antibodies and can also influence the duration of the immune response (Cox and Coulter, 1997). Second, targeting the delivery of the antigen to immune effector cells, generally via APCs. The most common way an adjuvant achieves this is to interact with antigen in such a way as to form multimolecular aggregates. These aggregates encourage uptake by macrophages and DCs and if an immunomodulatory adjuvant is present, will ensure that antigen and immunomodulator are delivered to the same APC. Such adjuvants are termed "particulate adjuvants". Third, generation of an antigen depot at

the site of injection, either short or long term, the latter giving either continuous or pulsed release of antigen. Depot formation is the major mechanism of adjuvanticity of mineral compounds, oil emulsions, liposomes and biodegradable polymer microspheres (Gupta and Siber, 1995). Fourth, immunomodulation, which refers to the ability of an adjuvant to modify the cytokine cascade, most commonly resulting in up-regulation of certain cytokines and down-regulation of others. These cytokines then act on helper lymphocytes to modulate immune responses. The response never swings totally in one direction or the other. The most notably skewed responses are produced by aluminium salts, which induce overwhelmingly Th2 type responses, and monophosphoryl lipid A (MPL), which induces predominantly Th1 type responses (Plotkin and Orenstein, Vaccines, 4$^{th}$ Edition, 2004).

The benefits of including a particular adjuvant in a vaccine formulation must be balanced against the potential risk of adverse reactions by inclusion of that adjuvant. Adjuvants should not induce local or systemic reactions, autoimmune diseases or hypersensitivity reactions. They should not be carcinogenic or teratogenic, and should be chemically defined allowing for consistent manufacture. Furthermore, adjuvants should elicit protective immune responses even with weak antigens, including polysaccharide-protein conjugates, with lower doses of antigens and with few injections. Adjuvants must be effective in infants and young children, ideally at birth, and elicit persistent high quality responses. Adjuvant should be stable with regard to adjuvanticity and toxicity and must be biodegradable and non-immunogenic by themselves (Gupta and Siber, 1995).

### 1.13.2 Freund's adjuvants

Freund's adjuvants (FA) are water-in-oil emulsions prepared with non-metabolisable oils in which the antigen is in aqueous phase droplets. A water-in-oil emulsion is an even dispersion of microdroplets of water in a continuous phase of oil. Such an emulsion can be formed with almost any antigen, regardless of its size, charge or other physical characteristics, provided it is water-soluble. A lot of energy is required for its production and the resulting emulsion becomes unstable unless an emulsifier is added. Freund's complete adjuvant (FCA) contains *Mycobacterium tuberculosis*, heat killed and dried, paraffin oil and the stabiliser mannide monooleate (Antibodies, A Laboratory Manual, Harlow and Lane, 1988). Freund's incomplete adjuvant (IFA) lacks the mycobacteria in FCA and therefore minimises the adverse side effects of FCA. For this reason IFA is used for booster injections and has also been used in human vaccine formulations (Petrovsky and Aguilar, 2004).

It is argued that antigens may become denatured during the emulsification process used to prepare water-in-oil emulsions (Kenney *et al.*, 1989) and therefore these adjuvants are probably less suitable for antigens where preservation of conformational epitopes is vital. After injection of the adjuvant antigen formulation, the antigen is continuously released from a depot formed at the injection site, and the mycobacterium in FCA attracts macrophages and other cells, enhancing uptake by these cells and thus enhancing the immune response. FAs are highly efficient for stimulating strong and prolonged immune responses (Antibodies, A Laboratory Manual, Harlow and Lane, 1988, Janeway *et al.*, Immunobiology, 6$^{th}$ edition, 2004).

CFA has been used widely for decades for producing antisera in animals. Although its toxicity was recognised immediately, a less toxic and equally effective alternative has not been found. CFA, and to a lesser extent IFA, induce local and systemic reactions. The injection site frequently develops hypersensitivity granulomas, which may ulcerate to form draining abcesses. Particles of the emulsion may spread through the body producing metastatic granulomas. This can be accompanied by wasting disease, adjuvant arthritis or other autoimmune conditions. It has been demonstrated that CFA can produce severe chronic pain and suffering in small animals (Bennett *et al.*, 1992, Leenars *et al.*, 1994). N-acetyl-muramyl-L-alanyl-D-isoglutamine (muramyldipepide, MDP) has been identified as a mycobacterial component with adjuvant activity in FCA. MDP itself is still too toxic, especially pyrogenic, for human use. Therefore, a number of derivatives of MDP have been investigated as non-toxic alternatives that retain MDPs adjuvant properties (Gupta and Siber, 1995, Petrovsky and Aguilar, 2004).

### 1.13.3 Ribi Adjuvant System

Ribi adjuvant system (RAS) is a preformed oil-in-water emulsion composed of monophosphoryl lipid A (MPL) and a synthetic trehalose dicorynomycolate (TDM), which simply requires mixing with aqueous antigen. RAS has been extensively investigated as a safe alternative to Freund's adjuvant, however these studies have given conflicting results with regard to adjuvanticity (Deeb *et al.*, 1992, Johnston *et al.*, 1991, Todd *et al.*, 1997). MPL is an immunostimulant, which has been used extensively as an adjuvant with vaccines administered parenterally. MPL is isolated from the LPS of *Salmonella minnesota* R595 and retains much of the immunostimulatory properties of the parent LPS without the inherent toxicity or pyrogenicity (Ismaili *et al.*, 2002). LPS, the first microbial component identified as a TLR agonist, stimulates the innate immune response by binding to the TLR4

complex, but is too toxic for clinical use as it is one of the main causes of septic shock in humans. The lipid A portion of LPS is responsible for both its adjuvant and toxic properties. Removal of the phosphate group from the reducing end sugar of the lipid A dissacharide reduces the toxicity of the molecule 100- to 1000-fold without appreciably affecting the adjuvanticity. Further removal of an ester-linked fatty acid group from the 3-position also reduces the pyrogenic properties, again without substantially affecting the adjuvant properties. The resulting 3-*O*-deacetylated MPL has proven to be a safe and effective vaccine adjuvant (Balbridge and Crane, 1999).

The immunostimulatory activity of MPL is mediated via binding to the TLR4 complex with subsequent activation of APCs and production of pro-inflammatory cytokines, in particular IL-12 by DCs and macrophages (Evans *et al.*, 2003, Ismaili *et al.*, 2002, McNeela and Mills, 2001). In common with LPS, MPL activates NF$\kappa$B, however, in contrast to LPS, MPL has been shown to induce rapid intracellular free calcium increases. It is currently thought that LPS and MPL activate different signaling pathways (Ismaili *et al.*, 2002). MPL has been shown to enhance the ability of macrophages and B cells to sensitise naïve T cells and confers to them the capacity to induce the development of both Th1 and Th2 cells (De Becker *et al.*, 2000). Presumably through the activation of these cells, vaccine antigens are more readily phagocytosed, processed and presented. These cells, also, release TNF-$\alpha$, IL-1 and GM-CSF in response to MPL. It is likely that these monokines lead to the recruitment and maturation of DCs in the lymph nodes, where DCs can efficiently present antigen to T cells. In addition, MPL either directly or indirectly stimulates the production of the Th1 cytokines IL-2 and IFN-$\gamma$. MPL has been shown to use TLR2 and TLR4 for the induction of TNF-$\alpha$, IL-10 and IL-12 by purified human monocytes, as well as by human peripheral blood mononuclear cells (Martin *et al.*, 2003). Additionally, stimulation of human monocytes by MPL leads to an up-regulation of surface expressed co-stimulatory molecules HLA-DR, CD80 and CD86 (Freytag and Clements, 2005, Ismaili *et al.*, 2002). MPL has been shown to effectively enhance both humoral and cellular immunity against antigens from hepatitis B virus, influenza virus and *C. tetani* when administered intranasally (Balbridge *et al.*, 2000).

### 1.13.4 Alumimium adjuvants

Aluminium adjuvants have been widely used in human vaccines since the 1930s, have an excellent safety record and are universally used with diphteria, tetanus, poliomyelitis and pertussis vaccines (Lindblad, 2004). Aluminium salts are insoluble, gel-like precipitates of

aluminium hydroxide ($Al(OH)_3$) or aluminium phosphate ($AlPO_4$), with a particle size ranging from 100 nm to 1000 nm. $Al(OH)_3$ and $AlPO_4$ have different physical characteristics to each other and also differ in their adjuvant properties (Gupta, 1998). $Al(OH)_3$ has been found to be a more potent adjuvant than $AlPO_4$ (Gupta and Siber, 1995). Two methods are used to prepare vaccines with aluminium adjuvants – in situ precipitation of aluminium compounds in the presence of the antigen, and adsorption of antigens onto preformed aluminium gels (Gupta, 1998). In the latter method, which is the most common, antigen is adsorbed to the gel by slow agitation at a pre-selected pH for a few hours or overnight, and is bound by electrostatic interaction. Adsorption of antigens on aluminium salts depends heavily on electrostatic forces between adjuvant and antigen, as well as other interactions including hydrophobic, van der Waals and hydrogen bonding. Other physical conditions affecting adsorption of antigens include pH, temperature, size of the gel particles, and ionic strength of the reaction mixture (Gupta, 1998). The amorphous nature of alumimium compounds contributes to their high surface area and high protein adsorption capacity, mainly for positively charged proteins.

Although aluminium adjuvants have immunostimulatory activities, such as attraction of eosinophils to the injection site and complement activation, adsorption of antigens to the gel is considered to be the key adjuvant activity. Adsorption of antigens to $Al(OH)_3$ increases the efficiency of APCs and up-regulates them as measured by IL-1 production. $Al(OH)_3$ activates complement, improves antigen trapping in lymph nodes and acts as a short-term (one to two weeks) depot for antigen release. Thus, the antigen is presented in a 'particulate' manner and the rate of antigen targeting is increased. Antigen adsorbed onto $Al(OH)_3$ is more readily taken up by human monocytes than free antigen. Alumimium adjuvants are known to induce strong Th2 responses, characterised by increased IL-4 production, but poor cell mediated responses. Strong IgE responses are also frequently observed. Aluminium adjuvants are inexpensive, safe and simple to formulate (Cox and Coulter, 1997, Lovgren-Bengtsson and Fossum, Methods in Microbiology, 2002, vol. 32).

### 1.13.5 Cholera toxin

#### 1.13.5.1 Structure and mode of action

CT is encoded by chromosomal genes in *Vibrio cholerae* and is an oligomeric protein toxin, which is secreted across the bacterial outer membrane into the extracellular environment (Spangler, 1992). CT is composed of two subunits, an enzymatically active A subunit with ADP-ribosyltransferase activity that is responsible for the toxicity, and a

pentameric B subunit that mediates binding of CT to its receptor on epithelial target cells. The A subunit is composed of a globular $A_1$ domain and an $A_2$ domain, the latter functioning primarily as an adaptor molecule by interacting with the B subunit (Williams *et al.*, 1999). The initial event in the pathogenesis of cholera is binding of the B subunit to the CT tissue receptor, GM1-ganglioside (Holmgren *et al.*, 1973, Lavelle *et al.*, 2004). GM1-ganglioside is a glycoshingolipid found ubiquitiously on all nucleated cells and is present in abundance on the luminal surface of intestinal epithelial cells (Marinaro *et al.*, 1995). Once inside the cells, CT is internalised into vesicles that are transported to the golgi. At this stage CT is still in the $A_2B_5$ form. Subsequently, the A and B subunits dissociate, and the A subunit is transported from the golgi to the endoplasmic reticulum, while the B subunit remains in the golgi and is later degraded. The A subunit is translocated to the cytosol, where it is activated and subsequently the $A_1$ fragment ADP-ribosylates the $\alpha$ subunit of a GTP binding protein, which regulates the activity of adenylate cyclase, and ultimately causes a sharp rise in intracellular levels of cAMP (McNeela and Mills, 2001, Pizza *et al.*, 2001, Spangler, 1992). The consequence of this, or other factors such as increased production of prostaglandins by the intoxicated cell, is excessive accumulation of electrolytes and water in the intestinal lumen (intestinal hypersecretion), clinically expressed as diarrhoea, and cell death. Neither the A subunit nor the binding B subunit are cytotoxic by themselves (Lönnroth *et al.*, 2003, Spangler, 1992).

### 1.13.5.2 Mechanism of CT adjuvanticity

Despite its pathophysiologic effects, CT is a powerful parenteral and mucosal adjuvant. Immunisation with antigen in the presence of CT via parenteral, musocal, peroral and transcutaneous routes result in substantial enhancement of mucosal IgA and serum IgG to the co-administered antigen (Lycke and Holmgren, 1986, McNeela and Mills, 2001). To induce immunity against a target antigen, CT must be administered by the same route and at the same time. The dose of CT required for adjuvant responses varies with different antigens (Marinaro *et al.*, 1995). The first critical step in the adjuvanticity of CT is thought to be binding of CT to the mucosal epithelial cells and subsequent increase in mucosal barrier permeability. This may allow CT and the co-delivered antigen to cross the musocal barrier and come into contact with cells of the immune system in the submucosa. Most studies indicate that CT induces strong $CD4^+$ Th2 responses to itself and to bystander antigens. This conclusion is based on T cell production of IL-4, IL-5, IL-6 and IL-10 with little IFN-$\gamma$, and higher titres of IgG1, rather than IgG2a, after immunisation with antigens in the presence of CT (Fujihashi *et al.*, 2002, Lavelle *et al.*, 2004). Moreover, this type of

response is markedly reduced in IL-4-knockout mice (Williams *et al.*, 1999). CT has also been shown to inhibit the production of IL-12p70 and the expression of the $\beta$1 and $\beta$2 chains of the IL-12 receptor, leading to the functional suppression of Th1 cell differentiation and a polarisation towards a Th2 type response (Braun *et al.*, 1999, Pizza *et al.*, 2001). Some studies have indicated mixed Th1/Th2 type responses to antigens administered with CT (Holmgren *et al.*, 2003, Lavelle *et al.*, 2004). CT has been shown to induce IL-1 by macrophages and up-regulate the expression of the co-stimulatory molecule B7-2 on murine macrophages and B cells (Gagliardi *et al.*, 2000, Kawamura *et al.*, 2003). CT has also been shown to up-regulate the expression of the co-stimulatory molecules B7-1, B7-2 and MHC class II molecules in human immature blood monocyte-derived DCs and to improve the ability of these cells to present antigen to memory T cells (Gagliardi *et al.*, 2002, Kawamura *et al.*, 2003). Recently, CT has been shown to interact with GM-1 gangliosides on DCs and induce nuclear translocation of NF$\kappa$B and terminal maturation of DCs in a GM-1 ganglioside-dependent fashion, potentially explaining the mechanism underlying the strong adjuvant activity of CT *in vivo*. Furthermore, CT lacks adjuvant activity in GM-1 ganglioside knockout mice (Kawamura *et al.*, 2003).

In addition to its powerful adjuvant activity, CT has also been investigated as an immunomodulator. Most immunisation studies with CT demonstrate that it enhances predominantly Th2 responses to co-administered antigen, these responses being characterised by production of IL-4, IL-5, IL-6 and IL-10 by $CD4^+$ T cells, and production of antigen-specific IgG1, IgA and IgE antibodies. However, anti-inflammatory effects of CT have also been documented, particularly the down-regulation of IL-12p70 and TNF-$\alpha$ production by DCs in response to LPS (Gagliardi *et al.*, 2000). Furthermore, CT can promote the induction of Tr cells to co-administered antigen by enhancing the production of IL-10 and inhibiting the activation of Th1-inducing DCs. Tr cells, generated by immunisation with antigen in the presence of CT have suppressor activity against Th1 cells *in vitro*. This may represent a mechanism evolved by *V. cholerae* to subvert protective Th1 responses *in vivo* (Lavelle *et al.*, 2002). The anti-inflammatory effects of CT and its ability to promote the induction of antigen-specific Tr cells highlights its potential as an immunomodulator for treatment of diseases mediated by dysregulated Th1 responses. (Lavelle *et al.*, 2004).

### 1.13.5.3 Separating CT toxicity from adjuvanticity

Clinical use of CT in humans is restricted due to its extreme toxicity and therefore attempts have been made to separate this toxicity from the adjuvanticity. Recently, site directed mutagenesis has allowed the generation of genetically detoxified derivatives of CT. Two CT mutants, one with a serine-to-phenylalanine substitution at position 61 of the A subunit (S61F), and a second with a glutamic acid-to-lysine substitution at position 112 of the A subunit (E112K), have been shown to lack both ADP ribosyltransferase activity and toxicity (Yamamoto *et al.*, 1997a, Yamamoto *et al.*, 1997b). S61F and E112K were found to be effective adjuvants and comparable to native CT when given parenterally or nasally. Furthermore, both the CT mutants and native CT resulted in the same Th2 type response against ovalbumin. Adjuvanticity of another CT mutant, which has a proline-to-serine substitution at position 106 of the A subunit (CTS106), was also found to have much reduced toxicity and excellent mucosal adjuvanticity (Pizza *et al.*, 2001). However, not all CT mutants retain their adjuvanticity and a case in point is the CTK63 mutant. This mutant has a serine-to-lysine substitution at position 63 of the A subunit, and although it has no detectable toxicity and maintains all other biological functions including receptor binding, CTK63 is ineffective as an adjuvant (Douce *et al.*, 1997, Pizza *et al.*, 2001). The possibility of nasal vaccines entering the CNS due to the neuronal connections of the olfactory epithelium/nerves with the olfactory bulbs has to be considered. The potential for neurotoxicity has major implications for the use of CT in humans. CT and the B subunit of CT have been shown to accumulate in the olfactory nerves/epithelium and olfactory bulbs of mice when given by the nasal route (Fujihashi *et al.*, 2002).

### 1.13.6 *E. coli* heat labile toxin

*E. coli* heat labile toxin (LT) is closely related both in structure and mode of action to CT. In common with CT, LT is composed of two subunits, an enzymatically active A subunit with ADP-ribosylase activity and a B subunit that mediates binding of LT to its receptor, GM1-ganglioside, on epithelial target cells (McNeela and Mills, 2001). LT mediates its adjuvant effects by increasing intestinal epithelial permeability, enhancing antigen presentation by a variety of cells and promoting isotype differentiation in B cells leading to increased IgA formation. LT also has complex stimulatory as well as inhibitory effects on T cell proliferation and cytokine production (Holmgren *et al.*, 2003). Despite the similarities in structure and mode of action, CT and LT elicit different responses to co-administered antigens. CT usually induces antigen-specific $CD4^+$ Th2 cells secreting IL-4, IL-5, IL-6 and IL-10, which provide helper signals for induction of antigen-specific S-IgA

as well as serum IgG1, IgA and IgE responses in mouse models. However, mucosal immunisation of mice with antigen and LT induces mixed $CD4^+$ Th1 and Th2 type responses, with subsequent mucosal S-IgA as well as serum IgG1, IgG2a and IgA responses (Freytag and Cements, 2005). Single amino acid substitutions have been introduced into LT in an attempt to separate toxicity from adjuvanticity. These have given rise to mutants such as LTK63, LTR72 and LTK7. LTK63 is non-toxic and has been shown to enhance Th1 and Th2 responses to nasally delivered co-administered pertussis antigens, whereas LTR72 is still partially toxic and has been shown to enhance Th2 responses at lower doses of adjuvant (McNeela and Mills, 2001).

### 1.13.7 Chitosan

Chitosan is a positively charged linear polysaccharide comprising copolymers of D-glucosamine and N-acetyl-D-glucosamine that is derived by the partial deacetylation of chitin. Chitin is found in the shells of crustaceans such as lobsters, prawns and crabs. Chitosan itself is found in some microorganisms and in yeast and fungi (Illum *et al.*, 2001). Compared with other natural polysaccharides, chitosan has a high biodegradability, low toxicity and is neither an irritant nor allergan in humans (Seferian and Martinez, 2001). Chitosan salts can bind strongly to negatively charged materials such as cell surfaces and mucus, thereby providing a longer contact time for drug transport across the nasal membrane before the formulation is cleared by the mucociliary clearance mechanism. Additionally, chitosan has been shown to decrease the transepithelial electrical resistance of Caco-2 monolayers and increase the paracellular transport of polar drugs by transiently opening the tight junctions between the epithelial cells. Chitosan is able to induce changes in F-actin distribution. Although F-actin is directly or indirectly associated with the proteins in the tight junctions, chitosan most probably allows for the paracellular transport of hydrophilic compounds by an indirect mechanism, whereby the integrity of the tight junctions is altered by changes in intracellular F-actin (Kotzé *et al.*, 1998a). Besides antigen uptake enhancement, chitosan has also been reported to have immune stimulating activity, such as increasing the accumulation and activation of macrophages and PMNs, promoting resistance to infections by microorganisms, inducing cytokines, and enhancing delayed type hypersensitivity and CTL responses. Activation of macrophages is initiated after uptake of chitosan by these cells. Production of IFN-$\gamma$ from macrophages has been observed following phagocytosis of chitosan particles (van der Lubben *et al.*, 2001a).

Chitosan is available in a range of molecular weights and degrees of deacetylation. A chitosan glutamate salt of a mean molecular weight of approximately 250 kDa and a degree of deacetylation of greater than 80% is commonly used for nasal delivery (Illum, 2003). Chitosan is insoluble in alkaline and neutral pH environments, but forms salts with inorganic and organic acids such as HCl, lactic acid, acetic acid and glutamic acid (Illum *et al.*, 2001). Chitosan derivatives, which are water soluble at neutral and basic pH values, have been described, and are especially useful for antigens that precipitate at acidic pH and for use in the alkaline environment of the large intestine and nasal cavity. In particular, derivatives with quaternary amino groups, such as N-trimethyl chitosan chloride are useful as absorption enhancers in neutral and alkaline environments, since they are water soluble and positively charged over a wide pH range (Kotzé *et al.*, 1998b).

## 1.14 RATIONALE AND OBJECTIVES OF THE STUDY

Immunisation strategies against *C. difficile* to date have focused on the toxins produced by this bacterium. However, this approach will not result in elimination of the carrier state and therefore is unlikely to halt the cycle of infection in hospitals and the community. Furthermore, there is the possibility of residual toxicity in vaccines based on inactivated toxins. A vaccine based on the SLPs is more likely to eliminate the organism and since the SLPs have no demonstrated toxicity, the likelihood of toxic side effects is greatly reduced. To be effective, a vaccine directed at the *C. difficile* SLPs must take account of the antigenic variation occurring in the protein. The Ribotype 1 strain chosen for this study accounts for the majority of *C. difficile* infections in the U.K. and Ireland **(Table 1.1)**, and thus a vaccine based on this strain would provide protection in the majority of cases.

The objectives of this study were to investigate the use of purified *C. difficile* SLPs as vaccine candidates to protect against CDAD in a hamster challenge model. Both passive and active immunisation regimens were investigated, which included both parenteral and mucosal vaccination strategies. Given the lack of information regarding the immune response to the *C. difficile* SLPs and the importance of this knowledge in rational vaccine design, the immune response to the SLPs was investigated in BALB/c mice. Following immunisation with the SLPs in combination with various vaccine adjuvants, serum and faecal IgG and IgA anti-SLP titres, as well as T cell responses, were analysed. Furthermore, the important role of DCs in directing this adaptive immune response was considered and the effect of the SLPs, independently and in combination with CT and Ribi adjuvants, on cytokine production from murine DCs was assessed *in vitro*.

# CHAPTER II

# MATERIALS AND METHODS

# CHAPTER II

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## 2.1 CHEMICALS

Bovine serum albumin (BSA), ammonium persulphate (APS), phorbol 12-myristate 13-acetate (PMA), β-mercpatoethanol, dimethylsulphoxide (DMSO), ampicillin, chloramphenicol, N,N,N',N'-tetramethlyenediamine (TEMED), Tris (hydroxymethyl) aminomethane, polyoxyethylenesorbitan monolaurate (Tween 20®), 30% $H_2O_2$, p-nitrophenyl phosphate tablets, nickel chloride, N-(2-Hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), urea, sodium thioglycolate, imidazole, arabinose, Luria-Bertani medium, agar (Select) and wide molecular weight range markers were from Sigma (Poole, Dorset, U.K. and St. Louis, MO, U.S.A.). Enhanced chemiluminescent (ECL) detection kit was from Amersham International (Aylesbury, U.K.). Complete® EDTA-free protease inhibitor cocktail was from Roche Diagnostics (East Sussex, U.K.). Ethanol, methanol, acetone, butanol, glycerol and acetic acid were from BDH Ltd. (Poole, U.K.). Electran 2.6 premix acrylamide: N,N'-methylenebisacrylamide (29.2:0.8) was from BDH, Poole, U.K. All buffer reagents for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were prepared in deionised water (Elga Prima Purelab Ultra). Electrophoresis grade agarose was from GIBCO BRL (Life Technologies, Paisley, Scotland). Recombinant enterokinase was from Merck Biosciences (Nottingham, U.K.). Polyethylene Glycol (MW 25000–40000) was from REATEC GmbH (purchased from Camida, Clonmel, Co. Tipperary, Ireland). CFSE (5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester) was from Molecular Probes (Eugene, OR, U.S.A.). Clindamycin HCl was from Duchefa (Haarlem, The Netherlands) and Sigma (Poole, Dorset, U.K. and St. Louis, MO, U.S.A). 1,2-phenylenediamine dihydrochloride (OPD, 2 HCl) tablets were from DakoCytomation (Copenhagen, Denmark). Streptavidin-HRP was from Kirkegaard and Perry Laboratories (Gaithersburg, MD, U.S.A.). Lipopolysaccharide (LPS) (*E. coli* serotype 127:B8) was from Alexis Biochemicals, U.K. Other reagents were analytical or general preparative grade and were from Sigma, Merck Biosciences or BDH.

## 2.2 ANTIBODIES

Goat anti-hamster IgG (H+L) HRP, goat anti-hamster IgG (H+L) alkaline phosphatase and goat anti-mouse IgG (H+L) alkaline phosphatase conjugates were from Southern Biotech (Birmingham, AL, U.S.A.). Biotinylated goat anti-mouse IgA was from Insight

Biotechnology Ltd. (Wembley, Middlesex, U.K.). Swine anti-rabbit IgG HRP and rabbit anti-human IgG HRP conjugates were from DakoCytomation (Copenhagen, Denmark). Human IgA was from Calbiochem (La Jolla, CA, U.S.A.). Mouse cytokine antibody pairs for the detection of IL-2, IL-4, IL-5, IL-10, IL-12p70, IFN-ϒ and TNF-α were from *e*Biosciences (San Diego, CA, U.S.A.) and R&D systems (Abingdon, U.K.). Mouse antibody pairs for the detection of TGFβ1 were from Promega (Madison, WI, U.S.A.) and R&D systems (Abingdon, U.K).

### 2.2.1 Rabbit anti-SLP antiserum

Polyclonal antibodies against combined high- and low-MW native *C. difficile* surface layer proteins (SLPs) and recombinant low-MW SLP were raised in rabbit using standard protocols. Briefly, 100 μg of each antigen preparation was emulsified in a 1:1 volume ratio with Freund's complete adjuvant (CFA) for the first inoculation and incomplete adjuvant (IFA) for two booster inoculations (days 14 and 28) and injected into two New Zealand white rabbits. Pre-immune and immune sera (day 38) were harvested from both rabbits and analysed by ELISA and Western blotting. Animal handling was carried out by staff at the BioResourse Unit, Trinity College Dublin.

## 2.3 ADJUVANTS

Imject® alum was from Pierce (Rockford, IL, U.S.A.). Cholera toxin (CT), from *Vibrio cholerae* Type Inaba 569B, was from Calbiochem (La Jolla, CA, U.S.A.). PROTASAN™ UP G 213 chitosan glutamate (molecular weight 150000-350000 g/mol, 75-90% deacetylated) was from Novamatrex (Drammen, Norway) and trimethylated chitosan (TMC) (molecular weight (g/mol) 244000, 21.9% quaternisation) was a gift from Dr. S.M. van der Merwe, Department of Pharmaceuticals, School of Pharmacy, Potchefstroom University for CHE, South Africa. Freund's complete and incomplete adjuvants and Ribi Adjuvant System (0.5 mg/ml monophosphoryl lipid A (MPL) and 0.5 mg/ml synthetic trehalose dicorynomycolate (TDM) emulsion) were from Sigma (Poole, Dorset, U.K. and St. Louis, MO, U.S.A).

## 2.4 *CLOSTRIDIUM DIFFICILE* CULTURE

### 2.4.1 *C. difficile* strains and growth conditions

Columbia blood agar, brain heart infusion (BHI) medium and cooked meat medium were from Oxoid (Basingstoke, Hampshire, U.K.). Fastidious anaerobe broth (FAB) was from LAB M (Bury, Lancs, U.K.). Difibrinated horse blood was from Biological Laboratories

Europe Ltd. *C. difficile* strains **(Table 2.1)** were isolated from patients in St. James's Hospital, Dublin, Ireland (Rachael Doyle, MD thesis, Trinity College Dublin 2004, Immune Response to *Clostridium difficile*-Associated Disease, call number TX-1-359) and typed by the Public Health Laboratory Service, Anaerobe Reference Unit, Public Health Laboratory, University Hospital of Wales. Strains were grown on Columbia blood agar plates (7% lysed horse blood) **(Figure 2.1)** supplemented with clindamycin HCl as a selective agent where appropriate [Appendix A]. Plates were incubated in an anaerobic atmosphere generated within an anaerobic jar by Gas Generating Kits (Oxoid, Basingstoke, Hampshire, U.K.) For liquid culture, starter cultures were grown overnight in pre-reduced fastidious anaerobe agar broth at 37°C. BHI medium (50 ml volumes) supplemented with 0.5% sodium thioglycolate [Appendix A] were inoculated with 2 ml of starter culture and grown under anaerobic conditions at 37°C to an $OD_{600}$ of approximately 1.

**TABLE 2.1 Profile of *C. difficile* clinical isolates used in this study**

| St. James's Hospital, routine microbiology laboratory ID number | Public Health Laboratory Service (PHLS) UK ID number | Ribotype | Toxin profile |
|---|---|---|---|
| 500 | R13537 | 1 | A and B |
| 536 | R12879 | 1 | A and B |
| 822 | R14637 | 1 | A and B |
| 324 | R12822 | 12 | A and B |
| 664 | R13711 | 31 | Neither |
| 862 | R13702 | 17 | B only |

### 2.4.2 *C. difficile* total lysate preparation

*C. difficile* from 3 day old Columbia blood agar plates were harvested into ice-cold 2X reducing sample buffer [Appendix A]. The resuspended material was lysed by sonication (5 x 60 sec pulses) in a Soniprep 150 Ultrasonic Disintegrator (SANYO, Leicester, U.K.), boiled at 100°C for 10 min and centrifuged at 14000 rpm (Eppendorf centrifuge 5417C, rotor F45-30-11) for 10 min at 4°C. The supernatant containing the lysate was analysed on 12 % SDS-PAGE gels and stored at -20°C.



**Fig. 2.1 *C. difficile* colonies following anaerobic culture on Columbia blood agar plate.** Typical colonies of *C. difficile* after 48 h anaerobic culture on a Columbia blood agar plate. Colonies are 2-3 mm in diameter, irregular, raised, opaque and grey/white after 48 h culture.

## 2.5 CELL CULTURE

### 2.5.1 Source of cell lines and cell culture reagents

THP-1, a human monocytic cell line derived from an acute monocytic leukaemia, was obtained from the European Collection of Animal Cell Cultures, ECACC (Porton Down, Salisbury, U.K.). RPMI-1640 medium, foetal calf serum (FCS) and Hank's balanced salt solution (HBSS) were obtained from GIBCO BRL (Life Technologies, Paisley, Scotland). Penicillin/streptomycin/L-glutamine prepared aliquots were purchased from Sigma (Poole, Dorset, U.K.).

### 2.5.2 Isolation of primary murine splenic cells

Primary spleen cells were isolated from mice by excising the spleen using aseptic technique and placing it in a sterile stainless steel mesh within a sterile Petri dish containing 5 ml of pre-warmed complete RPMI-1640 medium [Appendix A]. The spleen was mashed with a syringe plunger and the suspension drawn up through a 19-Gauge needle and expelled into a sterile container. Clumps were allowed to settle on ice for 10 min and the supernatant transferred to a clean sterile container and centrifuged for 10 min at 1000 rpm at room temperature. To lyse the red blood cells, the cell pellet was incubated with 5 ml of lysing buffer [Appendix A] for 5 min at room temperature with occasional shaking. 10 ml of pre-warmed complete RPMI-1640 medium was added and the suspension centrifuged at 1000 rpm for 10 min. Cells were washed twice more in the same manner. The final cell pellet was resuspended in 5 ml of complete medium for cell enumeration and seeding.

### 2.5.3 Isolation of primary bone marrow-derived murine dendritic cells

Bone marrow-derived immature dendritic cells (DCs) were prepared by Dr. Christine Loscher by culturing bone marrow cells obtained from the femurs and tibiae of BALB/c mice (10-14 weeks of age) in RPMI -1640 medium supplemented with 5% FCS and 10% supernatant from a GM-CSF-expressing cell line (J558-GM-CSF).

### 2.5.4 Maintenance of cell cultures

Cells were grown in complete RPMI-1640 medium [Appendix A] at 37°C in 5% $CO_2$. FCS was heat inactivated (56°C, 1 h) to inactivate complement and then aliquoted for storage at -20°C. Supplemented medium was stored at 4°C. Cells were routinely maintained in 75 $cm^2$ cell culture flasks (Nunclon, Roskilde, Denmark) at 37°C in 5% $CO_2$ in a humidified incubator. Visual examination was undertaken using phase contrast

microscopy. THP-1 cells were passaged upon reaching 80% confluence. For passage, the cell suspension was aspirated and the cells were washed twice with complete medium. Cells were resuspended in complete media for enumeration or seeding. Monocytic THP-1s ($1 \times 10^6$ cells per well in 24-well plates) were differentiated into macrophages with 100 nM PMA in complete medium for 72 h.

### 2.5.5 Cell freezing and revival

A cryopreservation solution was prepared containing 90% FCS and 10% DMSO [Appendix A]. THP-1 cells were harvested as for passage, the supernatant decanted and the cells resuspended in ice-cold cryopreservative medium at $2 \times 10^6$ cells/ml. Cell suspension aliquots (1 ml) were placed into sterile cryovials (Nunclon, Roskilde, Denmark) and the cryovials were then transferred to liquid nitrogen for long-term storage. For cell revival, cryovials removed from storage were quickly thawed in a 37°C water bath. Cells were washed with pre-warmed RPMI-1640 medium. Gentle centrifugation (1000 rpm, 3 min) was used to pellet the cells which were subsequently resuspended in 10 ml of RPMI supplemented with 20% FCS and transferred to 25 $cm^2$ tissue culture flasks and placed at 37°C, 5% $CO_2$ in a humidified incubator.

### 2.5.6 Cell enumeration and viability

To assess cell yield and viability 10 µl of a 0.4 % trypan blue solution (Sigma) was added to 90 µl of the cell suspension. Cells were counted on a Neubauer haemocytometer under a light microscope. Live cells appear white and non-viable cells appear blue. Enumerations were performed in duplicate. THP-1 cells, primary T-cells and primary DCs were seeded at a density of $2 \times 10^6$ cells/ml and used when less than 90% confluent.

### 2.5.7 Co-culture of cells with native *C. difficile* SLPs and other stimuli

Primary splenic cells from mice were adjusted to $2 \times 10^6$ cells/ml in complete medium and 100 µl added to wells of 96-well plates. Cells were stimulated with 20 µg/ml native *C. difficile* SLPs, 20 ng/ml PMA or culture media alone. Supernatants were collected after 24 h for IL-2 detection and after 72 h for IL-4, IL-5, IL-10, IFN-γ and TGF-β1 detection, and stored at -20°C until required. Duplicates were performed for the SLP treated cells.

Primary DCs were adjusted to $2 \times 10^6$ cells/ml in complete medium and 500 µl added to wells of 24-well plates. Cells were stimulated in triplicate with native *C. difficile* SLPs alone (1 µg/ml, 2 µg/ml, 10 µg/ml, 20 µg/ml and 40 µg/ml), with native *C. difficile* SLPs

(20 μg/ml) plus cholera toxin (1 μg/ml), with native SLPs (20 μg/ml) plus Ribi adjuvant (MPL and TDM emulsion) (5 μg/ml) or with CT (1 μg/ml) or Ribi (5 μg/ml) alone. LPS (100 ng/ml) was used as a positive control. Supernatants were collected after 24 h for IL-10, TGF-$\beta$, IFN-$\gamma$ and TNF-$\alpha$ detection, and stored at -20°C until required. For every experiment, control cells were incubated with culture medium alone. Representative results of three independent experiments are shown.

## 2.6 POLYACRYLAMIDE GEL ELECTROPHORESIS

### 2.6.1 Sample and molecular weight standard preparation

Following protein estimation, protein was resuspended in 5 μl to 20 μl of 2X reducing sample buffer [Appendix A] as required. Protein samples were boiled at 100°C for 10 min and centrifuged briefly (30 s) to remove any insoluble material. Sigma Broad Range molecular weight standards (5 μg) were run alongside samples.

### 2.6.2 SDS-PAGE

An ATTO system was used for all SDS-PAGE gels (ATTO Corporation, Japan), and protein samples were electrophoresed using a Consort electrophoresis power supply unit. Proteins were separated on reducing gels prepared using a discontinuous buffer system, as described by Laemmli (1970) and adapted by Sambrook and Gething (1989). Resolving and stacking acrylamide gels were prepared to the required percentage acrylamide in the order indicated in **Tables 2.2** and **2.3**. APS and TEMED were added last with gentle swirling of the mixture. Electrophoresis was carried out at 150 V, 25 mA per gel for approximately 1.5 h until the dye front had reached just above the gel base.

**TABLE 2.2 Composition of resolving gel for SDS-PAGE**

| Component | 10% | 12% |
|---|---|---|
| Acryl/Bis 30% | 6.7 ml | 8.0 ml |
| Tris 1.5M | 5.0 ml | 5.0 ml |
| $H_2O$ | 8.0 ml | 6.7 ml |
| SDS 10 % | 200 μl | 200 μl |
| APS 10% | 100 μl | 100 μl |
| TEMED | 10 μl | 10 μl |

**TABLE 2.3 Composition of stacking gel for SDS-PAGE**

| Component | Amount |
|---|---|
| Acryl/Bis 30% | 1.67 ml |
| Tris 1.0M | 1.25 ml |
| $H_2O$ | 6.98 ml |
| APS 10% | 50 µl |
| TEMED | 10 µl |

### 2.6.3 Western blotting

Western blotting was carried out using the semi-dry method for the transfer of electrophoresed proteins to immobilising membranes as described by Towbin *et al.* (1979) and was performed using an ATTO semi-dry transfer system (ATTO Medical Supplies, Japan). During SDS-PAGE, polyvinylidene fluoride (PVDF) transfer membrane (0.45 µm, Pall Life Sciences) was briefly saturated with methanol for 10-15 s and then equilibrated in transfer buffer [Appendix A] for approximately 20-30 min prior to semi-dry transfer. Whatmann 3 mm filter paper of the same dimensions was also saturated in transfer buffer prior to semi-dry blot sandwich assembly, which was assembled in the order cathode, filter paper, acrylamide gel, PVDF membrane, filter paper and anode. Electrophoretic transfer was performed at 100 mA per gel for 60 or 90 min as required. Following semi-dry transfer, the PVDF membrane was removed and processed for immunoblotting. The lane containing the molecular weight markers was stained with amido black [Appendix A] and de-stained in water.

### 2.6.4 Immunodetection and development

Following transfer, non-specific sites on the membrane were blocked with freshly prepared phosphate buffered saline (PBS)/5% dried skimmed milk/0.1% Tween 20® (blocking buffer) [Appendix A] for 1 h with gentle agitation at room temperature. Membranes were then washed three times with PBS/0.1% Tween 20® (washing buffer) [Appendix A] and incubated with appropriate primary antibodies, diluted as required in blocking buffer, for 2 h at room temperature or overnight at 4°C with gentle shaking in a sealed plastic bag. Following incubation with primary antibody, membranes were washed several times over 30 min with washing buffer. Membranes were then incubated with the relevant horseradish peroxidase-conjugated secondary antibody, diluted as required in blocking buffer, for 1 h

with shaking in a sealed plastic bag. Following incubation with secondary antibody, membranes were finally washed 4 to 5 times with washing buffer over 30 min with the final wash in PBS alone. Membranes were stored in PBS at 4°C if development could not be carried out immediately. Detection of immunoblots was performed using the enhanced chemiluminescence (ECL) method (Leong and Fox, 1990). Membranes were incubated for 1 min in a solution of iodophenol (400 μM), luminol (1.25 mM), and hydrogen peroxide (0.1% (v/v)) in 0.1 M Tris-HCl (pH 8.8). The membrane was removed after 1 min and placed between acetate sheets, which were then exposed to Kodak X-OMAT S film for the appropriate time period (range 10 s to 1 min). Exposed films were developed using an automatic developer (CURIX 60, AGFA, Type 9462/100/140, Agfa-Gevaert AG, Munich, Germany).

## 2.7 DNA MANIPULATION

### 2.7.1 Preparation of *C. difficile* genomic DNA

*C. difficile* from 3 day old BHI agar cultures was harvested into solution I [Appendix A] and the cells vortexed until fully resuspended. 500 μl of solution II [Appendix A] was added and mixed gently, followed by addition of 75 μl of 10% SDS. The mixture was then incubated in a 50°C water-bath for 10 min. 400 μl of 5 M sodium chloride and 100 μl of 3 M sodium acetate was added, mixed gently and the mixture incubated on ice for 30 min. Following centrifugation at 12000 rpm for 10 min the liquid fraction was harvested and the pellet discarded. The liquid fraction was split into 4 x 300 μl aliquots and 1.2 ml of 95% ethanol added to each, mixed well and the tubes placed at -80°C for 20 min. The tubes were then centrifuged at 12000 rpm for 5 min and the supernatant discarded. 100 μl of 70% ethanol was added to each pellet, without disturbing the pellet, and the tubes incubated at room temperature for 5 min. The ethanol was then gently pipetted off. The pellets were spun in an Eppendorf centrifuge at 12000 rpm for 10 sec and any remaining liquid was removed. The pellets were dried at room temperature for 15 min and 20 μl of sterile water added to each pellet. The DNA was stored at -20°C.

### 2.7.2 *Escherichia coli* DNA manipulation

Transformed *E. coli* colonies were analysed using a rapid procedure for screening of recombinant plasmids (Le Gouill and Déry, 1991). Briefly, single colonies were harvested with an automatic pipette by pressing the tips into the colonies, inoculating a plate for bacterial storage and recovery, and resuspending the remainder of the colonies in 16 μl of

lysis solution [Appendix A] by pipetting gently 4-5 times. Then, 3 µl of solution III [Appendix A] was deposited on the wall of the microcentrifuge tube. The samples were centrifuged for 4 min at 12000 rpm and the supernatants run on 1% agarose gels containing 0.5 µg/ml ethidium bromide [Appendix A]. Plasmids were purified using the GenElute™ Plasmid Miniprep Kit from Sigma (Poole, Dorset, U.K.). Restriction enzymes used were obtained from New England Biolabs (U.K.) or Promega (U.K.).

### 2.7.3 Preparation of competent *E. coli* cells for transformation

An overnight starter culture of the *E. coli* Rosetta™ host strain (a BL21 derivative) from Novagen (Madison, WI, U.S.A.) was grown in LB medium at 37°C. 2 ml of the starter culture was used to inoculate 200 ml of LB media and grown at 37°C with shaking at 200 rpm until mid-log phase was reached ($O.D._{600}$ 0.4-0.5). The culture was chilled on ice for 1 h and subsequently centrifuged at 3000 rpm at 4°C for 15 min. The resultant bacterial pellet was resuspended in 1/10 of the original volume of ice-cold 100 mM $MgCl_2$ [Appendix A], and the cells centrifuged as above. The resultant bacterial pellet was resuspended in 1/2 of the original volume of ice-cold 100 mM $CaCl_2$ [Appendix A], and incubated on ice for 1 h. The cells were centrifuged at 3000 rpm at 4°C for 15 min, and the final pellet resuspended in 20 ml of 100 mM $CaCl_2$/10 % glycerol. 0.5 ml aliquots were dispensed into cryovials, snap frozen in liquid nitrogen and stored at -80°C until use.

**TABLE 2.4 Genotypes of strains used for cloning and expression**

| Strain | Genotype |
|---|---|
| *E. coli* TOP 10 | F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74*rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str$^R$) *end*A1 *nup*G |
| *E. coli* Rosetta™ | F⁻ *ompT hsdS$_B$*($r_B^- m_B^-$) *gal dcm pRARE* (*arg*U, *ile*X, *gly*T, *leu*W, *pro*L) |
| *E. coli* DH5α | F′ Φ80d*lacZ*ΔM15 *recA1 endA1 gyrA26 thi-1 hsdR17 supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169* |

### 2.7.4 DNA amplification by polymerase chain reaction

Polymerase Chain Reactions (PCR) were performed in a Perkin-Elmer 2400 thermal cycler (Perkin-Elmer, U.K.) with Taq DNA Polymerase (Boerhinger Mannheim, Germany). Primers were from Qiagen (Cologne, Germany) or Sigma-Genosys (Haverhill, U.K.). For standard amplifications, in each reaction approximately 100 ng of chromosomal DNA was mixed with 1 μl of each specific primer (at 10 μM) in a reaction volume of 50 μl. Reactions were performed by denaturing DNA at 94°C for 1 min, annealing at 37°C for 30 s and extension at 68°C for 1.5 min. A total of 30 cycles was performed.

## 2.8 PRODUCTION OF SURFACE LAYER PROTEINS OF *C. DIFFICILE*

### 2.8.1 Recombinant Protein Production

#### 2.8.1.1 Cloning and expression of recombinant thioredoxin fusion low-MW SLP

The part of the *SlpA* gene coding for the low-MW SLP (from nucleotide number 73 to nucleotide number 1020) from a *C. difficile* Ribotype 1 strain (PHLS ID 13537), was amplified using the following PCR primers:

Forward, 500nf 5'- GATGATACAAAAGTTGAAACTGGTG- 3'
Reverse, 500nr 5'- TTAACTCTTAGTTGTAACTCTTTTTCCTTCTG- 3'

Under these conditions a single PCR product was obtained and was cloned into the pBAD/Thio-TOPO vector **(Figure 2.2)** and transformed into TOP10 *E. coli* cells **(Table 2.4)** (Invitrogen, The Netherlands). Colonies were analysed for the presence or absence of the recombinant plasmid using the method of Le Gouill and Déry (1991) for rapid screening of recombinant plasmids **(Section 2.7.2)**. The identity of the cloned insert was confirmed by sequencing using the dideoxynucleotide chain termination method (Department of Biochemistry, University of Cambridge, U.K.). Both strands of the construct were completely sequenced and matched that of the published sequence. Transformants of *E. coli* Rosetta™ strain **(Table 2.4)** containing the recombinant plasmid with the amplified part of the *SlpA* gene, were grown at 37°C in LB broth supplemented with ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml). Overexpression of the fusion protein was induced by adding arabinose to a final concentration of 0.2% [Appendix A] for 6 h at 37°C. The low-MW SLP was expressed as a soluble thioredoxin fusion protein. The induced cells were harvested by centrifugation (5000 x g, 10 min, 4°C), washed once with 20 mM Tris HCl pH 8.5 [Appendix A] and lysed by sonication (6 x 30 s) in a Soniprep 150

Ultrasonic Disintegrator (SANYO, Leicester, U.K.). Sonicated material was centrifuged to remove debris (12000 x g, 25 min, 4°C) and filtered (0.45 μM) to clarify.

### 2.8.1.2 Purification of recombinant low-MW SLP

The clarified lysate was applied to an anion exchange column (MonoQ HR 5/5) attached to an AKTA***FPLC*** system (GE Healthcare, Bucks, U.K.). The recombinant low-MW SLP was eluted with a linear gradient of 0-0.3 M NaCl in 20 mM Tris HCl pH 8.5, [Appendix A] at a flow rate of 1ml/min. Peak fractions were pooled and dialysed against 20 mM Tris HCl pH 7.8, 500 mM NaCl, for nickel affinity chromatography. The dialysate was applied to a $Ni^{2+}$ charged 1 ml HiTrap column (GE Healthcare) and the recombinant low-MW SLP eluted with 50 mM imidazole at a flow rate of 1 ml/min. Peak fractions were dialysed against recombinant enterokinase (rEK) cleavage buffer [Appendix A] and the histidine thioredoxin patch cleaved with 1 U rEK (Novagen, Madison, WI, U.S.A.) per 50 μg recombinant fusion protein for 16 h at room temperature with gentle shaking. The rEK was removed by batchwise binding to Ekapture Agarose (100 μl of 50% agarose slurry per 2 U rEK) (Novagen, Madison, WI, U.S.A.). The cleaved recombinant low-MW SLP was dialysed into the nickel chromatography start buffer and passed through a final $Ni^{2+}$ charged HiTrap column. Peak fractions corresponding to the pure low-MW SLP were analysed on 12% SDS-PAGE gels stained with Coomassie blue. The N-terminal amino acid sequence of the recombinant protein was determined by Edman degradation. Briefly, 10 μg of recombinant protein was transferred to polyvinylidene difluoride (PVDF) membrane (ProBond™) and stained with Coomassie blue. Sequencing was then carried out by Mike Weldon, Department of Biochemistry, University of Cambridge.

### 2.8.1.3 Cloning of recombinant thioredoxin fusion high-MW SLP

The part of the *SlpA* gene coding for the high-MW SLP (from nucleotide number 1021 to nucleotide 2268) from a *C. difficile* Ribotype 1 strain (PHLS ID 13537) was amplified using the following PCR primers:

Forward, 500cf 5'- GCTGCAAAGGCTTCAATTGCTGATG- 3'
Reverse, 500cr4 5'- CAATTTTATTATACCTTAAAATTATAGAATAGTAGAC- 3'

Under these conditions a single PCR product was obtained and was cloned into the pBAD/Thio-TOPO vector **(Figure 2.2)** (Invitrogen, The Netherlands) and transformed into TOP10 *E. coli* cells (Invitrogen, The Netherlands). Colonies were analysed for the

presence or absence of the recombinant plasmid using the method of Le Gouill and Déry (1991) for rapid screening of recombinant plasmids **(Section 2.7.2)**. The identity of the cloned insert was confirmed by sequencing using the dideoxynucleotide chain termination method (Department of Biochemistry, University of Cambridge, U.K.). Both strands of the construct were completely sequenced and matched that of the published sequence.

### 2.8.2 Production of native *C. difficile* SLPs

#### 2.8.2.1 Crude S-layer preparation

Crude S-layers were produced essentially as described before (Cerquetti *et al.*, 2000). Briefly, *C. difficile* BHI cultures, grown to an $O.D._{600}$ of 1, were harvested at 6000 rpm for 10 min at 4°C and the supernatant discarded. The cell pellet was washed once with ice-cold 50 mM Tris HCl pH 7.4 [Appendix A] and centrifuged at 6000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in room temperature 8 M urea/50 mM Tris HCl pH 8.3 [Appendix A], supplemented with Complete® protease inhibitor cocktail, and incubated at 37°C for 1 h. The mixture was centrifuged at 12000 rpm at 4°C for 30 min. The supernatant, containing the SLPs, was dialysed overnight against 20 mM Tris HCl pH 8.5 with three changes of buffer. The SLP preparation was concentrated by placing it in dialysis tubing (BioDesignDialysis Tubing, MW cut-off 8000, BioDesign Inc., Carmel, New York, U.S.A.) in a dish with polyethylene glycol (MW 25000-40000) until the volume was reduced four to five-fold. The concentrated SLP preparation was stored at -20°C until required.

#### 2.8.2.2 Refined SLP preparation

The crude SLPs were further purified by anion exchange chromatography. Briefly, the crude SLP preparation was applied to a MonoQ HR 10/10 anion exchange column attached to an AKTA***FPLC*** system (GE Healthcare). The pure SLPs were eluted with a linear gradient of 0-0.3 M NaCl in 20 mM Tris HCl pH 8.5 at a flow rate of 4 ml/min. Peak fractions corresponding to pure SLPs were analysed on 12% SDS-PAGE gels stained with Coomassie blue.

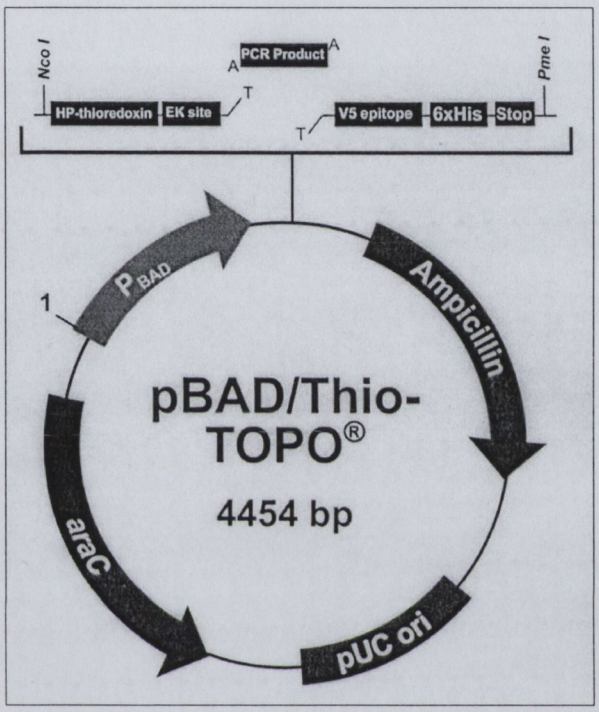



**Fig. 2.2 Features of the pBAD/Thio-TOPO vector.** Reproduced from pBAD/TOPO® ThioFusion™ Expression System instruction manual (Version E, 010302, 25-0257), Invitrogen, The Netherlands.

### 2.8.2.3 Endotoxin testing of native SLPs

The amount of endotoxin present in the native SLP preparation was determined using the chromogenic Limulus Amoebocyte Lysate (LAL) quantitative QCL-1000 assay (Cambrex Bio Science, Berkshire, U.K.) according to the manufacturers' instructions. Briefly, a 96-well plate was equilibrated to 37°C in a heating block and 50 µl of protein sample or endotoxin standard pipetted into appropriate wells. 50 µl of LAL was added to each well and the plate incubated at 37°C for 10 min. 100 µl of substrate solution was then added and the plate incubated for 6 min and the reaction was stopped with 10% SDS. The absorbance was read at 405 nm on a Wallac Victor2 1420 microplate reader (Perkin-Elmer Lifesciences) and the amount of endotoxin present in the samples determined from the standard curve. Assays were run in duplicate and included a reagent diluent only well. Endotoxin testing was carried out on two representative preparations of SLPs and subsequent SLP preparations were carried out under the same conditions.

### 2.8.3 Determination of protein concentration

The concentration of protein preparations was determined by the method of Bradford (1976) using Bio-Rad Protein Assay Concentrate (Bio-Rad, U.K.). Assay concentrate was freshly diluted 1:5 with distilled water and mixed well prior to each assay. A BSA standard curve was prepared in the appropriate buffer from a 20 mg/ml BSA stock solution [Appendix A] using serial dilutions to yield standards at 20, 10, 5, 2.5, 1.25, 0.625, 0.312, and 0.0625 µg/ml BSA. 5-10 µl of sample or standard was mixed with 1 ml of the diluted BioRad staining solution, vortexed and incubated for 5 min at room temperature. The absorbance of protein solutions were read at 595 nm in triplicate in a Tecan Spectra Fluor Plus spectrophotometer. A specifically designed Excel macro averaged readings, constructed a standard curve and calculated the individual protein concentrations with reference to the standard curve automatically. A commercial BSA solution at a concentration of 2 mg/ml (BSA Protein Standard, Sigma, Poole, U.K.) was used to monitor accuracy and reproducibility of the Bradford method.

### 2.8.4 Gel filtration chromatography

Protein samples were dialysed into a 20 mM TrisHCl, 0.15 M NaCl, pH 8.0 start buffer [Appendix A] and generally concentrated to a volume of 250 µl using an Amicon Centricon concentrator (Millipore, Bedford, M.A., U.S.A.) with a MW cut-off of 10000. 200 µl of protein samples were then applied to either a Superdex 75 or a Superdex 200 gel filtration column (GE Healthcare), which had been pre-equilibrated with the start buffer,

and the proteins eluted isocratically. The Superdex 75 column separates proteins in the range 3 kDa to 75 kDa and was calibrated with albumin (63.3 kDa), ovalbumin (42.7 kDa), chymotrypsinogen A (19.5 kDa) and ribonuclease A (15.5 kDa). The Superdex 200 column separates proteins in the range 10 kDa to 600 kDa and was calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), catalase (211 kDa), aldolase (163 kDa) and ovalbumin (42.7 kDa) (calibration standards were from GE Healthcare). A calibration curve for each column was constructed by measuring the elution volumes of these standards and calculating their corresponding $K_{av}$ values (an elution volume parameter) using the formula:

$$K_{av} = (V_R - V_O)/(V_C - V_O)$$

Where,

$V_R$ = elution volume of the protein
$V_O$ = void volume of the column
$V_C$ = geometric bed volume in mls.

The $K_{av}$ value for each standard was then plotted against the log of its molecular weight. Molecular weights of sample proteins were calculated by comparing the $K_{av}$ of the sample with the values obtained for the known calibration standards.

## 2.9 ANIMALS

Female Golden Syrian hamsters (*Mesocricetus auratus*) were obtained from Charles River Laboratories (Kent, U.K.). BALB/c mice were obtained from Harlan (Bichester, U.K). The animals were housed in the Specific Pathogen Free room in the BioResouce Unit, Trinity College Dublin, and were fed a standard laboratory diet *ad libitum*. All animal procedures were conducted under protocols approved by the Irish Department of Health and Children and the Trinity College BioResources Unit Committee.

## 2.10 HAMSTER MODEL OF *C. DIFFICILE* INFECTION

Hamsters were given a 2 mg dose of clindamycin HCl orogastrically to predispose them to *C. difficile* infection and challenged orogastrically with $10^5$ CFU 4 h later. A gavage needle was used for orogastric delivery of clindamycin and *C. difficile*. From the day after infection, hamsters were observed two-hourly in a blinded fashion for approximately 72 h,

and approximately four times a day at regular intervals thereafter. Hamsters were graded as follows: 0, normal; 1, loose faeces or wet perianum, activity close to normal; 2, reduced activity, still responding to stimuli, tender abdomen; 3, hunched, inactive, tender abdomen, loss of balance, ruffled fur. Hamsters were sacrificed at grade 3 by intraperitoneal (i.p.) injection with 1 ml of pentobarbitone (Euthatal or Dolethal, from Boileau and Boyd, Parkmore Industrial Estate, Longmile Road, Dublin 12, Ireland). Time of sacrifice or last time seen alive (whichever was earlier) was considered the end-point. Where indicated, samples of caecum, colon, small intestine, liver and kidney were taken for histological analysis. Briefly, the tissues were fixed in 10% formalin, processed to paraffin embedded blocks, sectioned at 5 μm and stained with haemoatoxylin and eosin. To confirm *C. difficile* as the causative agent of disease, perianal swabs were taken from a representative number of symptomatic hamsters per experiment and cultured anaerobically for four days on Columbia blood agar plates containing 50 μg/ml clindamycin.

## 2.11 IMMUNISATION REGIMENS

### 2.11.1 Passive immunisation of hamsters

Anti-SLP treated hamsters were given 100 μl of rabbit anti-SLP serum orogastrically at several time points before or after infection, depending on the experiment. The challenge inoculum was pre-incubated with 100 μl of anti-SLP serum for 30 min at 37°C. Antiserum was co-administered with 0.1 M sodium carbonate buffer, pH 9.6 [Appendix A] to neutralise stomach acid. Control hamsters were treated identically, but were given an irrelevant rabbit antiserum raised against the maltose-binding protein of *Escherichia coli* (Déirdre Ní Eidhin, unpublished). A *C. difficile* only group, which received no antiserum, was included in each experiment.

### 2.11.2 Active immunisation of hamsters

Five different active immunisation regimens were tested in hamsters **(Table 2.5)**. For intraperitoneal (i.p.) immunisation with alum, 50 μl of alum (aluminium hydroxide 45 mg/ml; magnesium hydroxide 40 mg/ml) was added drop-wise with mixing to the appropriate amount of native SLP in 50 μl of 20 mM Tris HCl pH 8.5 [Appendix A]. The resultant 100 μl alum/antigen mixture was injected i.p. with a 19-gauge needle. For i.p. immunisations with Ribi (MPL + TDM), 100 μl of Ribi was mixed with the appropriate amount of native SLPs in 100 μl of 20 mM Tris HCl pH 8.5 and injected i.p. using a 19-gauge needle. For intranasal (i.n.) immunisation, 5 μg of CT adjuvant was mixed with the appropriate amount of native SLPs in 50 μl of 20 mM Tris HCl pH 8.5 and was delivered

into the nostrils using a micropipettor, half of the dose in each nostril. Hamsters were challenged with *C. difficile* approximately 14 days after receiving their final boost.

**TABLE 2.5 Immunisation regimens for hamsters with *C. difficile* SLPs**

| Route (on days) | SLPs per dose | Adjuvant used | Number of animals/group |
|---|---|---|---|
| i.p. prime (0); i.p. boosts (21, 42) | 0 μg, 10 μg, 25 μg, 50 μg, 100 μg and 200 μg | Alum prime + boosts x 2 | 3 |
| | 25 μg | None, SLP only control | 3 |
| i.p. prime (0); i.p. boosts (21, 43) | 0 μg, 25 μg, 50 μg and 75 μg | Ribi (MPL + TDM) prime + boosts x 2 | 5 |
| i.p. + i.n. prime (0); i.n. boosts (21, 43) | 75 μg i.p. + 100 μg i.n. prime; 100 μg i.n. boosts | Ribi (MPL + TDM) + CT prime; CT boosts x 2 | 3 |
| | None, adjuvant only control | Ribi (MPL + TDM) + CT prime; CT boosts x 2 | 3 |
| i.n. prime (0); i.n. boosts (14, 28) | 100 μg | CT prime + boosts x 2 | 6 |
| | None, adjuvant only control | CT prime + boosts x 2 | 6 |
| i.n. prime (0); i.n. boosts (14, 35, 49) | 100 μg | CT prime + boosts x 3 | 6 |
| | None, adjuvant only control | CT prime + boosts x 3 | 6 |

### 2.11.3 Active immunisation of BALB/c mice

Four different active immunisation regimens were tested in BALB/c mice **(Table 2.6)**. For i.p. immunisations with Ribi (MPL + TDM), 100 μl of Ribi was mixed with 50 μg of native SLPs in 100 μl of 20 mM Tris HCl pH 8.5, and injected i.p. with a 19-guauge needle. For i.n. immunisations with CT, 1 μg of CT was mixed with 50 μg of native SLPs in 20 μl of 20 mM TrisHCl pH 8.5, and was delivered into the nostrils using a micropipettor, half of the dose in each nostril. For i.n. immunisation with chitosan

glutamate, 50 μg of SLP in 20 mM TrisHCl pH 8.5 was added to 100 μg of chitosan glutamate and 0.1 M HCl added dropwise until the chitosan glutamate went into solution. Trimethylated chitosan (100 μg) was mixed with 50 μg of SLPs in 20 μl of 20 mM Tris HCl pH 8.5. Doses were delivered into the nostrils using a micropipettor, half of the dose in each nostril. Mice were not challenged with *C. difficile*.

**TABLE 2.6 Immunisation regimens for BALB/c mice with *C. difficile* SLPs**

| Route (on days) | SLPs per dose | Adjuvant used | Number of animals/group |
|---|---|---|---|
| i.n. prime (0); i.n. boosts (21, 42) | 50 μg | CT prime + boosts x 2 | 5 |
| | 50 μg | None, SLP only control | 5 |
| | None, CT only control | CT prime + boosts x 2 | 5 |
| i.p. + i.n. prime (0); i.n. boosts (21, 42) | 50 μg i.p. + 50 μg i.n. prime; 50 μg i.n. boosts | Ribi (MPL + TDM) + CT prime; CT boosts x 2 | 5 |
| | None, adjuvant only control | Ribi (MPL + TDM) + CT prime; CT boosts x 2 | 5 |
| i.n. prime (0); i.n. boosts (21, 42) | 50 μg i.n. prime; 50 μg i.n. boosts | Chitosan glutamate prime + boosts x 2 | 5 |
| | None, adjuvant only control | Chitosan glutamate prime + boosts x 2 | 5 |
| i.n. prime (0); i.n. boosts (21, 42) | 50 μg i.n. prime; 50 μg i.n. boosts | Trimethylated Chitosan prime + boosts x 2 | 5 |
| | None, adjuvant only control | Trimethylated Chitosan prime + boosts x 2 | 5 |

### 2.11.4 Sampling of blood and faeces

To evaluate the immune response, samples of blood and faeces were obtained from naïve animals and at different time points during and after immunisation, depending on the experiment. Hamsters were bled from the saphenous vein following sedation by subcutaneous injection with 150 μl of Hypnorm (Janssen Animal Health, Bucks, U.K.) and yielded approximately 50-100 μl of blood per hamster. Mice were bled from the tail vein

and yielded approximately 200 µl of blood per mouse. Blood samples were left to clot overnight at 4°C and serum was obtained by centrifugation. Freshly voided faeces (1-2 pellets per hamster; 2–3 pellets per mouse) were collected in Eppendorf tubes and stored at -20°C until processed. For processing, 5 µl of ice-cold PBS, supplemented with Complete® protease inhibitors, was added per 1 mg of faeces and incubated for 5 min on ice. The pellet was broken up with a pipette tip, vortexed for 2 min, and centrifuged at 14000 rpm at 4°C for 10 min. The supernatants were transferred into clean tubes and stored at -20°C until required.

## 2.12 AGGLUTINATION ASSAY

Two-fold serial dilutions (1:2 to 1:4,096) of anti-SLP serum were prepared in duplicate in 96 well U-bottom microtitre plates in PBS in 50 µl final volumes. *C. difficile* suspension was adjusted to an $O.D._{600}$ = 1, and 50 µl added to antiserum dilutions. PBS and rabbit pre-immune serum served as negative controls. Plates were incubated for 24 h at 4°C and the degree of agglutination scored. Endpoint titres were defined as the reciprocal of the highest dilution of serum causing strong agglutination.

## 2.13 ENZYME LINKED IMMUNOSORBENT ASSAYS

### 2.13.1 Rabbit serum ELISA

96-well Maxisorp plates (Nunclon, Roskilde, Denmark) were coated overnight in a moist chamber at 4°C with 100 µl of either native SLPs or recombinant low-MW SLP at 1 µg/ml in 0.1 M carbonate buffer (pH 9.6) [Appendix A]. Wells were washed three times with PBS/0.1% Tween 20® (washing buffer) [Appendix A] and were blocked for 1 h at 37°C with 200 µl of PBS/2% dried skimmed milk (blocking buffer) [Appendix A]. Following three washes with washing buffer, 200 µl of two-fold serial serum dilutions (rabbit anti-SLP or rabbit anti-low-MW SLP) were added and plates incubated for 1 h at room temperature. Serum dilutions ranged from 1:1000 to 1:512000. Wells were washed three times with washing buffer and 200 µl of swine anti-rabbit HRP (diluted 1:1000 in blocking buffer) was added per well and plates incubated for 1 h at 37°C. Following three washes with washing buffer, bound antibody was detected by incubation with 100 µl of HRP substrate (OPD + $H_2O_2$) [Appendix A] per well for 20 min at room temperature in the dark. The reaction was stopped with 50 µl of 2.5 M $H_2SO_4$ [Appendix A] per well and OD read immediately at 490 nm on a Wallac Victor2 microplate reader. All determinations were carried out in triplicate. Pre-immune rabbit serum served as a negative control. A conjugate only control was also included.

### 2.13.2 Hamster and mouse serum IgG ELISA

96-well Maxisorp plates were coated overnight in a moist chamber at 4°C with 100 µl of native crude SLPs at 2 µg/ml in 0.05 M carbonate buffer (pH 9.6) [Appendix A]. Plates were washed three times with washing buffer [Appendix A] and blocked for 1 h with blocking buffer [Appendix A]. Plates were washed three times with washing buffer and serum samples, diluted as required, were added in duplicate and incubated for 1 h in a moist chamber at 37°C. Following five further washes, bound antibody was detected with goat anti-hamster IgG alkaline phosphatase conjugate or goat anti-mouse IgG alkaline phosphatase conjugate as required, followed by p-nitrophenyl phosphate substrate [Appendix A]. Reactions were allowed to develop for 30 min and stopped with 50 µl of 3 M NaOH [Appendix A]. Optical densities were read immediately at 405 nm on a Wallac Victor2 microplate reader.

### 2.13.3 Mouse serum and faecal IgA ELISA

96-well maxisorp plates were coated overnight at 4°C with 100 µl of native SLPs at 1 µg/ml in 0.1 M carbonate buffer (pH 9.6). Wells were washed three times with washing buffer [Appendix A] and were blocked for 1 h at 37°C with blocking buffer [Appendix A]. Serum (diluted 1:100 and 1:1000 in blocking buffer) or faecal supernatants (diluted 1:10 in blocking buffer) were added to wells and plates incubated for 1 h at 37°C. Wells were washed three times with washing buffer and 100 µl of biotinylated goat anti-mouse IgA (diluted 1:4000 in blocking buffer) was added to wells and plates incubated for 1 h at 37°C. Wells were washed three times with washing buffer and 100 µl of streptavidin-HRP (diluted 1:200 in blocking buffer) was added to wells and plates incubated for 1 h at 37°C. Following three washes with washing buffer, bound antibody was detected by incubation with 100 µl of HRP substrate [Appendix A] per well for 20 min at room temperature in the dark. The reaction was stopped with 50 µl of 2.5 M $H_2SO_4$ [Appendix A] per well and optical densities read immediately at 490 nm on a Wallac Victor2 microplate reader.

### 2.13.4 Hamster and Mouse CT ELISAs

96-well maxisorp plates were coated overnight at 4°C with 100 µl of CT at 2 µg/ml in 0.05 M carbonate buffer (pH 9.6) (hamster) or 1 µg/ml in 0.1 M carbonate buffer (pH 9.6) (mouse). Wells were washed three times with washing buffer [Appendix A] and were blocked for 3 h at 37°C with blocking buffer [Appendix A]. Following three further washes

serum dilutions were added to wells and plates incubated for 1 h at 37°C. Wells were washed five times with washing buffer and 100 µl of biotinylated goat anti-hamster IgG (diluted 1:4000 in blocking buffer) was added to wells and plates incubated for 1 h at 37°C. Wells were washed five times with washing buffer and 100 µl of p-nitrophenyl phosphate substrate [Appendix A] added per well for 60 min at room temperature in the dark. The reaction was stopped with 50 µl of 3 N NaOH [Appendix A] per well and optical densities read immediately at 405 nm on a Wallac Victor2 microplate reader.

### 2.13.5 Cytokine ELISAs

Immunoassays for IL-2, IL-4, IL-5, IL-10, IL-12p70, TNF-α and IFN-ϒ were carried out using paired antibodies according to the manufacturers' instructions and were optimised where necessary. Briefly, 96-well Maxisorp plates were coated overnight with 100 µl of the relevant capture antibody at 2 µg/ml (1.5 µg/ml for IL-10) in Dulbecco's PBS [Appendix A] at 4°C in a moist chamber. After washing three times with wash buffer (1X PBS, pH 7.0, 0.05% Tween 20®) [Appendix A], wells were blocked with 200 µl of assay diluent (1X PBS, pH 7.0, 10% FCS) [Appendix A] for 1 h at room temperature. After repeating the washing step, 100 µl of mouse T-cell or dendritic cell supernatants (see **Section 2.5.7**) or standards was added to wells and incubated at room temperature for 2 h, or overnight at 4°C where greater sensitivity was required. After washing three times, 100 µl of the relevant biotinylated detection antibody, diluted 1 µg/ml (0.5 µg/ml for IL-10) in assay diluent, was added to each well and plates incubated for 1 h at room temperature in a moist chamber. Wells were washed five times with wash buffer and 100 µl of streptavidin-HRP (1:1000 in assay diluent; 1:2000 for IL-10) added to each well and plates incubated for 30 min at room temperature in a moist chamber. Finally, wells were washed seven times and 100 µl of HRP substrate [Appendix A] added per well and incubated for 30 min in the dark. The reaction was stopped by addition of 50 µl 2.5 N $H_2SO_4$ [Appendix A] per well.

Immunoassays for TGFβ1 were carried out using paired antibodies according to the manufacturers' instructions. Paired antibodies from Promega were used to measure TGF-β1 production from SLP re-stimulated murine splenic T cells; Paired antibodies from R&D Systems were used to measure TGF-β1 production from DCs. Briefly, 96-well Maxisorp plates were coated overnight at 4°C with 100 µl of the capture antibody (4 µg/ml in PBS pH 7.2-7.4, R&D Systems; 1:1000 in carbonate coating buffer pH 9.7, Promega)

[Appendix A]. Plates were washed three times with washing buffer (0.05% Tween 20® in PBS pH 7.2-7.4, R&D Systems; 0.05% Tween 20® in TBS pH 7.6, Promega) [Appendix A], and blocked for 1 h at room temperature with 5% Tween 20® in PBS pH 7.2-7.4 (R&D Systems) or for 35 min at 37°C with 1X blocking solution (Promega). Latent TGFβ1 in cell culture supernatants was activated by the addition of 1 N HCl [Appendix A] for 10 min at room temperature and subsequently neutralised by the addition of 1.2 N NaOH/0.5 M HEPES (R&D Systems) or 1 N NaOH (Promega) [Appendix A]. The washing step was repeated and samples and standards diluted in reagent diluent [Appendix A] were added to the wells and incubated for 2 h at room temperature (R&D Systems) or for 90 min at room temperature with shaking at 600 rpm (Promega). The washing step was repeated and 100 µl of biotinylated anti-TGFβ diluted to 200 ng/ml in reagent diluent (R&D Systems) or 1:1000 in 1X sample buffer (Promega) was added to the wells and incubated for 2 h at room temperature (with shaking at 600 rpm, Promega). The washing step was repeated and 100 µl of streptavidin-HRP diluted 1:200 in reagent diluent (R&D Systems) or 1:100 in 1X sample buffer (Promega) was added per well and incubated for 20 min at room temperature (R&D Systems) or for 2 h at room temperature with shaking at 600 rpm (Promega). The washing step was repeated and 100 µl of a 1:1 mixture of room temperature tetramethylbenzidine (TMB) and $H_2O_2$ added to each well and incubated for 20 min at room temperature in the dark. The reaction was stopped with 50 µl of 2 N $H_2SO_4$ [Appendix A] per well (R&D Systems) or with 100 µl of 1 M HCl [Appendix A] per well (Promega).

Optical densities were read immediately at 450 nm on a Wallac Victor2 microplate reader. Sensitivity of the cytokine immunoassays was generally in the range 16 pg/ml to 1000 pg/ml. Cytokine concentrations in the samples were determined from standard curves.

## 2.14 *C. DIFFICILE* PHAGOCYTOSIS ASSAY

### 2.14.1 CFSE staining of *C. difficile*

*C. difficile* (two to three loopfuls) from three day old cultures on Columbia blood agar plates were resuspended into 1 ml of pre-warmed RPMI-1640 medium containing 5 µM 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) and incubated for 10 min at 37°C in the dark. Excess CFSE was removed by conjugation to FCS by washing the stained bacteria with RPMI/50% FCS [Appendix A] at 3000 rpm for 5 min. The bacteria were washed twice more with RPMI/10% FCS and bacterial concentration determined using $O.D._{600}$.

### 2.14.2 *C. difficile* phagocytosis assay

PMA differentiated THP-1 cells were washed twice with RPMI/10% FCS two to three hours prior to infection to remove detached cells and debris. CFSE stained *C. difficile* were mixed with the THP-1s at a Multiplicity of Infection (MOI) of 400 and incubated at 37°C in 5% $CO_2$ for 2 h. Cells were then washed three times with room temperature PBS to remove detached THP-1s and non-phagocytosed *C. difficile*. THP-1s were detached with 250 μl per well of pre-cooled detaching buffer [Appendix A] for 20 min at 200 rpm at 4°C, and washed three times at 3000 rpm at 4°C with pre-cooled PBS. Cells were resuspended in 300 μl of pre-cooled PBS for FACS analysis.

The effect of rabbit anti-SLP, rabbit anti-low MW-SLP, preimmune, irrelevant and a selection of human antisera on phagocytosis, were analysed by co-incubation of the sera with the *C. difficile* and THP-1s. Where indicated, the sera were heat inactivated at 56°C for 30 min to assess the opsonising activity in the sera due to complement. *E. coli* DH5α (pKFW408) expressing a modified GFP (Dr. Kenneth F. Whelan, Trinity College Dublin, unpublished) was used as a positive control for phagocytosis. Results are representative of at least three independent experiments.

### 2.14.3 FACS analysis of *C. difficile* phagocytosis by THP-1 cells

Green fluorescent emission of the phagocytosed *C. difficile* was assessed by flow cytometry analysis. Single THP-1s were gated using FSC and SSC. 30,000 events were acquired using a FACSCalibur and data was analysed with Cell Quest software (Becton Dickinson). The fluorescence of cell-surface attached *C. difficile* was quenched immediately prior to acquisition by addition of 0.8 mg/ml crystal violet.

## 2.15 DATA ANALYSIS

*In vitro* experiments were conducted a minimum of three times except where otherwise stated. Statistical analysis of results involved calculation of the summary statistics (mean, median, range, standard deviation (SD), standard error of the mean (SEM) and 95% confidence intervals), and student's t-test. Results are expressed as mean ± SEM. Groups of animals of were compared using non-parametric two tailed analyses (Kruskal-Wallis or Mann Whitney) as described in the results sections. Cytokine concentrations were compared by one-way ANOVA. Where differences were found, the Tukey multiple comparisons test was used to identify differences between individual groups. Correlation analysis was carried out using Spearman's rank correlation.

# CHAPTER III

# MOLECULAR CLONING OF DIFFERENT REGIONS OF *SLPA*, PRODUCTION OF NATIVE AND RECOMBINANT PEPTIDES, AND INITIAL CHARACTERISATION

# CHAPTER III

## 3.1 INTRODUCTION

Many Gram positive and Gram negative bacteria possess an S-layer. Particular attention has focused on S-layers present on the cell envelopes of pathogenic bacteria, as they may be potential virulence factors and therefore targets for vaccine development. The role of the *C. difficile* S-layer in establishing, maintaining and spreading infection has remained largely unexplored. Unlike the well-recognised *C. difficile* virulence factors, toxin A and toxin B, the S-layer of this pathogen has not been studied extensively as a potential virulence factor, and the specific biological functions of the SLPs in the context of *in vivo* models of *C. difficile* infection have not yet been determined.

The protein subunits of S-layers interact with one other and to the under-wall layer with hydrogen and ionic bondings, and hydrophobic interactions (Takeoka *et al*, 1991). The *C. difficile* S-layer is not covalently linked to other major structures and therefore the isolation of S-layers from cells walls has been achieved by using chaotropic agents such as urea, guanidine hydrochloride and EDTA (Cerquetti *et al.*, 1992, Cerquetti *et al.*, 2000, Takumi *et al.*, 1992). Low pH has also been used successfully to isolate the *C. difficile* SLPs (Calabi *et al.*, 2001). Gel filtration and anion exchange chromatography techniques have been used to isolate the SLPs from cell wall preparations (Cerquetti *et al.*, 1992, Hagiya *et al.*, 1992, Takeoka *et al.*, 1991). Liquid chromatography coupled to diode array detector and electrospray ionisation mass spectrometry has been used to carry out detailed biochemical analysis of the *C. difficile* SLPs (Mauri *et al.*, 1999).

In practice many clostridial proteins are poorly expressed in *E. coli*. It is thought that this phenomenon might be due to the fact that *E. coli* has a higher GC content than clostridia and consequently several types of codons common in clostridial coding sequences are rarely used in *E. coli*. For example, the codons ATA, AGA and CTA are rarely used in *E. coli* while at the same time they are abundant in clostridial genes (Zdanovsky and Zdanovskaia, 2000). The problem of unusual codon usage in clostridia can be overcome by using an expression host containing a plasmid with tRNAs that are rare in *E. coli* or by synthesising the gene to remove the codons (Kane, 1995). The former experimental approach was used successfully for the expression of the *C. difficile* SLPs in this work.

The cloning of an affinity tag into a target gene has revolutionised the purification of recombinant proteins, as such tags have greatly facilitated purification. However, this approach is not successful in all cases and many recombinant proteins are expressed as insoluble proteins in inclusion bodies. Thioredoxin fusion proteins are useful for producing recombinant proteins in *E. coli* in a soluble form. Simultaneously, they can be manipulated to provide for a simple, one-step purification via nickel affinity chromatography. To create a nickel binding domain in the thioredoxin fusion protein, the glutamate residue at position 32 and the glutamine residue at position 64 are mutated to create histidine residues. When the mutated thioredoxin folds, the histidines at positions 32 and 64 interact with a native histidine at position 8 resulting in the formation of a "histidine patch" on the surface. This patch has a strong and specific affinity for nickel ions (Lu *et al.*, 1996). A thioredoxin fusion tag was employed in this study in order to improve the yield of soluble recombinant protein while simultaneously providing a tag for purification of the protein.

In this chapter, the native SLPs of *C. difficile* from a Ribotype 1 strain isolated from a patient with *C. difficile* associated diarrhoea were purified to a high degree for use in an *in vivo* model of infection and for initial crystallography analysis. Recombinant low and high-MW SLPs were over-expressed and the low-MW SLP was purified for initial crystallography analysis.

## 3.2 OBJECTIVES

1. To clone the part of *slpA* gene encoding for the low-MW SLP and over express the low-MW SLP of *C. difficile*.
2. To clone the part of *slpA* gene encoding for the high-MW SLP of *C. difficile*.
3. To purify the recombinant low-MW SLP of *C. difficile* to homogeneity.
4. To purify and separate the native SLPs of *C. difficile* to homogeneity.
5. To compare the immunoreactivity of recombinant and native low-MW SLPs.
6. To prepare recombinant and native SLPs of *C. difficile* in order to determine initial crystallography conditions to produce crystals suitable for X-ray crystallography.

## 3.3 RESULTS

### 3.3.1 Cloning of the parts of the *slpA* gene encoding the low-MW SLP and the high-MW SLP, and overexpression of the low-MW SLP.

Knowledge of the nucleotide sequence of the *slpA* gene and cleavage of the precursor protein permitted PCR cloning. The parts of the *slpA* gene encoding for both the low-MW and high-MW peptides were cloned from the genomic DNA of *C. difficile* (Ribotype 1, PHLS UK ID number 13537). The signal sequence was inferred from published N-terminal sequence and forward and reverse PCR primers were deduced from the published sequence (EMBL database accession number AJ300676). The complete coding region of the part of the *slpA* gene encoding for the low-MW peptide, excluding the signal sequence, was amplified by PCR. A single product of the expected size of 948 bp was obtained following PCR **(Figure 3.1, A)**. The complete coding region of the part of the *slpA* gene encoding for the high-MW peptide was also amplified by PCR. A single product of the expected size of 1456 bp (1247 bp + 209 bp from the downstream reverse primer) was obtained following PCR **(Figure 3.2, A)**. The products were cloned into the pBAD/Thio-TOPO vector via TA cloning. Following transformation of the cloning hosts, competent TOP10 *E. coli*, the identity of the cloned inserts was confirmed by sequencing using the dideoxynucleotide chain termination method (Department of Biochemistry, University of Cambridge, U.K.). Proof reading *Pfu* polymerase does not give the 3' thymidine overhangs necessary for TA cloning and non-proof reading *Taq* polymerase was instead used for PCRs. Several inserts of each recombinant plasmid were sequenced in order to get the correct sequence. Both strands of each construct were completely sequenced and matched those of the published sequence. The inserts were in frame with the vector encoded thioredoxin fusion peptide. Following transformation of the expression host, competent Rosetta *E. coli*, induction of recombinant proteins was optimised. Induction of the recombinant low-MW SLP was found to be optimal at 6 h at a final concentration of 0.2% arabinose. The recombinant fusion low-MW SLP migrated as a band of between 45 kDa and 55 kDa on a 12% SDS-PAGE gel, the low-MW SLP accounting for 33 kDa and the thioredoxin fusion tag accounting for 13 kDa **(Figure 3.1, B)**. From 1 L of induced *E. coli* Rosetta, approximately 1-2 mg of *C. difficile* low-MW SLP was purified as a soluble N-terminal thioredoxin fusion protein. Induction of the recombinant high-MW SLP was found to be optimal at 4 h at a final concentration of 0.1% arabinose. The recombinant fusion high-MW SLP migrated as a band of between 55 kDa and 66 kDa on a 12% SDS-PAGE gel, the high-MW SLP accounting for 47 kDa and the thioredoxin fusion tag accounting for 13 kDa **(Figure 3.2, B)**.

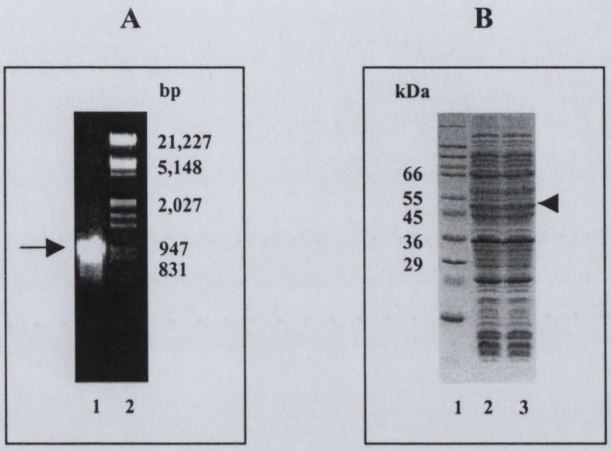



**Fig. 3.1 (A) Agarose gel electrophoresis of PCR amplified part of the *slpA* gene of *C. difficile* encoding the low-MW SLP.** The PCR amplified part of the s*lpA* gene was analysed on a 1% agarose. Lane 1, amplified PCR product of 948 bp (indicated with an arrow); Lane 2, DNA marker, sizes indicated in bp on the right hand side. **(B) SDS-PAGE analysis of expression of recombinant low-MW SLP of *C. difficile*.** Expression of the recombinant low-MW SLP was analysed on a 12% SDS-PAGE gel stained with Coomassie blue. Lane 1, molecular masses in kDa; Lanes 2 and 3, 10 µl of lysate of a 6 h induced culture. The recombinant low-MW SLP is visible between 45 kDa and 55 kDa and is indicated with an arrow. An uninduced culture (not shown) was used for comparison. Molecular masses of markers, in kDa, are indicated on the left.

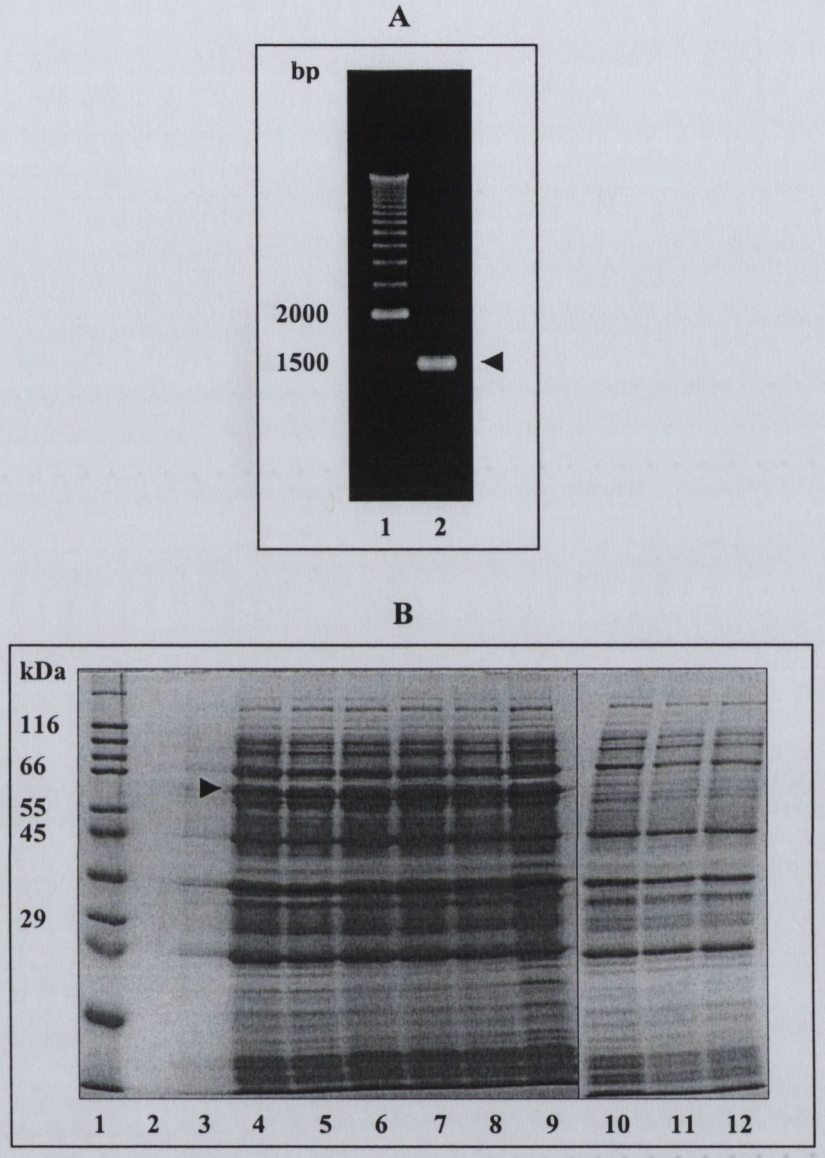



**Fig. 3.2 (A) Agarose gel electrophoresis of PCR amplified part of the *slpA* gene of *C. difficile* encoding the high-MW SLP.** The PCR amplified part of the *slpA* gene was analysed on a 1% agarose gel. Lane 1, DNA marker, sizes are indicated in bp on the left hand side. Lane 2, amplified PCR product of 1456 bp (indicated with arrow). **(B) SDS-PAGE analysis of expression of recombinant high-MW SLP of *C. difficile*.** Expression of the recombinant high-MW SLP was analysed on 12% SDS-PAGE gel stained with Coomassie blue. Lane 1, Molecular weight markers. Masses of markers, in kDa, are indicated on the left; Lanes 4 to 9, lysate (10 µl) of a 4 h culture induced with 0.02%, 0.04%, 0.08%, 0.1%, 0.2% and 0.4% arabinose respectively; Lanes 10 to 12, lysate (10 µl) of 1 h, 2 h and 4 h non-induced cultures respectively. The recombinant high-MW SLP is visible at approximately 60 kDa and is indicated with an arrow.

### 3.3.2 Purification of recombinant low-MW SLP.

The recombinant low-MW SLP was purified by a combination of anion exchange **(Figure 3.3, A)** and $Ni^{2+}$ affinity chromatography **(Figure 3.3, B)**. Analysis of fractions collected from the anion exchange and $Ni^{2+}$ affinity columns, by SDS-PAGE plus Coomassie staining, revealed fractions containing increasingly pure protein at each step of the purification scheme **(Figure 3.4, lanes 3-6)**. Recombinant low-MW SLP migrated as a single band of approximately 33 kDa **(Figure 3.4, lanes 6 and 9)**. Pure fractions were pooled and consisted of recombinant protein of a high level of purity as determined by SDS-PAGE and Coomassie blue staining. The N-terminal amino acid sequence of the recombinant low-MW SLP was determined to be LALDDTK by Edman degradation (Mike Weldon, Department of Biochemistry, University of Cambridge). The first three amino acids, LAL, are vector encoded and remained after enterokinase cleavage of the thioredoxin fusion peptide. DDTK are the first four amino acid residues of the mature Ribotype 1 *C. difficile* low-MW SLP (Calabi *et al.*, 2001), indicating that the recombinant low-MW peptide was intact at its N-terminus.

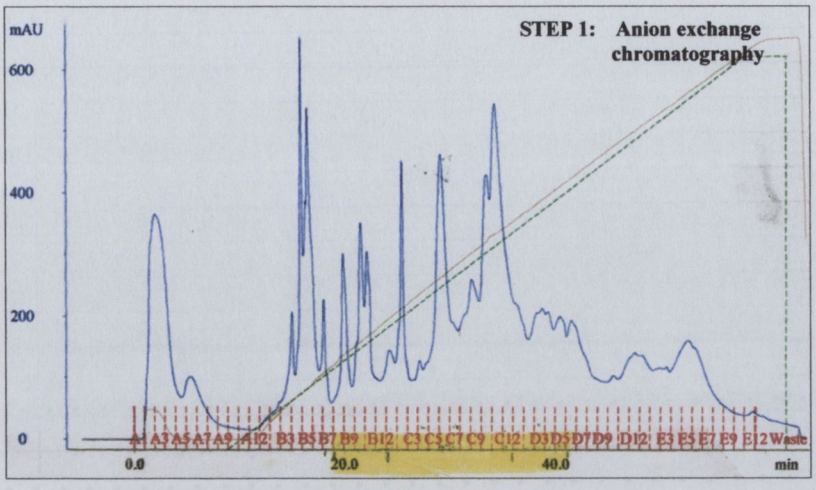
### 3.3.3 Removal of the thioredoxin fusion protein by enterokinase digestion.

Removal of the thioredoxin fusion protein from the recombinant low-MW SLP was achieved by digestion with enterokinase (1 U enterokinase/50 μg protein) for 16 h at room temperature. To terminate the reaction, the enterokinase was removed from the mixture by batchwise binding to Ekapture agarose. Analysis of the reaction products was carried out by SDS-PAGE and Coomassie blue staining. Removal of the thioredoxin fusion protein resulted in a 13 kDa decrease in the molecular mass of the recombinant protein **(Figure 3.4, lanes 4 and 5)**. Thioredoxin cleavage was successful, with 100% of the protein resolving as a single band on the gel **(Figure 3.4, lane 5)**. Following cleavage of the thioredoxin fusion protein, the recombinant low-MW SLP was passed through a final nickel affinity column with resultant pure protein present in the flow-through **(Figure 3.4, lane 6)**.

### 3.3.4 Purification of the native *C. difficile* SLPs.

Purification of the native SLPs was achieved by a combination of a crude 8 M urea preparation and anion exchange chromatography. A crude S-layer preparation made with 8 M urea **(Figure 3.5, B, lane 2)** was applied to a MonoQ HR10/10 anion exchange column and the pure SLPs eluted with an NaCl gradient **(Figure 3.5, A)**. In the chromatography trace shown, fractions B11 and B12 contained the pure SLPs. From a 400 ml culture of *C.*

A




B

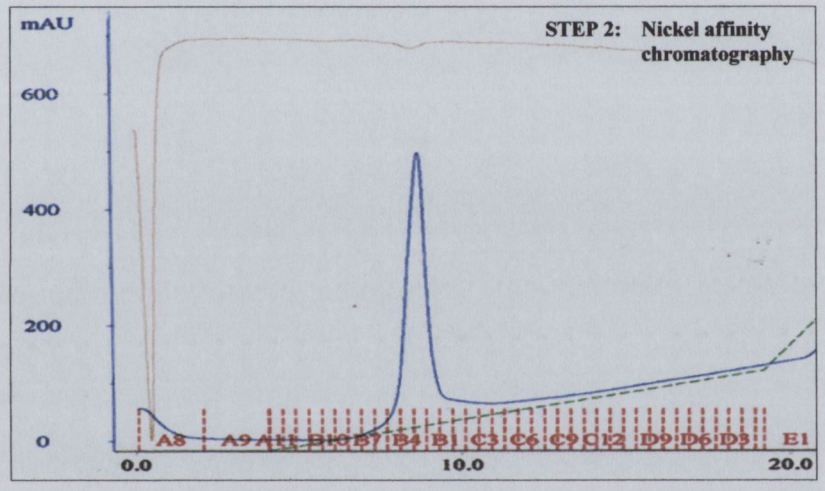



**Fig. 3.3 Purification of the recombinant low-MW SLP of *C. difficile* by anion exchange and affinity chromatography.** (A) Partial purification of the recombinant low-MW SLP of *C. difficile* by anion exchange chromatography on a MonoQ HR 10/10 column attached to an *AKTA*FPLC system. A 0-0.5 M NaCl gradient was applied to the column and the protein eluted at approximately 0.2 M NaCl. Fractions C5-C7 contained the partially purified protein in this chromatography. (B) The second step in the purification process, affinity chromatography of the partially purified protein on a nickel charged HiTrap column attached to an *AKTA*FPLC system. A 0-100 mM imidazole gradient was applied to the column and the recombinant protein eluted at approximately 50 mM imidazole. Fraction numbers, indicated on the x-axis, were monitored automatically at OD 280 nm (blue line). Gradients are represented by the dashed green line.

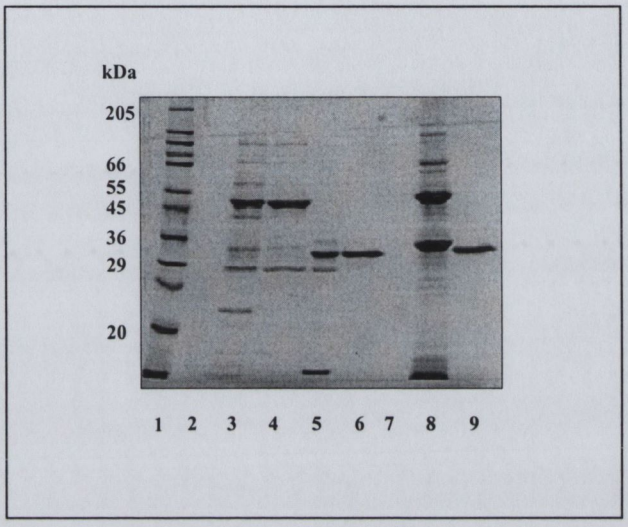



**Fig. 3.4 SDS-PAGE analysis of purification of the recombinant low-MW SLP of *C. difficile*.** Pooled fractions (10 µl) from the sequential steps in the purification of the recombinant low-MW SLP were analysed on a 12% SDS-PAGE gel stained with Coomassie blue, as follows: lane 1, molecular weight markers; lane 3, post anion-exchange column; lane 4, post nickel affinity column; lane 5, post enterokinase cleavage of histidine patch fusion protein; lane 6, post final nickel affinity column; lanes 8 and 9 show native SLPs of *C. difficile* and recombinant low-MW SLP of *C. difficile* respectively, side by side. Molecular masses of markers, in kDa, are indicated on the left.

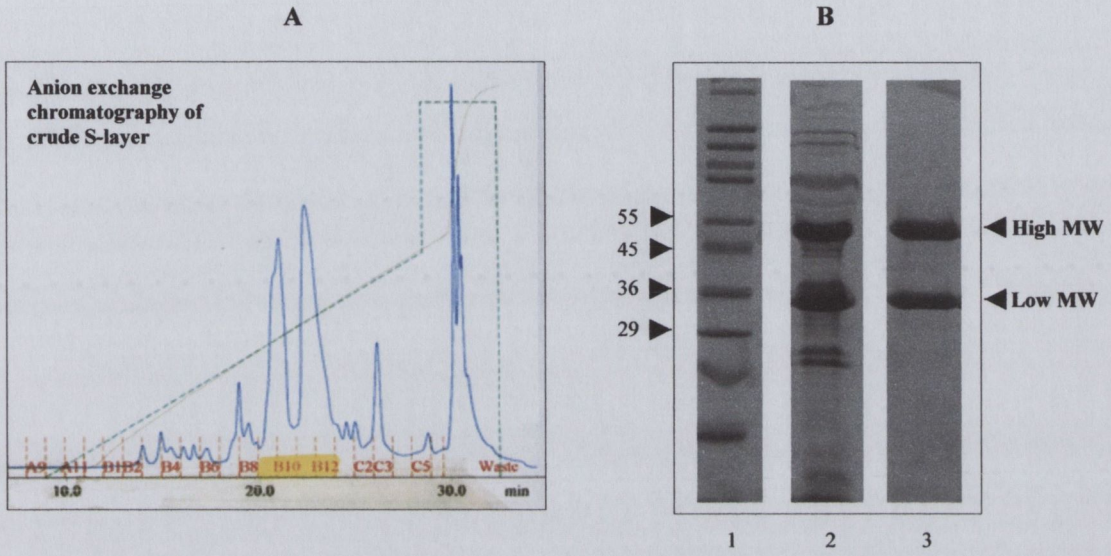



**Fig. 3.5 Purification of *C. difficile* native SLPs by anion exchange chromatography.** (A) Anion exchange chromatography trace of a crude 8 M urea S-layer preparation applied to a MonoQ HR 10/10 anion exchange column attached to an *AKTA*FPLC system. Fraction numbers, indicated on the x-axis, were monitored automatically at OD 280 nm (blue line). Pure SLPs were eluted with a 0-0.3 M NaCl gradient (dashed green line). (B) 12% SDS-PAGE gel, stained with Coomassie blue, of 10 µg of the crude preparation (lane 2) and 10 µg of fraction B11 from the anion exchange chromatography shown in panel A (lane 3). Molecular weight markers are in lane 1, with molecular masses, in kDa, indicated on the left.

*difficile* approximately 6-8 mg of the two native SLPs was co-purified. The purity was estimated as > 98% by SDS-PAGE and Coomassie staining **(Figure 3.5, B, lane 3)**. Endotoxin content of the refined SLP preparation was determined using the Limulus Amoebocyte Lysate (LAL) quantitative QCL-1000 assay. As expected for a Gram-positive organism, no endotoxin contamination was found. The co-purified native SLPs were separated from each other using anion exchange chromatography in the presence of 8 M urea. Using this method, the low- and high-MW SLPs were successfully separated from one another **(Figure 3.6)**.

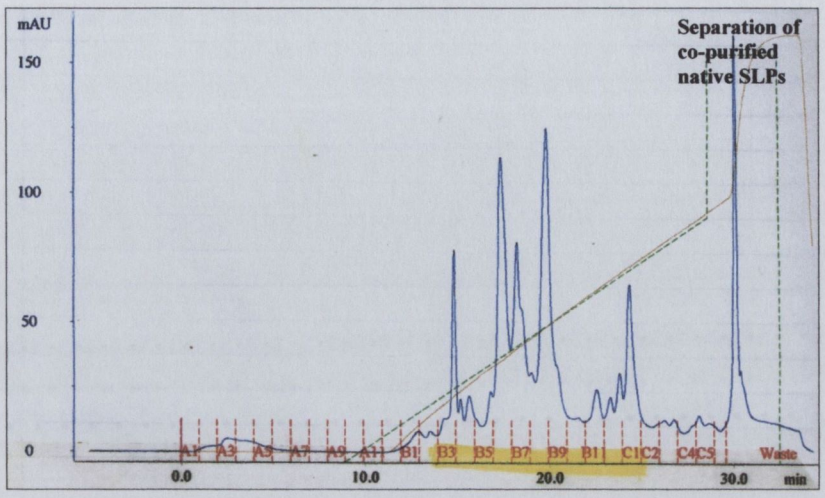
### 3.3.5 Western blot analysis of the SLPs with convalescent serum from a patient infected with *C. difficile* and rabbit antiserum raised against the recombinant low-MW SLP.

The identity of the recombinant low-MW SLP was further confirmed by Western blotting, using convalescent serum from a patient who had been infected with *C. difficile* of the same PCR ribotype. The serum reacted strongly with the recombinant protein **(Figure 3.7, A)**, indicating that antigenic components were not lost during the generation of the recombinant protein. Relative optical density determined by densitometric scanning of the autoradiogram (Kodak 1D Image Analysis System) showed no major difference in the strength of reaction between the native and recombinant SLPs with the serum. Following cleavage of the thioredoxin fusion peptide, the recombinant low-MW SLP still reacted strongly with the patient's convalescent serum **(Figure 3.7, B)**. A faint band is visible between 45 kDa and 55 kDa and may represent a small amount of uncleaved recombinant protein that was not visible with Coomassie blue staining **(Figure 3.4, lanes 5 and 6)**. When examined by Western blotting, rabbit antiserum raised against the recombinant low-MW SLP reacted strongly with the protein **(Figure 3.7, C)**. Rabbit antiserum raised against the purified native SLPs reacted equally with the low- and high-MW SLP **(Figure 3.7, D)**.

### 3.3.6 Gel Filtration Analysis of the SLPs.

Gel filtration analysis was used to investigate the self-assembly behaviour of the purified SLPs, and also as a polishing step in the purification process for proteins being sent for crystallography. When the co-purified native SLPs were analysed on a Superdex 200 gel filtration column, two peaks were obtained, indicating the purified SLPs were forming two kinds of aggregate. The two aggregates were determined to be 222.5 kDa and 102 kDa in size **(Figure 3.8, A)**. On a second analysis of a different native SLP preparation on a Superdex 200 gel filtration column, two aggregates were again found. The molecular

A




B

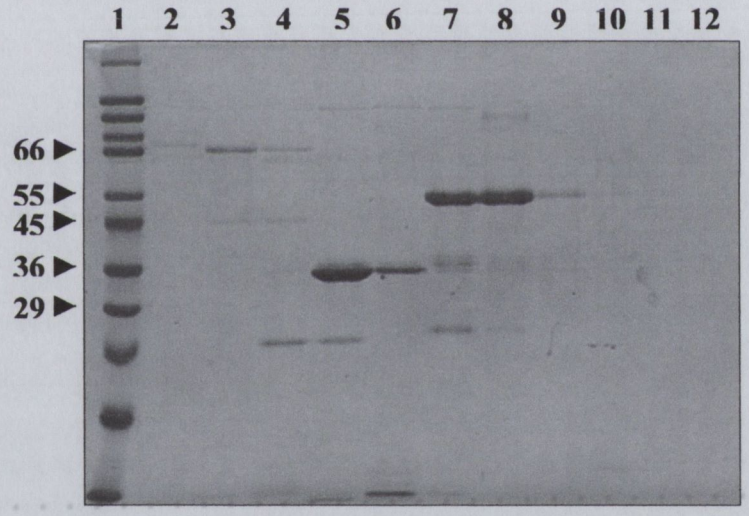



**Fig. 3.6** **Separation of the co-purified native SLPs of *C. difficile*.** (A) Anion exchange chromatography trace of a crude 8 M urea S-layer preparation applied to a MonoQ HR 10/10 anion exchange column attached to an *AKTA*FPLC system. All buffers contained 8 M urea. Fraction numbers, indicated on the x-axis, were monitored automatically at OD 280 nm (blue line). Separated SLPs were eluted with a 0-0.3 M NaCl gradient (dashed green line). (B) Fractions B3-C1 (10µl of each, lanes 2-12 respectively) were run on a 12% SDS-PAGE gel and stained with Coomassie blue. Fraction B6 contained very pure native low-MW SLP and fractions B8 and B9 contained very pure native high-MW SLP. Molecular masses of markers, in kDa, are indicated on the left.

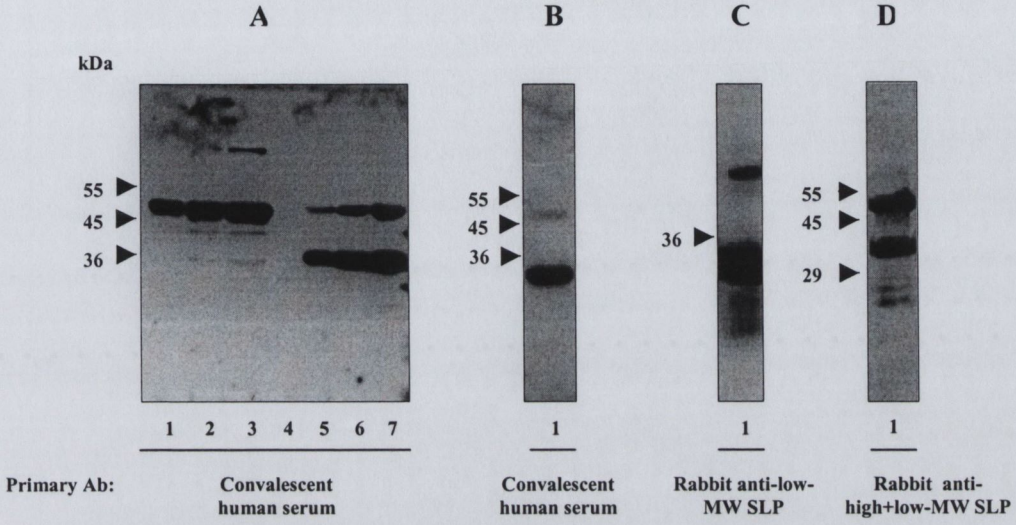



**Fig. 3.7 Western Blot analysis of SLPs with convalescent serum from a patient infected with *C. difficile* and rabbit antiserum raised against the SLPs.** (A) Western blot of recombinant low-MW SLP, prior to cleavage of the thioredoxin fusion tag, 4 μg, 8 μg and 16 μg (lanes 1, 2 and 3 respectively), and native SLPs, 4 μg, 8 μg and 16 μg (lanes 5, 6 and 7 respectively). Proteins were probed with convalescent serum (1:2000) from a patient infected with *C. difficile* of the same PCR ribotype as the proteins (Ribotype 1). (B) Western blot of the recombinant low-MW SLP (4 μg), after cleavage of the thioredoxin fusion protein, with convalescent serum (1:2000) from a patient infected with *C. difficile* of the same PCR ribotype as the proteins. The secondary antibody used in A and B was rabbit anti-human IgG HRP. (C) Western blot of the recombinant low-MW SLP (16 μg) with rabbit anti-recombinant low-MW SLP serum (1:10000). (D) Western blot of the native SLPs combined (10 μg) with rabbit anti-SLP serum (1:20000). The secondary antibody used in C and D was swine anti-rabbit IgG conjugated to HRP. Molecular masses of markers, in kDa, are indicated on the left of each panel.

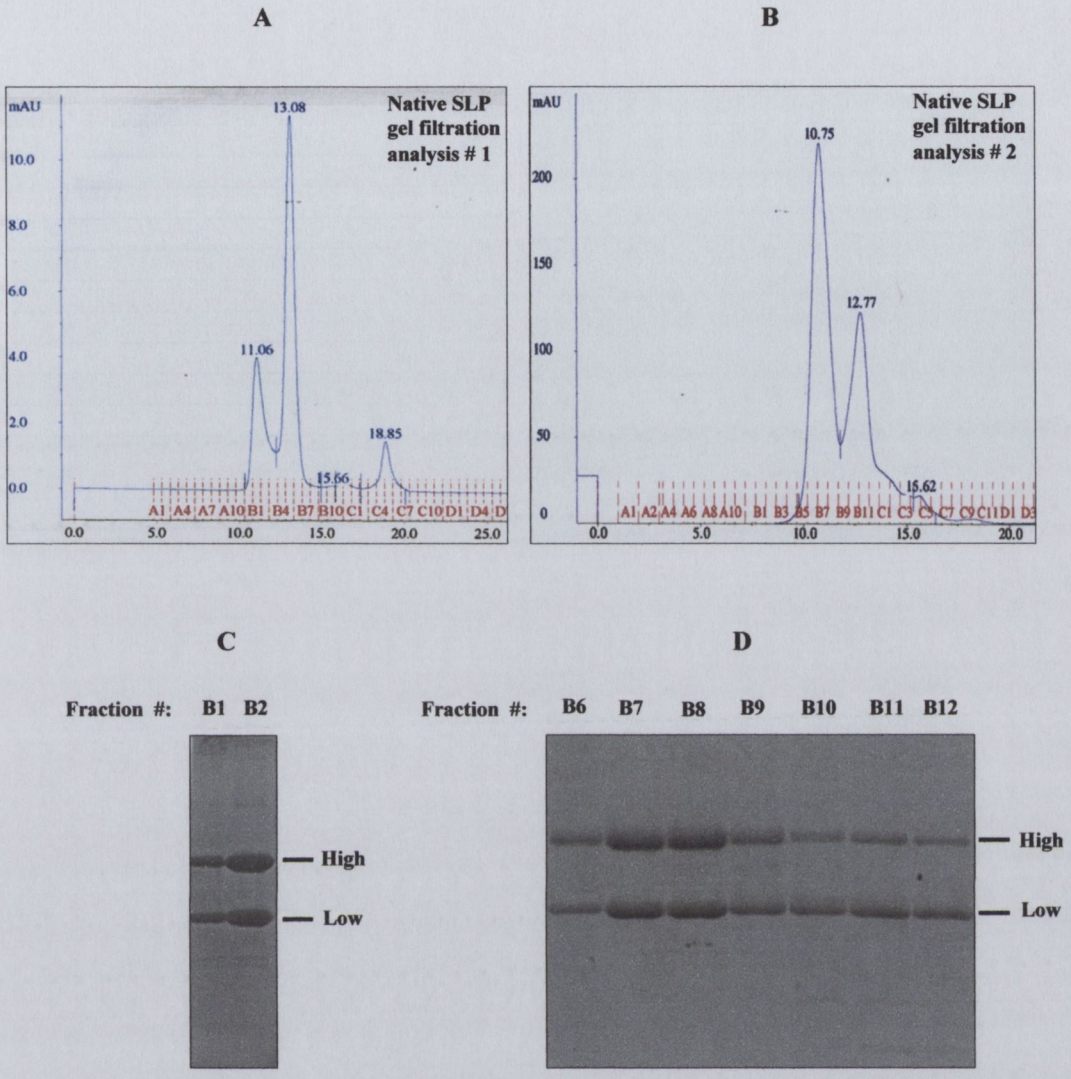



**Fig. 3.8** **Gel filtration analysis of the native SLPs of *C. difficile*.** (A) and (B) show gel filtration analyses of two different native SLP preparations applied to a Superdex 200 column attached to an *AKTA*FPLC system. The proteins were eluted isocratically, and fractions, indicated on the x-axis, were monitored automatically at OD 280 nm (blue line). The numbers on top of the peaks are the elution volumes of the proteins and molecular weights were determined from these as outlined in **Chapter II, Section 2.8.4.** (C) Fractions B1 and B2 (10 µl of each) from the gel filtration chromatography shown in panel A on a 12% SDS-PAGE gel stained with Coomassie blue. (D) Fractions B6-B12 inclusive (10 µl of each) from the gel filtration chromatography shown in panel B on a 12% SDS-PAGE gels stained with Coomassie blue.

masses in this case were different to the first analysis and were determined to be 250.8 kDa and 114.8 kDa respectively **(Figure 3.8, B)**. Both the low and high-MW SLPs were present in both protein aggregates as determined by SDS-PAGE and Coomassie blue staining **(Figure 3.8, C and D)**. The amount of protein in each of the aggregates differed between analyses, indicating a kind of unstable aggregate. Minor peaks were evident in both analyses and most likely represent small amounts of contaminating proteins.

The recombinant low-MW SLP was also analysed on a Superdex 75 gel filtration column **(Figure 3.9)**. The relative purity of the protein was established by the presence of a single sharp symmetric peak, with a calculated size of 40.79 kDa. The size of the protein as determined by SDS-PAGE plus Coomassie blue staining was approximately 33 kDa. The calculated size of the low-MW SLP based on the published amino acid sequence is 33.365 kDa (ExPASy Compute PI/MW tool). The discrepancy between size determined by gel filtration and SDS-PAGE may be due to limitations in the accuracy of molecular weight determination by gel filtration. It should also be noted that SDS-PAGE is not always accurate for molecular mass determination, particularly with acidic proteins such as the SLPs. The Superdex 75 column separates proteins in the range 3 kDa to 75 kDa. Had the recombinant protein existed as a dimer or some other multimer, it would have exhibited a much lower elution volume on a column that separates in the range 3 kDa to 75 kDa. It is therefore probable that the protein exists as a monomer.

### 3.3.7 Identification of initial crystallisation conditions for the *C. difficile* SLPs.

Following trials, initial crystallisation conditions have been identified for both the recombinant low-MW SLP and the native SLPs combined. These conditions are being refined with the intention of producing crystals suitable for structure solution by X-ray crystallography. Crystallisation and structural studies were carried out by Dr. Claire Naylor, Birkbeck College, University of London. **Figure 3.10 (A)** shows a typical recombinant low-MW SLP crystal and **Figure 3.10 (B)** shows a preliminary X-ray diffraction pattern obtained from such a crystal. However, crystals that diffracted were multiple crystals, and the resolution of 10.6 Å obtained from the recombinant low-MW crystal was not of sufficient quality to gather interpretable diffraction data. Additional material has been prepared for crystallisation and on-going studies are focused on obtaining single crystals suitable for diffraction.

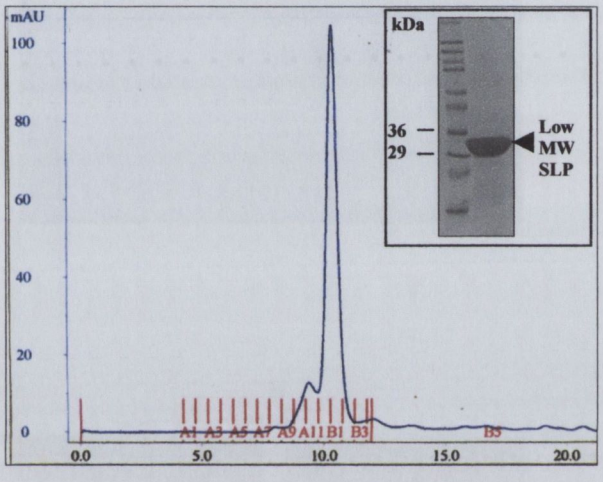
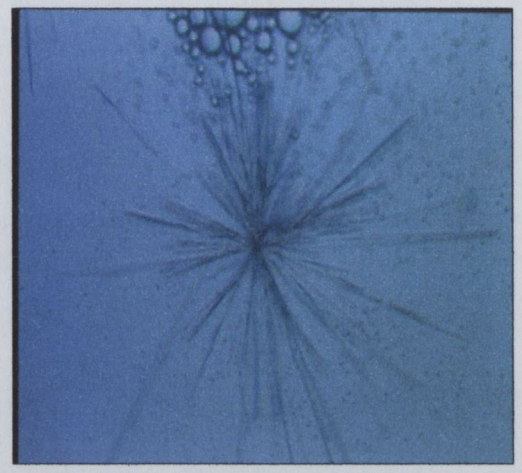



**Fig. 3.9 Gel filtration analysis of the recombinant low-MW SLP.** Gel filtration analysis of the purified recombinant low-MW SLP was carried out on a Superdex 75 gel filtration column attached to an *AKTA*FPLC system. The protein was eluted isocratically, and fractions, indicated on the x-axis, were monitored automatically at OD 280 nm (blue line). Inset: Fractions A12 and B1, from the gel filtration chromatography shown, were pooled, acetone precipitated and run on a 12% SDS-PAGE gel stained with Coomassie blue.

A



B

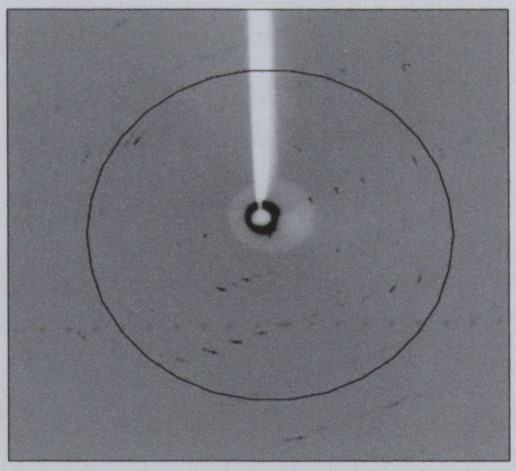


**Fig. 3.10 Typical crystal of recombinant low-MW SLP from *C. difficile* and x-ray diffraction pattern obtained from such a crystal.** (A) A typical recombinant low-MW SLP crystal is depicted (Dr. Claire Naylor, Birkbeck College, London). (B) Diffraction pattern for such a crystal. Crystals were sent to the European Synchrotron Radiation Facility, Grenoble, France, for diffraction tests. Some ordered rows of diffraction spots are visible and were obtained at 10.6 Å resolution.

## 3.4 DISCUSSION

The first step towards elucidating the potential role of the SLPs was the development of a reliable method of production, purification and initial characterisation of these proteins. This chapter describes the development of a reliable method of producing high quality SLPs for use in *in vitro* and *in vivo* studies, in order to gain an insight into the functions of these antigens.

Recombinant low and high-MW SLPs from *C. difficile* were over expressed in *E. coli* and the low-MW SLP purified to homogeneity. The recombinant SLPs were expressed in a strain (*E. coli* Rosetta™ (Novagen) an *E. coli* BL21 derivative) specifically chosen for the fact that it contained a plasmid, pRARE, encoding tRNAs which are rare in *E. coli*, namely AGG, AGA, AUA, CUA, CCC and GGA, thus overcoming problems due to the different codon bias of *C. difficile*. The recombinant SLPs were over expressed in this strain without difficulty. Interestingly, successful production and purification of hexahistidine-tagged recombinant low- and high-MW SLPs of *C. difficile* in a common *E. coli* expression strain (BL21 (DE3)) was reported in the literature at this time (Calabi *et al.*, 2001). The codon bias of *C. difficile* was not taken into account in this system, indicating that this issue may not be of critical importance in the expression of *C. difficile* SLPs. However, the yield of recombinant protein in this study was not revealed. To take account of the knowledge that *C. difficile* uses rare codons and in order to obtain the necessary yield for *in vivo* studies, expression in *E. coli* Rosetta was the method of choice for this study.

Reverse PCR primer design for the part of the *slpA* gene encoding for the high-MW peptide was troublesome, due to the very high AT content of the sequence. After several unsuccessful attempts at primer design based on the *slpA* sequence, a successful reverse primer was designed based on the sequence downstream of the *slpA* gene (sequenced by Dr. Déirdre Ní Eidhin). This primer was 38 bp in length and started 209 bp downstream of the *slpA* nonsense codon. The over expressed high-MW SLP was not purified, however it is intended to use the construct to make protein truncates in order to identify immunodominant regions of the protein in further studies. The low-MW SLP construct will be used in a similar manner.

The recombinant SLPs were expressed as fusion proteins with *E. coli* thioredoxin at their N-terminus. Due to affinity of thioredoxin histidine patch fusion proteins for nickel ions, in theory it should have been possible to purify the protein to homogeneity using a single

affinity chromatography step. In practice, however, this was not possible and a three-step purification scheme incorporating anion exchange and two affinity chromatography steps was developed. As seen in **Figure 3.4**, the protein was eluted after the final step in the purification process with minimal contaminating proteins. There are multiple possible reasons for the failure of the affinity tag to work effectively. As seen in **Figure 3.3 (B)**, the affinity tag bound weakly to the nickel charged resin, so that the recombinant protein eluted at a concentration of just 50 mM imidazole. It is possible that the affinity tagged recombinant SLP was in competition for resin functional groups with contaminating *E. coli* proteins, which may interact with the functional groups via ionic interaction. Such interaction would occupy column binding sites and mask a large column surface area. This resulted in poor binding of the recombinant fusion protein to the resin and in many weakly bound contaminating proteins co-eluting with the recombinant protein **(Figure 3.4, lane 4)**. This problem was overcome with a number of modifications. First, an initial anion exchange step was incorporated into the purification scheme to eliminate as many contaminating *E. coli* proteins as possible. Second, imidazole was omitted from the $Ni^{2+}$ affinity column binding buffer and the low-MW SLP was eluted with low concentrations of imidazole. Third, the same $Ni^{2+}$ affinity purification step was carried out twice, both prior to and following fusion protein cleavage. In this way, contaminants that bound and eluted with the fusion protein on the first column bound again along with the cleaved fusion partner. The recombinant protein was recovered in a highly purified form in the flow-through fractions of the second affinity column.

It is likely that the ability of thioredoxin to act as a successful fusion partner is intimately linked to its structural stability. It was possible that the recombinant protein was re-assembling into lattice type structures, as it does in nature, and therefore making the thioredoxin "histidine patch" inaccessible to the nickel ions on the resin. With subsequent gel filtration analysis, it became clear that the recombinant low-MW SLP was in fact a monomer, and so was not reassembling into lattice type structures. However, this information was unknown when optimising the purification scheme. Had a conventional histidine tag been employed, purifying under denaturing conditions might have been used to investigate this possibility. Denaturing conditions cannot be used to purify thioredoxin histidine patch fusion proteins, as the histidine patch tertiary structure, and therefore its ability to bind nickel, is destroyed under these conditions.

Due to the poor affinity of the thioredoxin fusion protein for nickel ions, a three-step purification process was necessary. Due to the number of steps in the process, the yield of recombinant protein was substantially reduced, as some target protein was lost at each step. As sufficient recombinant protein for *in vivo* studies could not be produced in this system, it was necessary to develop a method of producing sufficient quantities of highly purified native forms of the *C. difficile* SLPs. Purification of native SLPs was based on published methods (Cerquetti *et al.*, 2000, Hagiya *et al.*, 1992, Takeoka *et al.*, 1991) but with some modifications. Using a combination of 8 M urea extraction with anion exchange chromatography, a semi-automated method of producing large quantities of native *C. difficile* SLPs was developed. This method resulted in the production of very pure, endotoxin-free SLPs in the quantities necessary for *in vivo* studies. As *C. difficile* is a Gram-positive organism, it does not have endogenous endotoxin. However, due to extraneous sources of endotoxin, such as glassware, water and Gram-negative bacteria, contamination with exogenous endotoxin was a possibility. Endotoxin is one of the most potent modulators of the innate immune system, inducing the production of an array of inflammatory cytokines including IL-1, IL-6, IL-8 and TNF-α and is capable of inducing lethal septic shock in experimental animals (Jones, 2001). The presence of endotoxin in the antigen preparation for subsequent immunisation studies was highly undesirable and would have resulted in skewed immune responses in any *in vivo* studies carried out.

The antigenicity of native and recombinant proteins was compared by Western blotting, using serum from a patient recovering from infection with *C. difficile* of the same ribotype. It was possible that glycosylation and/or other post-translational modifications would be required for immunoreactivity. The patient's serum reacted equally with the recombinant and native SLP, indicating that the recombinant protein had retained its antigenicity. When analysed side by side on a 12% SDS-PAGE gel stained with Coomassie blue, the recombinant low-MW SLP has an apparent molecular weight 1-2 kDa smaller than that of the native low-MW SLP **(Figure 3.4, lanes 8 and 9)**. It was possible that this discrepancy was as a result of enterokinase cleavage of the thioredoxin fusion protein occurring incorrectly several amino acids into the recombinant protein. N-terminal amino acid sequencing proved this not to be the case, with the first seven amino acids including the vector encoded 'LAL', followed by the first four amino acids of the low-MW SLP 'DDTK'. It is most likely that glycosylation and/or other post-translational modifications, that are present in the native SLP but not in the recombinant version, are responsible for

this difference in electrophoretic behaviour of the proteins. It is now generally accepted that both the low- and high-MW SLPs of *C. difficile* are glycosylated.

A previous study of the SLPs of *C. difficile* also revealed discrepancies in molecular weights as determined by SDS-PAGE and liquid chromatography/mass spectrometry. Mauri *et al.*, (1999) found that by SDS-PAGE analysis the low- and high-MW SLPs had molecular masses of 36 kDa and 47 kDa respectively. However, by liquid chromatography coupled to mass spectrometry, molecular masses were determined to be 34.258 kDa and 39.545 kDa respectively. The authors demonstrated that both protein subunits were glycosylated and concluded that the discrepancy was probably due to the different electrophoretic migration of glycosylated proteins. Calabi *et al* (2001) also demonstrated that both the low- and high-MW SLPs from a Ribotype 1 *C. difficile* strain were glycosylated.

Interestingly, rabbit antiserum raised against the purified SLPs reacted equally with the low-MW SLP and the high-MW SLP. This was different to the pattern of reactivity seen with human serum from a patient recovering from *C. difficile* infection, where stronger reactivity was seen against the low-MW SLP. The low-MW SLP is known to be the more immunogenic of the two major *C. difficile* SLPs (Pantosti *et al.*, 1989). The SLPs have been shown to form two superimposed lattices on the surface of the bacterium (Cerquetti *et al.*, 2000), with both the low and high MW SLPs exposed at the cell surface as determined by surface iodination and immunogold labelling and indirect immunofluorescence (Takeoka *et al.*, 1991). Several studies have suggested that the low-MW SLP is located on the exterior surface of the bacteria (Cerquetti *et al.*, 2000, Karjalainen *et al.*, 2002, Karjalainen *et al.*, 2001). The equal reactivity against both the high and low-MW SLPs by the rabbit antiserum suggests the possibility that both are equally immunogenic but that differences in antibody reactivity in infection may relate to arrangement of the components on the bacterium surface.

It is a recognised feature of S-layers that the isolated subunits self-assemble into regular arrays similar to those observed in the native cell walls. The native SLPs from other *C. difficile* strains have been shown to spontaneously reassemble *in vitro* (Cerquetti *et al.*, 2000, Takeoka *et al.*, 1991, Takumi *et al.*, 1992). The ability of purified SLPs to self-assemble is due to the fact that these proteins are acidic with a large proportion of hydrophobic amino acids (Takeoka *et al.*, 1991, Mastrantonio *et al.*, 1995). On gel

filtration of the anion exchange purified native SLPs from this Ribotype 1 strain two multimers of different size were observed. The predicted masses (from amino acid sequences) of the low and high-MW SLPs are 33 kDa and 45 kDa respectively (Calabi *et al.*, 2001). In one gel filtration analysis, two aggregates of 225 kDa and 102 kDa were evident **(Figure 3.8, A)**. Two similar sized aggregates of 250.8 kDa and 114.8 kDa were observed in a second gel filtration analysis of another SLP preparation from the same *C. difficile* strain **(Figure 3.8, B)**. It is not immediately obvious what kind of aggregates these may be, but both the low and high-MW subunits are present in all as determined by SDS-PAGE **(Figure 3.8, C** and **D)**. Molecular weight determination by gel filtration tends to deviate from that of SDS-PAGE, as factors such as glycosylation do not interfere in gel filtration, and for this reason it is not possible to accurately decipher how many of each subunit constitutes these aggregates. They most likely represent a kind of unstable aggregate, where the dynamic favours the reformation of the surface layer lattice structure in the absence of a denaturing agent. On gel filtration analysis, the recombinant low-MW SLP appeared as a monomer. This is in contrast to the native low-MW SLP, which has been shown to exist as a dimer (Cerquetti *et al.*, 1992, Takeoka *et al.*, 1991). The monomeric form of this protein, along with its high level of purity, demonstrated by the single, symmetrical peak observed on gel filtration, made it an ideal candidate for x-ray crystallography.

Solving the three-dimensional structure of the SLPs may help to elucidate their potential role or roles in the pathogenicity of *C. difficile*. The SLPs from *C. difficile* have not been structurally characterised in this manner to date. Given its history of success, X-ray crystallography is regarded as the most widely used tool for structure determination efforts. Both the recombinant low-MW SLP and the native SLPs were prepared for initial crystallography analysis. A prerequisite to any study of a protein's three-dimensional structure is the need to produce protein of sufficiently high purity. To ensure a high level of purity, gel filtration was used as a final polishing step for proteins sent for crystallography analysis. Another major challenge of the x-ray diffraction structure determination approach lies in obtaining suitable three-dimensional crystals, and such has been the case with the *C. difficile* SLPs. Crystals of the recombinant low-MW SLP obtained to date have not been suitable for structure solution by X-ray crystallography and are being further refined in order to produce suitable crystals. Ideally crystals should diffract to less than 4 Å resolution, while the recombinant low-MW SLP crystal diffracted to 10.6 Å. Crystals of the native SLPs obtained to date are pseudocrystals and are similarly

unsuitable for X-ray diffraction. SLPs in general terms are not structurally well characterised, mainly due to the high degree of heterogeneity among SLPs. Therefore, structures are not available for the molecular replacement that is vital for the elucidation of any protein model. Future work on the elucidation of the three-dimensional structure of the SLPs will require the production of selenomethionyl replacement crystals for phase determination (Doublié, 1997).

In summary, the SLPs of *C. difficile* are major antigens of this pathogen and it is reasonable to assume they may be involved in virulence in some way. This possibility has not been tested in an *in vivo* setting to date. The objective of this work was to produce SLPs, which could be used for structural analysis and ultimately immunisation studies in an *in vivo* model of infection. Highly pure SLPs in the quantities required were produced for *in vivo* studies, which are described in subsequent chapters. A recombinant low-MW SLP and the native forms of the co-purified SLPs have had preliminary crystallographic analysis performed by our collaborators. The conditions are currently being refined in order to produce crystals suitable for structure solution by x-ray crystallography. A combination of *in vivo* studies and structural analysis may result in the specific functions of these proteins being solved.

# CHAPTER IV

# PASSIVE IMMUNISATION OF HAMSTERS AGAINST *CLOSTRIDIUM DIFFICILE* INFECTION USING ANTIBODIES TO SURFACE LAYER PROTEINS

# CHAPTER IV

## 4.1 INTRODUCTION

*C. difficile*-associated diarrhoea (CDAD) is a worldwide problem with major incidence in the elderly and hospitalised populations. A recent prospective study estimated the annual cost of managing CDAD in the United States of America at over US $1.1 billion (Kyne *et al.*, 2002). The human and economic impacts highlight the need for novel preventive approaches against CDAD.

Passive immunisation is generally used when exposure to a pathogen has already occurred and there is not enough time to induce protective immunity, or prophylactically when a susceptible individual is likely to be exposed to a pathogen. In the context of *C. difficile* infection, passive immunisation has the potential to provide a simple means of protecting susceptible individuals from infection prior to likely exposure to the pathogen. Additionally, passive transfer of antibodies could be used to treat individuals with *C. difficile* toxin-induced disease. Individuals at risk of CDAD are well defined and include history of antibiotic use (especially ampicillin (amoxicillin), cephalosporins and clindmycin), advanced age, antineoplastic agents, gastrointestinal surgery or manipulation, extended hospital stay, serious underlying illness and immunocompromised individuals (Kyne *et al.*, 1999, Oldfield *et al.*, 2004, Poutanen and Simor, 2004, Surawicz and McFarland, 1999). Administration of a dose of passive antibody prior to admission to hospital and subsequent likely exposure to *C. difficile* has potential to provide a means of protecting such at risk individuals.

Two toxins secreted by the bacterium (toxins A and B) mediate the pathogenesis of disease (Kelly and LaMont, 1998) and an IgG response against toxin A is correlated with recovery in humans (Kyne *et al.*, 2001). Purified inactivated toxins and antibodies against the toxins have been shown to protect against CDAD in the hamster model (Giannasca *et al.*, 1999, Lyerly *et al.*, 1991). However, vaccines based on the toxins are unlikely to eradicate infection, maintaining the pool of asymptomatic carriers and allowing for possible transmission of *C. difficile*. The major surface proteins of *C. difficile* present a good alternative potential target for passive immunotherapy development.

Adhesion to the intestinal epithelium is considered an important primary step for gut colonisation by *C. difficile*. Based on their location at the outer surface of the bacterium, it

has been proposed that the *C. difficile* SLPs are involved in binding of the bacterium to the intestinal epithelium (McCoubrey and Poxton, 2001). Direct binding of the bacterium to Caco-2 and HT-29 colonic epithelial cell lines, as well as to primary intestinal epithelial cells, has recently been shown (Cerquetti *et al.*, 2002, Drudy *et al.*, 2001). Native forms of SLPs and a recombinant high-MW SLP are able to bind directly to human gastrointestinal tissue sections (Calabi *et al.*, 2002). Moreover, antisera against whole bacteria and the high-MW SLP can reduce binding of *C. difficile* to the gastric epithelial cell lines HT-29 and Hep-2 respectively (Drudy *et al.*, 2001, Calabi *et al.*, 2002). SLPs have been reported to elicit a strong IgG response in patients infected with *C. difficile* (Pantosti *et al.*, 1989, Drudy *et al.*, 2004) and high IgM anti-SLP levels have been associated with a reduced risk of recurrent CDAD in humans (Drudy *et al.*, 2004).

It has been recognised since the 1980's that a lethal enterocolitis can be induced in hamsters by the administration of clindamycin (Fernie *et al.*, 1983, Libby *et al.*, 1982). This phenomenon is the basis for the hamster model of *C. difficile* infection, in which animals are administered a dose of clindamycin to clear their gut flora and are subsequently challenged with *C. difficile*. In this model an acute lethal disease develops within two to three days if highly toxigenic strains are used (Corthier *et al.*, 1991, Borriello *et al.*, 1987), and it is a valuable tool for studying protective effects, or otherwise, of novel preventative approaches against CDAD. This acute lethal outcome does not directly parallel clinical presentation in humans, which results in severe disease (fulminant colitis with ileus, toxic megacolon, perforation and death) in only 3% of patients (Kelly and LaMont, 1998, Borriello *et al.*, 1987). Thus, the hamster model provides an extremely stringent test for any protective effect of the anti-SLP serum against CDAD. Given the limitations of the disease model, and the need to minimise the numbers of animals used for development of therapeutic agents, the passive immunisation approach was a logical first step in investigating the SLPs are potential vaccine candidates.

The aim of this study was to test the protective capacity of passively administered rabbit anti-SLP serum against CDAD in the lethal hamster challenge model outlined above, and to investigate the mechanism of action of the antiserum. To achieve this, high-specificity, high-titre antibodies to the SLPs of a Ribotype 1 *C. difficile* strain were raised in rabbit. Administration of the anti-SLP serum prolonged survival of hamsters following challenge with $10^5$ CFU *C. difficile*. A potential mechanism of action was shown to be through enhanced phagocytosis of *C. difficile*.

## 4.2 OBJECTIVES

1. To raise high-specificity, high-titre rabbit antibodies to the SLPs of a Ribotype 1 *C. difficile* strain.
2. To test the protective capacity of rabbit anti-*C. difficile* SLP serum to protect against *C. difficile* infection in a lethal hamster challenge model.
3. To investigate phagocytosis of *C. difficile* by a human monocytic/phaogcytic cell line in the absence and presence of rabbit anti-SLP serum.

## 4.3 RESULTS

### 4.3.1 Western blot analysis of rabbit anti-*C. difficile* SLP serum.

Based on their location on the outer bacterium surface and their *in vitro* capacity to bind to human gastrointestinal tissues (Calabi *et al.*, 2002), SLPs represent a strong vaccine candidate for targeting *C. difficile* colonisation and CDAD. To develop an anti-SLP serum for passive immunisation experiments, we initially purified SLPs from a Ribotype 1 *C. difficile* strain (PHLS UK ID number R13537) to homogeneity from a crude S-layer preparation by anion exchange chromatography as described in Chapter III **(Section 3.3.4)**. The pure SLPs were then used to raise rabbit polyclonal antibodies, which reacted strongly with both the high- and low-MW SLPs by immunoblotting against a crude SLP preparation **(Figure 4.1)**. Several less intense bands are visible below the low and high-MW SLP bands and are most likely degradation products **(Figure 4.1, lanes 2 and 3)**. Pre-immune rabbit serum showed no reactivity against the crude S-layer preparation within a wide range of concentrations (1:1000, 1:5000, 1:10000, 1:15000, 1:20000) (1:15000 is shown in **Figure 4.1, lane 1**). To ensure no anti-toxin antibodies were present in the rabbit antiserum, immunoblotting against a total *C. difficile* lysate was carried out. The anti-SLP serum did not recognise the *C. difficile* toxins (toxins A and B have molecular weights of 308 kDa and 270 kDa, respectively) as demonstrated by this method **(Figure 4.1 lane 3)**. Due to the limited amount of pre-immune serum available, an irrelevant rabbit serum raised against the maltose-binding protein of *Escherichia coli* (Dr. Déirdre Ní Eidhin, unpublished) was used as a control serum in place of pre-immune serum in the *in vivo* experiments where indicated. The irrelevant serum (diluted 1:500 and 1:1000) showed little reactivity against a total *C. difficile* lysate by immunoblotting and exposing the membrane for 10 min (Dr. Matthew McCabe) **(Figure 4.1, lanes 4 and 5)**.

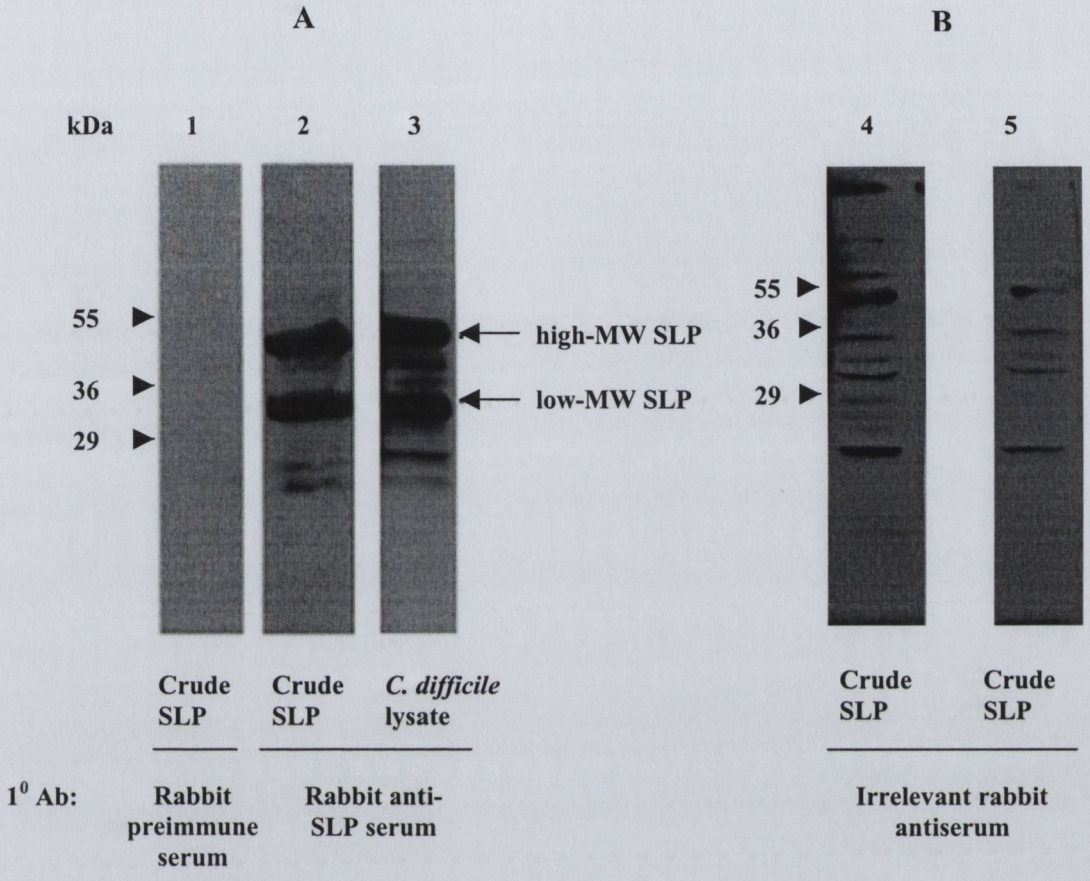



**Fig. 4.1 Western blot analysis of a crude *C. difficile* S-layer preparation and a *C. difficile* lysate probed with rabbit pre-immune, anti-SLP or irrelevant serum.** A Ribotype 1 crude *C. difficile* S-layer preparation (lanes 1 and 2) and a *C. difficile* lysate (lanes 3, 4 and 5) (PHLS UK ID number R13537) were resolved on a 12% SDS-PAGE gel, transferred to PVDF membrane and immunoblotted with rabbit pre-immune serum, 1:15000 (lane 1), rabbit anti-SLP serum, 1:15000 (lanes 2 and 3) or irrelevant rabbit antiserum, 1:500 and 1:1000 (lanes 4 and 5, respectively). The secondary antibody used was swine anti-rabbit IgG HRP. Molecular masses of markers, in kDa, are indicated on the left of each panel. The blot was developed with ECL and exposed for 10 seconds (panel A) or 10 minutes (panel B).

### 4.3.2 *In vitro* agglutination of *C. difficile* by rabbit anti-SLP serum.

To corroborate that the anti-SLP serum is capable of recognising SLPs on the surface of intact whole *C. difficile*, *in vitro* agglutination experiments were conducted. Two-fold serial dilutions of anti-SLP or pre-immune antiserum (1:8 to 1:4096) were tested for their capacity to agglutinate whole *C. difficile in vitro*. The anti-SLP antibodies readily agglutinated whole *C. difficile* cells, with a titre of 512 as compared to a titre of 8 for the pre-immune serum. No agglutination of *C. difficile* was seen with the negative control (PBS) **(Table 4.1)**.

### 4.3.3 Post-*C. difficile* challenge survival of hamsters passively immunised with anti-SLP.

The ability of passively administered anti-SLP serum to protect against CDAD was tested in a lethal hamster challenge model. In total, three passive immunisation experiments were carried out, the first two in parallel. All animals were predisposed to *C. difficile* infection by clindamycin treatment, followed by passive immunisation with anti-SLP serum, pre-immune serum or irrelevant serum. A *C. difficile*-only group, which received no serum, was included in each experiment. All groups were subsequently challenged with *C. difficile*.

Initially, hamsters were predisposed to infection with 0.5 mg clindamycin and challenged with either $10^2$ or $10^3$ *C. difficile*. Percentage post-challenge survival of these hamsters is shown in **Figure 4.2 (A)**. All hamsters challenged with $10^2$ *C. difficile* (n = 5) that received anti-SLP serum died from CDAD within five days post-challenge (median = 83.2 h, range (65.2 h - 131.7 h)) **(Figure 4.2, A (i),** green line**)**. Two out of three control hamsters treated with pre-immune rabbit serum remained well throughout this experiment **(Figure 4.2, A (i),** blue line**)**. Both control hamsters that received $10^2$ *C. difficile* only showed severe classical symptoms of CDAD, but one of these animals subsequently recovered and survived **(Figure 4.2, A (i),** purple line**)**. No *C. difficile* colonies were isolated on anaerobic culture of faeces from the three surviving hamsters. **Figure 4.2 (B (i))** shows post-challenge weight profiles for these hamsters. Surviving hamsters from the pre-immune serum treated group (blue symbols) maintained a steady weight throughout the experiment, while the surviving hamster from the *C. difficile* only group (purple symbols) initially dropped weight before slowly recovering to almost pre-challenge weight.

**TABLE 4.1 *In vitro* agglutination of *C. difficile* with rabbit anti-SLP serum [a]**

| Serum dilutions | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 | 1/256 | 1/512 | 1/1024 | 1/2048 | 1/4096 |
|---|---|---|---|---|---|---|---|---|---|---|
| Rabbit preimmune serum | +++ | ++ | + | - | - | - | - | - | - | - |
| Rabbit anti-SLP serum | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + |
| PBS | - | - | - | - | - | - | - | - | - | - |

[a] Endpoint titres were defined as the reciprocal of the highest dilution of serum causing strong agglutination (+++ = strong agglutination; ++ = medium agglutination; + = weak agglutination).

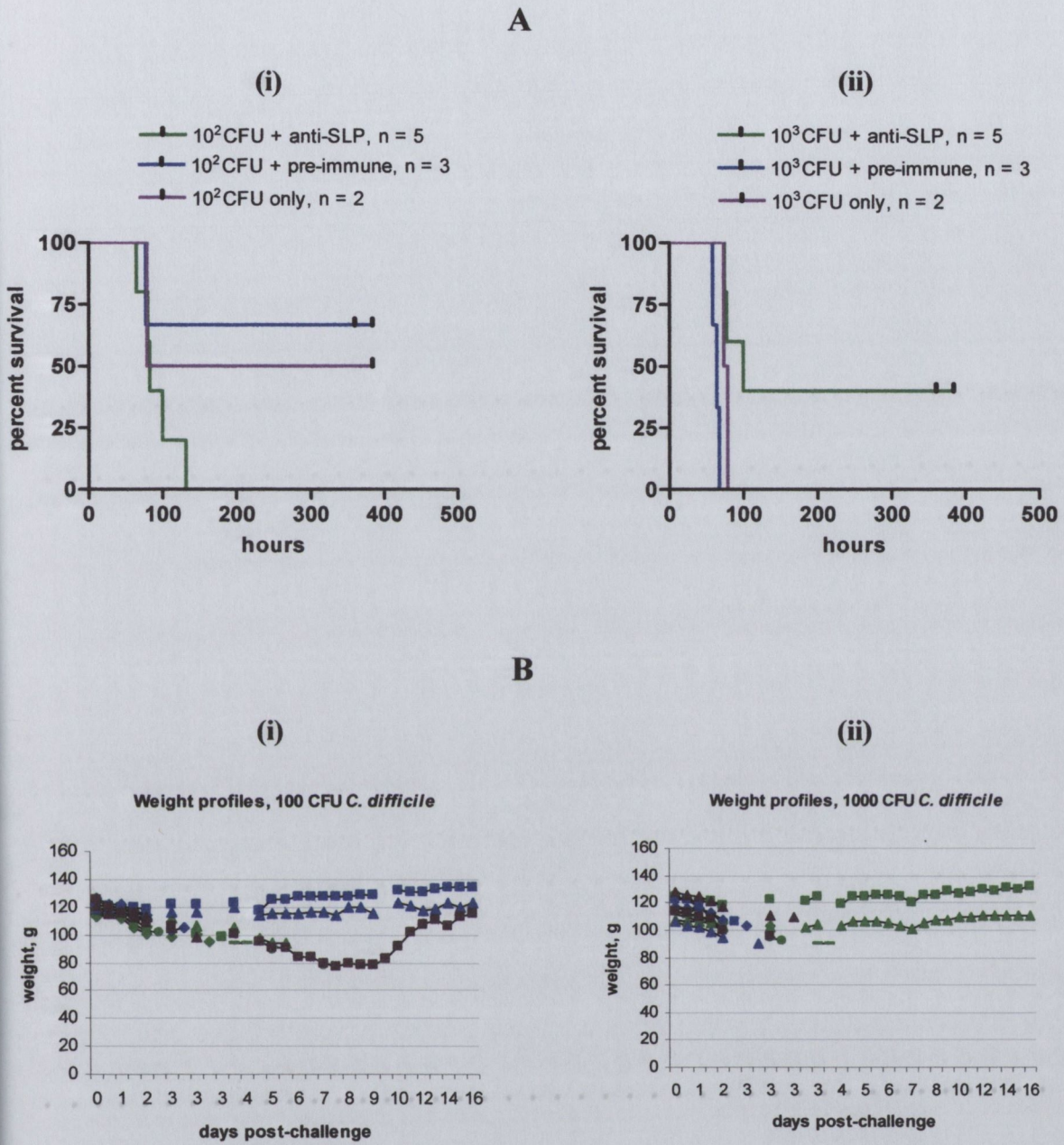



**Fig. 4.2 Post-challenge survival and weight profiles of hamsters passively immunised with anti-SLP and challenged with *C. difficile*.** (A) Post-challenge survival. Hamsters were predisposed to *C. difficile* infection with 0.5 mg clindamycin and challenged 3 h later with $10^2$ or $10^3$ *C. difficile*, which had been pre-mixed with 100 µl of rabbit anti-SLP serum (green line, n = 5) or rabbit pre-immune serum (blue line, n = 3) or no serum (purple line, n = 2). Three further 100 µl doses of serum were administered at 7 h, 11 h and 24 h of infection. Time zero = time of administration of *C. difficile*. (B) Post *C. difficile* challenge weight profiles. Note: Weights were taken at multiple times on each day from day zero to day nine.

Two out of five hamsters challenged with $10^3$ *C. difficile* treated with anti-SLP rabbit serum remained well throughout the experiment **(Figure 4.2, A (ii),** green line**)**. Anaerobic culture of faeces from these two animals revealed no *C. difficile* colonies. All control hamsters (n = 3) that received $10^3$ *C. difficile* and pre-immune rabbit serum died from CDAD within three days post-challenge (median = 64.0 h, range (58.3 h, 67.2 h)) **(Figure 4.2, A (ii),** blue line**)**. Hamsters that received $10^3$ *C. difficile* only (n = 2) died from CDAD within three and a half days of infection (median = 75.8 h, range (73.2 h, 78.3 h)) **(Figure 4.2, A (ii),** purple line**)**. **Figure 4.2 (B (ii))** shows post-challenge weight profiles for these hamsters. Surviving hamsters recovered their weight to greater than pre-challenge levels, indicating that they were unlikely to succumb to infection at this stage.

Given the anomalous survival of control hamsters in the initial passive immunisation experiments, it was not possible to conclude whether the anti-SLP serum had protective effects against CDAD or not. However, since there was a trend towards protection at the higher inoculum, another passive immunisation experiment incorporating a number of modifications was carried out. In this experiment, animal numbers were increased and hamsters were given 2 mg clindamycin to predispose them to infection. The inoculum was increased to $10^5$ *C. difficile* and was pre-incubated with the anti-SLP serum prior to infection. Four additional doses of serum were administered. **Figure 4.3 (A)** shows percentage post-challenge survival for this experiment. **Figure 4.3 (B)** shows the post-challenge weight profiles of hamsters from all three groups and illustrates that there was no weight advantage in the anti-SLP treated hamsters. Hamsters from all experimental and control groups developed diarrhoea within two to five days post-challenge, showing no significant difference in time to first symptoms **(Figure 4.4, A)**. However, post-challenge survival **(Figure 4.4, B)** was significantly prolonged in animals treated with anti-SLP serum as compared to animals treated with irrelevant serum (P = 0.0281) and untreated animals (P = 0.0283) (summary statistics, **Table 4.2**). Seven out of eight (irrelevant serum treated) and three out of four (*C. difficile* only) control hamsters as compared to two of eight anti-SLP treated hamsters had died at 72 h post-challenge.

### 4.3.4 Histological analysis of caecum and colon from hamsters passively immunised with anti-SLP and challenged with *C. difficile*.

The typical pathology seen in the hamster model of CDAD was confirmed on histological examination of caecum and colon sections. Slides were read in a blinded fashion by

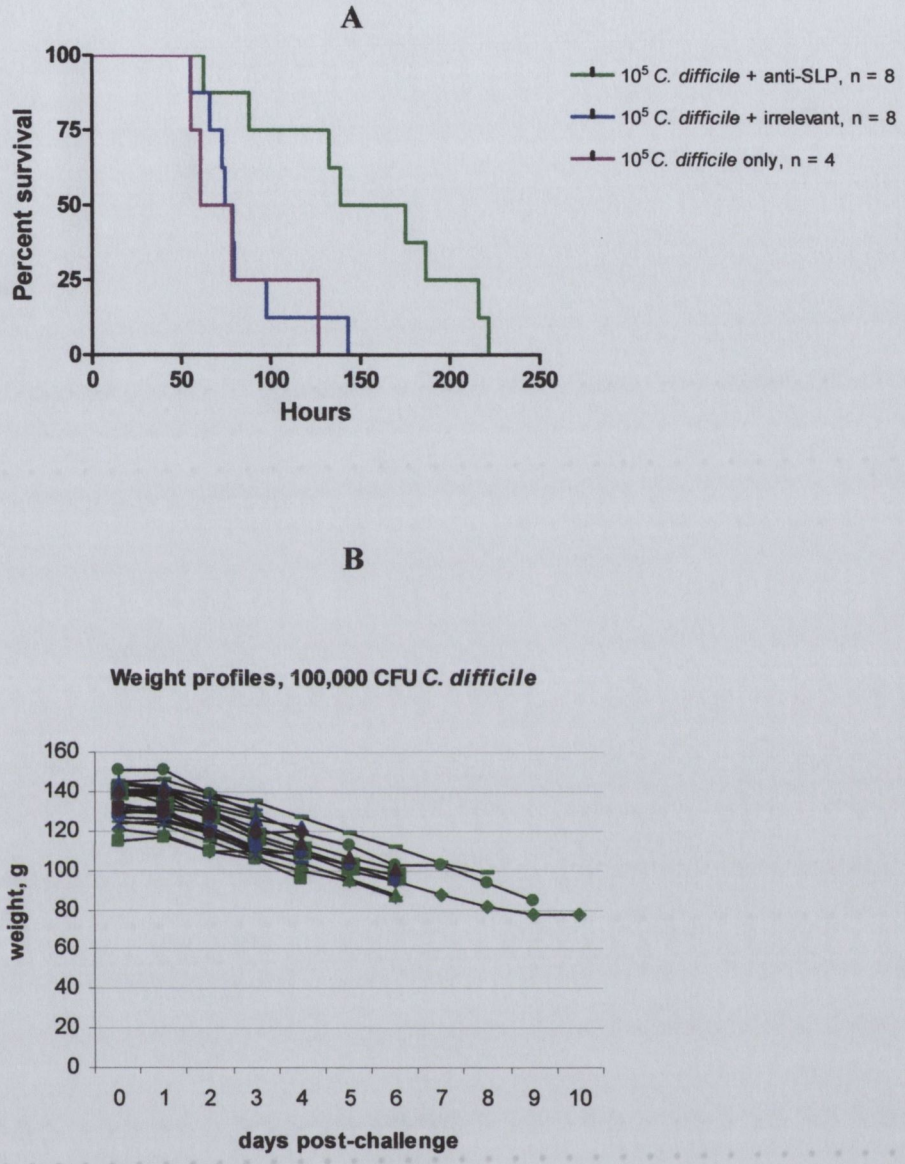



**Fig. 4.3 Post-challenge survival and weight profiles of hamsters passively immunised with anti-SLP serum and challenged with $10^5$ *C. difficile*.** (A) Post-challenge survival. Hamsters were predisposed to *C. difficile* infection with 2 mg of clindamycin and challenged 4 h later with $10^5$ *C. difficile*, which had been pre-mixed with 100 µl of rabbit anti-SLP serum (green line, n = 8) or rabbit pre-immune serum (blue line, n = 8) or no serum (purple line, n = 4). Four further 100 µl doses of serum were administered at –7 h, 6 h, 17 h and 24 h of infection. Time zero = time of administration of *C. difficile*. (B) Post *C. difficile* weight profiles.

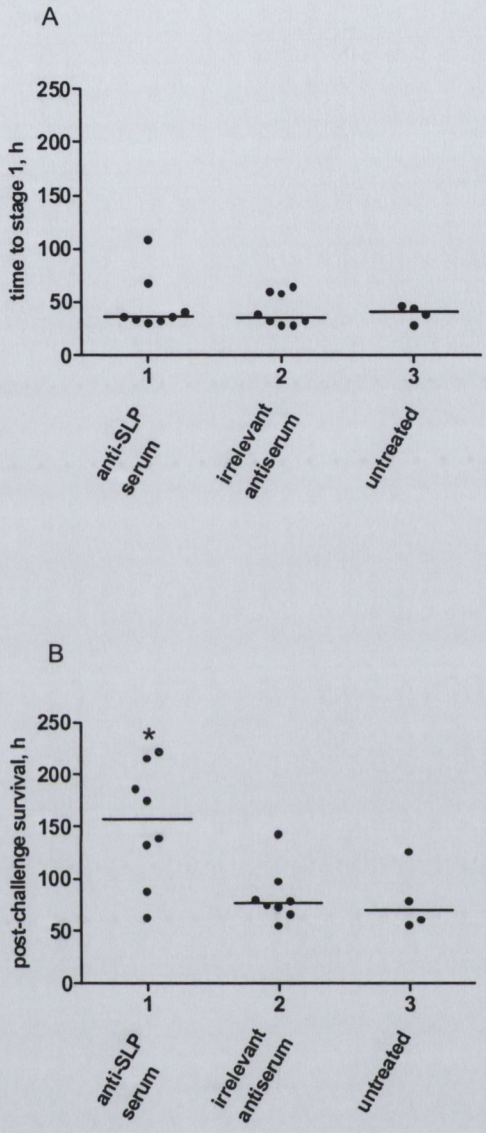



**Fig. 4.4 Passive immunisation with anti-SLP prolongs survival of *C. difficile* infected hamsters.** Following clindamycin treatment hamsters were given rabbit anti-SLP serum (group 1, n = 8) or rabbit irrelevant serum (group 2, n = 8) or no serum (group 3, n = 4). All groups received $10^5$ *C. difficile*. Time zero = time of administration of *C. difficile*. Dots (•) represent individual hamsters; bars represent median values for each group. (A) Time to first symptoms. (B) Post-challenge survival of hamsters. Treatment groups are on the x-axis and post-challenge survival (h) is on the y-axis. Differences in means were calculated using a non-parametric two-tailed Mann Whitney test.

**TABLE 4.2 Hamster post-*C. difficile* challenge survival[a] summary statistics**

| Group[b] | Median survival, h | Survival range, h | 95% CI for mean survival, h |
|---|---|---|---|
| 1 (n = 8) | 156.9 | 62.5, 221.5 | 104.4, 200.6 |
| 2 (n = 8) | 76.6 | 55.2, 143.0 | 61.9, 105.0 |
| 3 (n = 4) | 69.6 | 55.3, 126.4 | 43.9, 116.6 |

[a]Post-challenge survival measured from time of administration of *C. difficile*.

[b]Group 1 received anti-SLP serum plus $10^5$ CFU, group 2 received irrelevant serum plus $10^5$ CFU, group 3 received $10^5$ CFU only.

Dr. George McDonald, pathologist, Department of Histopathology and Morbid Anatomy, Trinity College Dublin.

**Figure 4.5, panels (A), (C)** and **(D)** show caecum from hamsters that received pre-immune serum and $10^2$ *C. difficile* and remained well throughout the experiment. Focal full thickness loss of the epithelium was evident in some areas as seen in **(A)** and **(C),** while normal epithelium remained in other areas **(D)**. **Figure 4.5 (B)** shows colon from a hamster treated with pre-immune serum and $10^2$ *C. difficile* that remained well throughout the experiment. Again, some areas of normal epithelium were observed, but extensive damage was also obvious. **Figures 4.5 (E)** and **(F)** show colon from a control hamster that received $10^2$ *C. difficile* only and recovered despite having had advanced severe classical symptoms of CDAD. Some areas of epithelial damage were evident in tissue from this hamster, but large areas of completely normal epithelium remained, such as that shown in the top left hand quadrant of **(E)** and also in **(F)**.

**Figure 4.6** shows colon **(A)**, **(D)** and **(F)** and caecum **(B)**, **(C)** and **(E)** from hamsters treated with anti-SLP serum and subsequently given $10^3$ CFU *C. difficile* that remained well throughout the experiment. Some areas of normal colonic epithelium were observed **(A)** but large areas of extensive damage were also evident **(D)**. Extensive loss of the epithelium was observed in the caecal mucosa, as seen in **(B)**, **(C)** and **(E)**. Mucin is seen streaming from the openings of the damaged crypts in **(C)**. Normal epithelium adjacent to severely desquamated epithelium is seen in **(E)**. This type of pattern was evident throughout the colon and caecum sections.

**Figure 4.7** shows caecum and colon sections from hamsters that died from CDAD. (A) and (B) show caecum from an anti-SLP treated hamster that received $10^5$ *C. difficile*, and, from a control hamster that received irrelevant serum, respectively. (C) and (D) show colon from an anti-SLP treated hamster that received $10^5$ *C. difficile*. Since all pathology samples were taken post-mortem, severe desquamation of the epithelium and acute inflammation was evident in all sections, regardless of whether they received anti-SLP serum or not. Dilated blood vessels, as seen between the arrows in **(A)**, were seen in a number of sections. Acute inflammation, as seen in **(B)**, was evident in all sections. Desquamation of the epithelium and the full depth of the crypts was seen, along with areas of haemorrhage in many sections, as illustrated in **(C)** (arrow). Lymphoid aggregates, as seen between the arrows in **(D)**, were also observed in a number of sections.

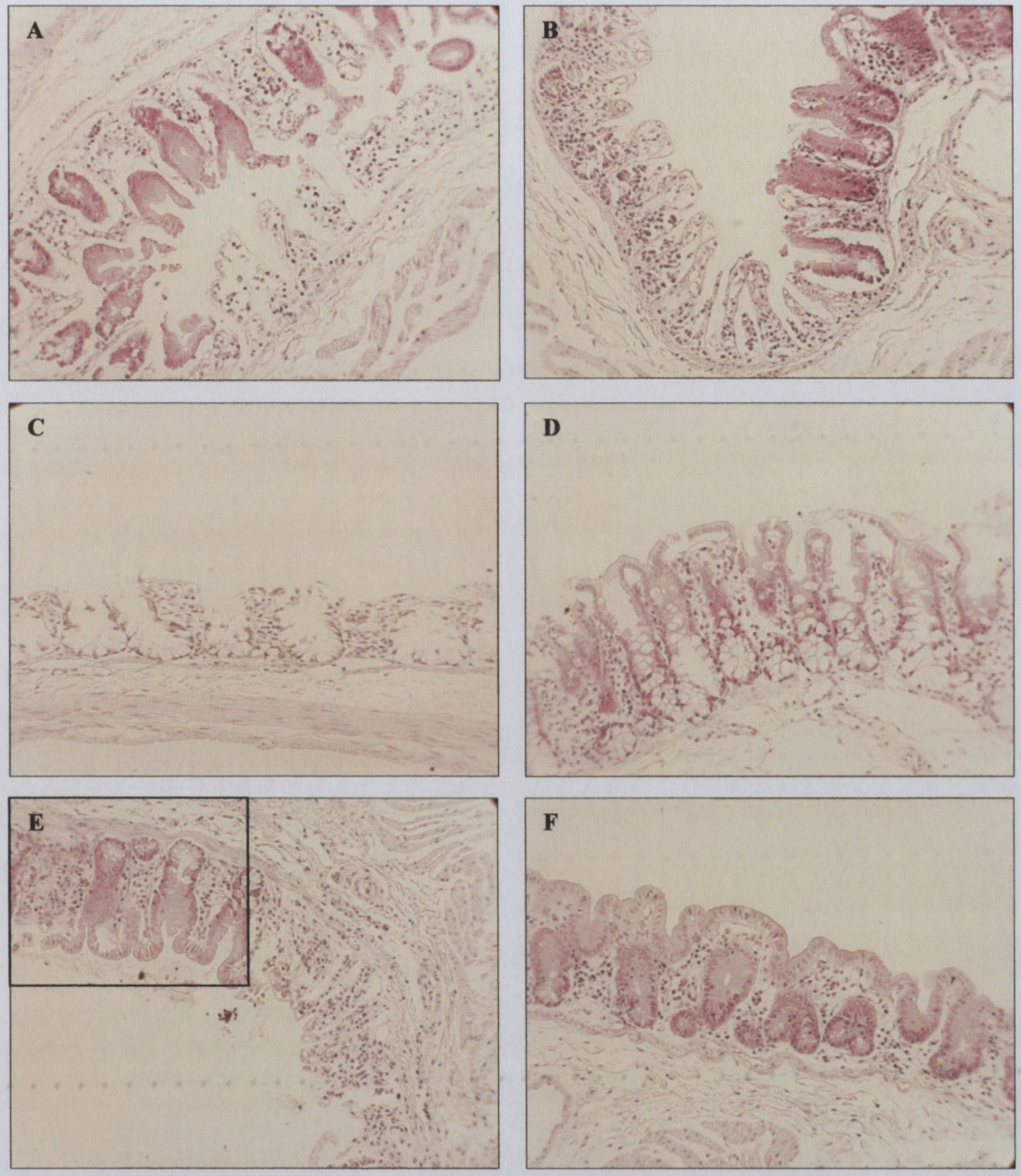



**Fig. 4.5 Histology of colon and caecum from control hamsters passively immunised with pre-immune serum and infected with *C. difficile* or unimmunised *C. difficile* only controls.** Tissues were sectioned at 5 μm, stained with haematoxylin and eosin, and slides photographed at 200X magnification. (A) Caecum from a control hamster, which received preimmune serum and $10^2$ *C. difficile* and remained well. Panels B (colon), C and D (caecum) are from a control hamster, which received $10^2$ *C. difficile* and pre-immune serum and remained well. E and F show colon from a control hamster, which received $10^2$ *C. difficile* only and recovered from severe CDAD symptoms.

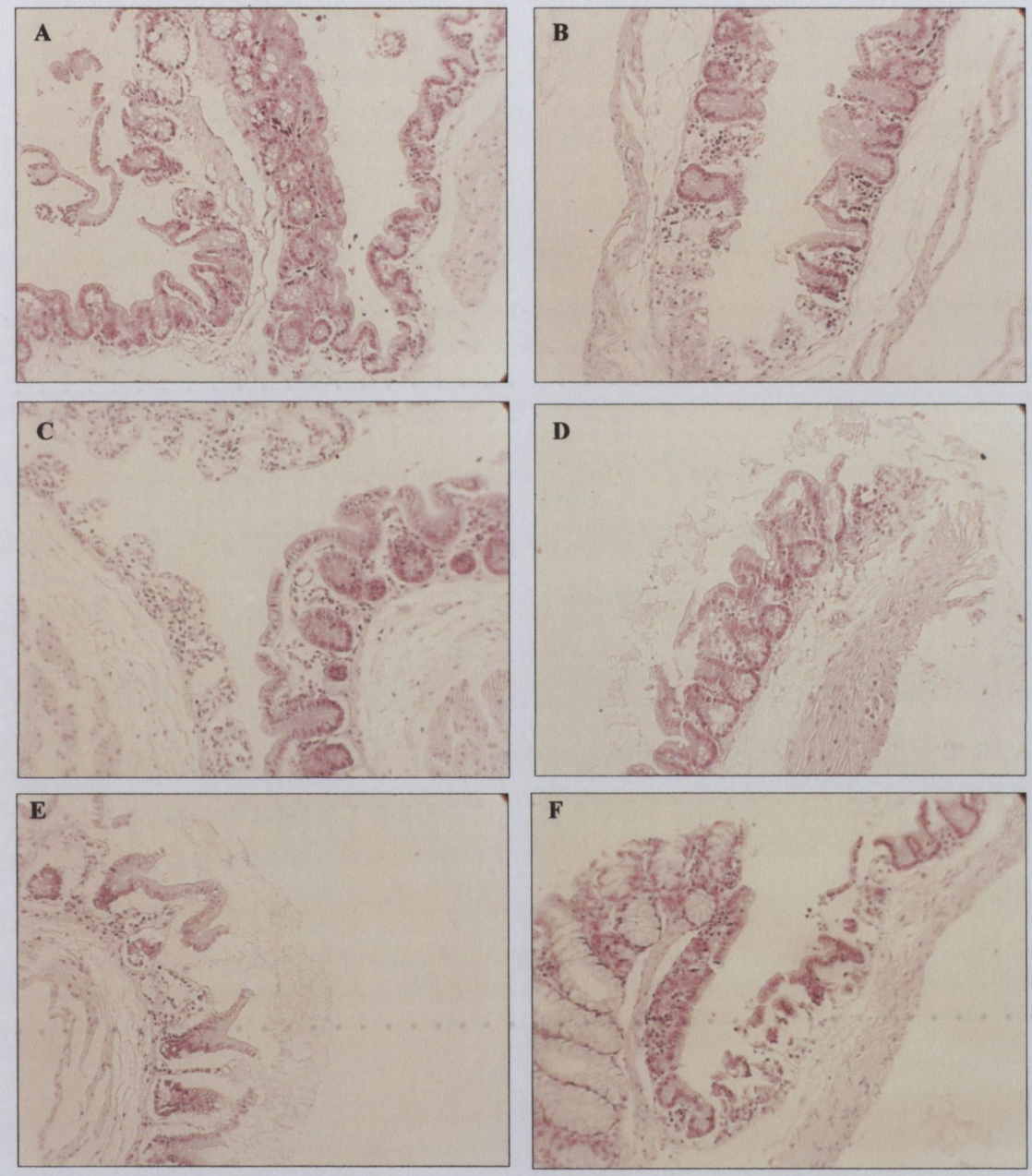



**Fig. 4.6 Histology of colon and caecum from hamsters passively immunised with anti-SLP and infected with *C. difficile*.** Tissues were sectioned at 5 μm, stained with haematoxylin and eosin, and slides photographed at 200X magnification. Panels A, D and F (colon) and panels B, C and E (caecum) from hamsters that received anti-SLP serum and $10^3$ *C. difficile* and remained well.

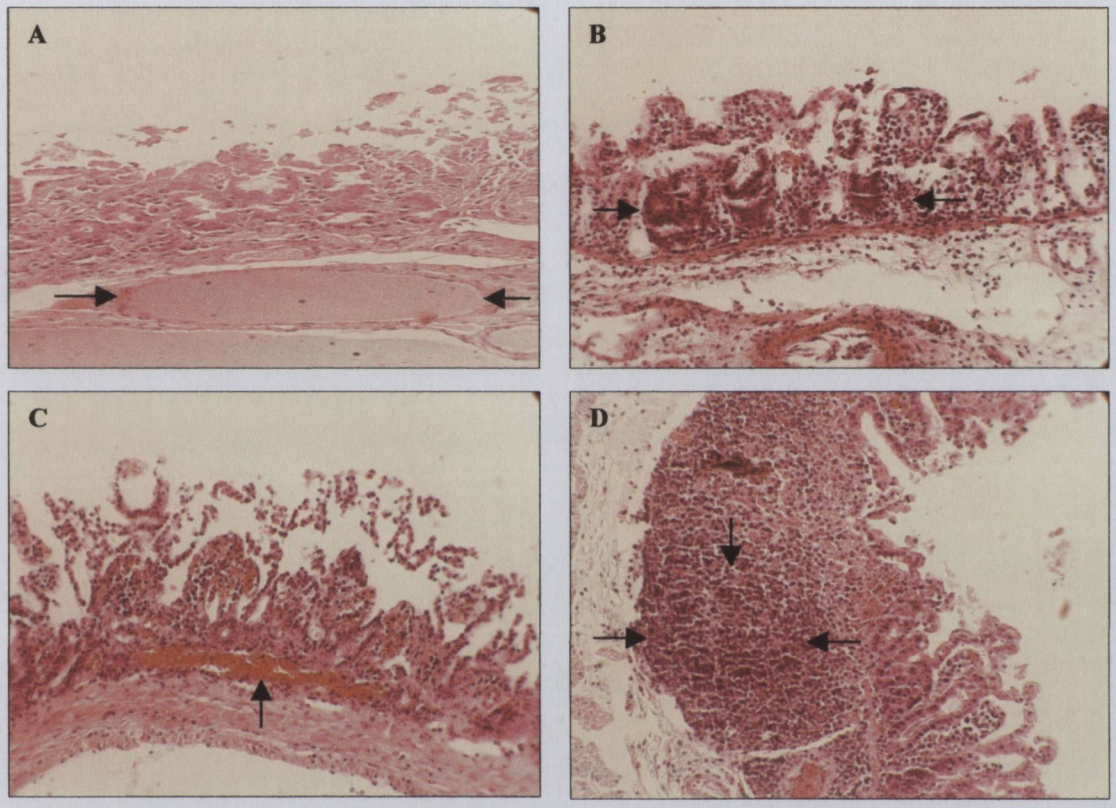



**Fig. 4.7 Histology of colon and caecum from hamsters passively immunised with anti-SLP and infected with *C. difficile*.** Tissues were sectioned at 5 μm, stained with haematoxylin and eosin, and slides photographed at 200X magnification. (A) Caecum from an anti-SLP treated hamster, which received $10^5$ *C. difficile* and died from *C. difficile* infection. An enlarged blood vessel is indicated with an arrow. (B) Caecum from a control hamster, which received irrelevant serum and $10^5$ *C. difficile* and died from *C. difficile* infection. An infiltrate of acute inflammatory cells is visible between the arrows. Panels C and D show colon from an anti-SLP treated hamster, which received $10^5$ *C. difficile* and died from *C. difficile* infection. An area of haemorrhage (C) and a lymphoid aggregate (D) are indicated with arrows.

The level of epithelial damage and acute inflammation observed on histological analysis of colon and caecum sections, even in hamsters that remained well, illustrates the severity of the hamster model of CDAD and hence its stringency as a test of the protective effects of the anti-SLP serum.

### 4.3.5 Serum IgG antibodies to *C. difficile* antigens in surviving hamsters.

ELISA analysis was used to assess if circulating anti-SLP antibodies were present and may have been responsible for the surviving hamsters in the first and second passive immunisation experiments. As shown in **Figure 4.8**, hamsters treated with pre-immune serum had low serum anti-SLP titres (bars 3 and 4 respectively). As one of the *C. difficile* only hamsters showed severe symptoms of CDAD and subsequently recovered, high anti-SLP titres might have been expected. Interestingly, this was not the case (**Figure 4.8,** bar 5). A definitely raised titre was seen in one of the surviving hamsters treated with anti-SLP serum and a slightly raised titre in the second (**Figure 4.8,** bars 1 and 2 respectively). This is unexpected, as these hamsters developed no or only slight symptoms of *C. difficile* infection, and should have had minimal exposure to *C. difficile* as the inoculum was co-administered with the anti-SLP serum. Western blot analysis was used to assess if the same surviving hamsters had developed antibodies against another common antigen or antigens that might have afforded them protection from *C. difficile*. To achieve this, a *C. difficile* lysate **(Figure 4.9, A)** was probed with serum from the five surviving hamsters. A high molecular weight band was recognised by the sera from all five hamsters at a dilution of 1/100 and most likely represents antibody to the toxins (toxins A and B have molecular weights of 308 kDa and 270 kDa respectively). It is possible that these anti-toxin antibodies afforded some level of protection to the hamsters. A variety of lower molecular weight bands were recognised by the sera from all five hamsters, but no one antigen appeared to be dominant **(Figure 4.9, B)**.

### 4.3.6 Anti-SLP enhanced *C. difficile* phagocytosis.

Considering the effect of the anti-SLP serum on post-challenge survival of hamsters in the final passive immunisation experiment, a potential mechanism of action of the antiserum would be by increasing phagocytosis of *C. difficile*. To investigate this possibility, phagocytosis of fluorescent stained *C. difficile* by differentiated THP-1 monocytes was assessed in the presence and absence of the rabbit anti-SLP serum. Using this technique, 100% of *C. difficile* were consistently stained with CFSE **(Figure 4.10, A).** CFSE-*C. difficile* fluorescence could be readily quenched by the addition of 0.8 mg/ml crystal violet

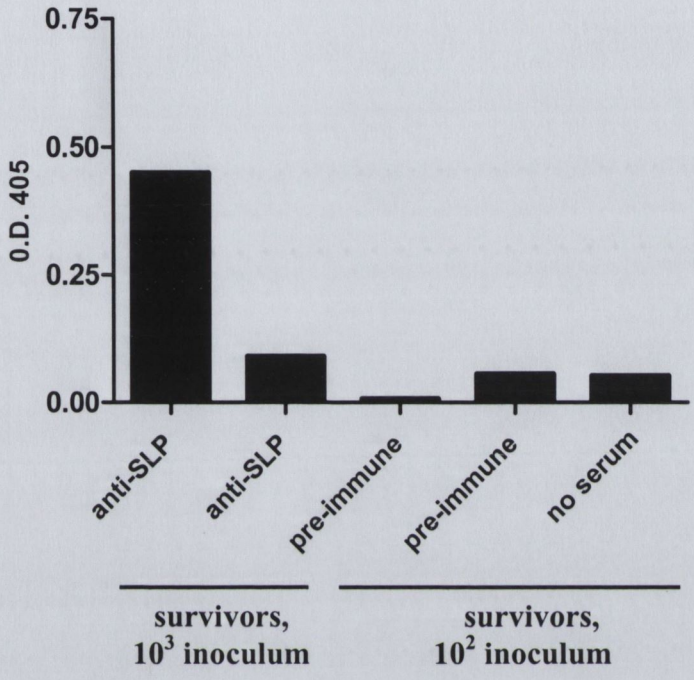



**Fig. 4.8 Serum IgG anti-SLP levels in surviving hamsters, passively immunised with anti-SLP or pre-immune serum or unimmunised *C. difficile* only control.** Serum samples were taken two weeks post-infection from surviving hamsters in the first passive immunisation experiments. The sera (diluted 1/50) were analysd by ELISA for anti-SLP titres. Each bar represents a single hamster and individual treatments are shown on the x-axis in the following order: - hamsters treated with anti-SLP serum and $10^3$ *C. difficile* (bars 1 and 2); hamsters treated with preimmune serum and $10^2$ *C. difficile* (bars 3 and 4); hamster treated with $10^2$ *C. difficile* alone (bar 5).

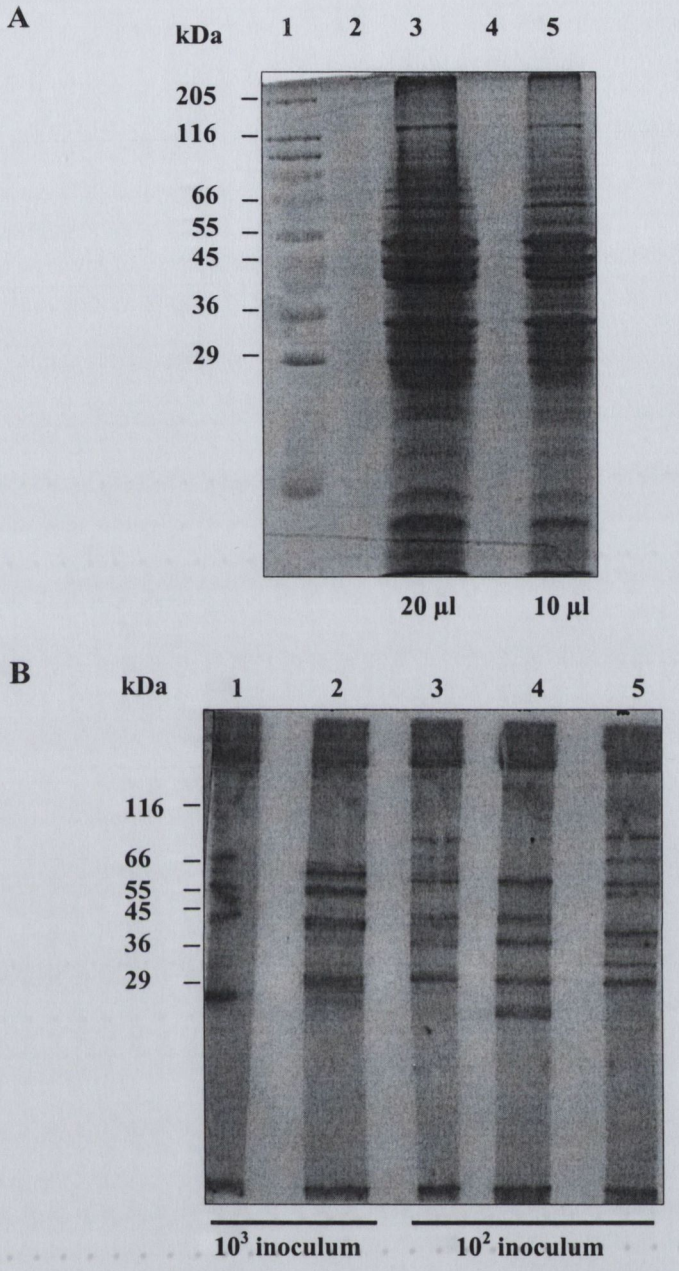



**Fig. 4.9 Western blot of *C. difficile* lysate probed with serum from surviving hamsters immunised with anti-SLP or pre-immune serum or unimmunised *C. difficile* only control.** (A) Ribotype 1 (UK PHLS # R13537) *C. difficile* lysate (lanes 3 (20 µl) and 5 (10 µl)) on 12% SDS-PAGE stained with Coomassie blue. Molecular masses of markers in kDa (lane 1) are indicated on the left. (B) Western blot of *C. difficile* lysate (10 µl/lane) probed with serum from surviving hamsters in the first passive immunisation experiments. Hamster sera were taken two weeks post-infection and were diluted 1:100. Anti-SLP serum and $10^3$ *C. difficile* treated hamster (lanes 1 and 2); Pre-immune serum and $10^2$ *C. difficile* treated hamsters (lanes 3 and 4); $10^2$ *C. difficile* treated hamster (lane 5). The secondary antibody used was goat anti-hamster IgG HRP. The Western blot was developed with ECL and exposed for 5 min.

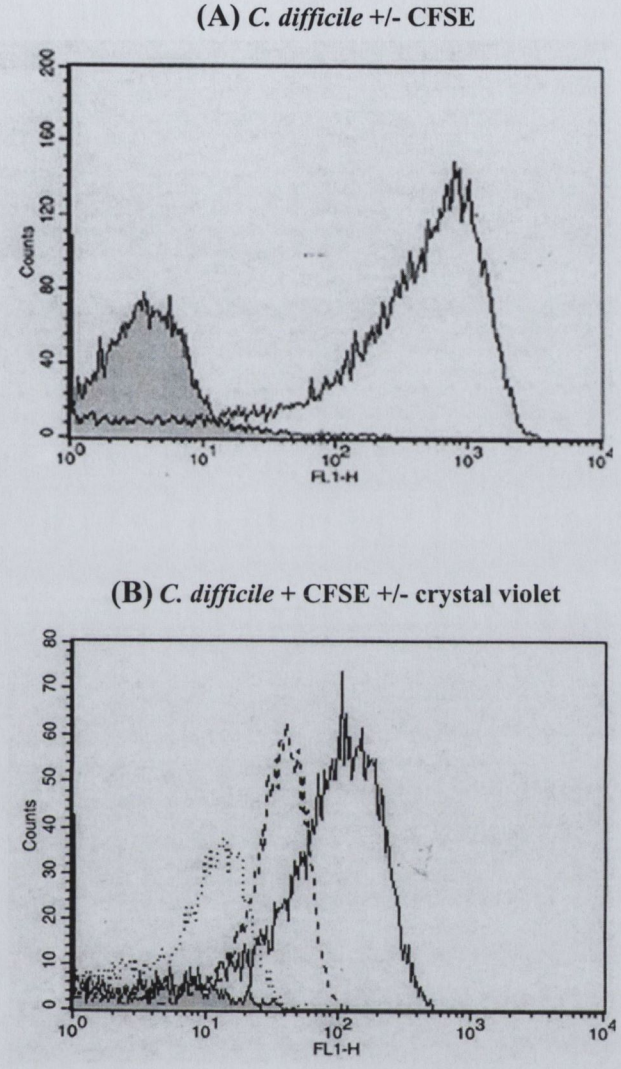



**Fig. 4.10 CFSE staining of *C. difficile*.** (A) Unstained *C. difficile* (grey histogram), CFSE stained *C. difficile* (open histogram). (B) Crystal violet quenching of CFSE-*C. difficile* fluorescence. CFSE-*C. difficile* (open histogram), plus 1.6 mg/ml crystal violet (open histogram, dashed line), plus 0.8 mg/ml crystal violet (open histogram, dotted line), unstained *C. difficile* (grey histogram). Single THP-1 cells were gated using FSC and SSC. Generally, 30,000 events were acquired using a FACSCalibur and data was analysed with Cell Quest software (Becton Dickinson). Graphs are representative of at least three independent experiments.

**(Figure 4.10, B)** to eliminate fluorescence from cell-surface attached *C. difficile*. A low number of THP-1 cells were shown to phagocytose CFSE-*C. difficile* (17.97% THP-1 cells) **(Figure 4.11, A).** The addition of the anti-SLP serum resulted in markedly increased phagocytosis of *C. difficile* by the THP-1 cells (69.19%) as compared to the irrelevant serum **(Figure 4.11, B)**. The addition of an antiserum raised against the recombinant low-MW SLP (Chapter III, **Figure 3.3**) also resulted in increased phagocytosis of *C. difficile* **(Figure 4.11, C)**, indicating that the anti-low-MW SLP serum might function *in vivo* in the same manner as the anti-SLP serum. To eliminate the possibility that the increase in phagocytosis was due to complement in the sera, the anti-SLP serum was heat treated (56°C, 30 min) to inactivate complement before the phagocytosis assay. No major contribution of complement to the phagocytosis was found **(Figure 4.12, A)**. These results suggest that the anti-SLP antibodies may exert at least some of their protective effect *in vivo* by enhancing phagocytosis of *C. difficile*. The Ribotype 1 strain (PHLS UK ID number R13537) used for the phagocytosis assay produces both toxin A and toxin B, and it was possible that the toxins were contributing to the inhibition of phagocytosis seen in the absence of an opsonin. To investigate this possibility, a Ribotype 17 toxin B-only strain (PHLS UK ID number R13702) and a Ribotype 31 toxin negative strain (PHLS UK ID number R13711) were assessed in the phagocytosis assay. All non-opsonised *C. difficile* were poorly ingested by the THP-1 cells, regardless of their toxin status **(Figure 4.12, B).** This result suggested that the *C. difficile* toxins were not an anti-phagocytic factor in this system. *E. coli* DH5α (pKFW408) expressing a modified GFP was used as a positive control for phagocytosis in each experiment **(Figure 4.12, C)**.

### 4.3.7 Cross-reactivity of SLPs from *C. difficile* clinical isolates with anti-SLP.

Cross reactivity of different *C. difficile* strains with the Ribotype 1 anti-SLP serum was investigated. To achieve this, lysates of six clinical isolates of *C. difficile* from four different ribotypes were resolved on SDS-PAGE and immunoblotted with the anti-SLP serum. The six *C. difficile* strains were isolated from different episodes of CDAD in St. James's Hospital, Dublin (Dr. Rachel Doyle) and typed by the Public Health Laboratory Service, Anaerobe Reference Unit, Public Health Laboratory, University Hospital of Wales. As seen in **Figure 4.13 (A)** lysates from the Ribotype 1 clinical isolates tested have similar patterns on a 12% SDS-PAGE gel stained with Coomassie blue. It is likely that these are identical strains (sequencing of the SLPs from these three strains revealed 100% sequence identity (Dr. Déirdre Ní Eidhin, manuscript in preparation). As expected, the anti-SLP serum showed strong antigenic reactivity with both the low and high-MW SLPs

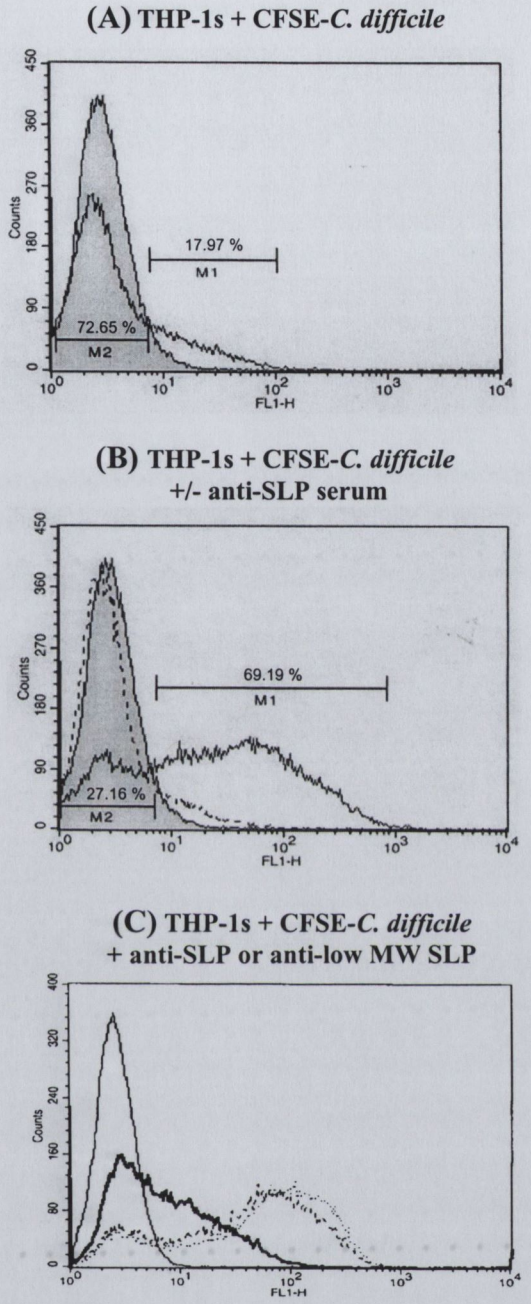



**Fig. 4.11** ***C. difficile*** **phagocytosis by THP-1 cells in the presence of rabbit anti-SLP and anti-low-MW SLP serum.** (A) THP-1 cells alone (grey histogram), THP-1 cells plus CFSE-*C. difficile* (open histogram). (B) Phagocytosis of CFSE-*C. difficile* by THP-1 cells in the presence of 12.5% rabbit anti-SLP serum (open histogram) or 12.5% irrelevant antiserum (open histogram, dashed line), THP-1 cells alone (grey histogram). (C) Phagocytosis of CFSE-*C. difficile* by THP-1 cells in the presence of 12.5% rabbit anti-SLP serum (open histogram, dashed line) or 12.5% anti-low-MW SLP serum (open histogram, dotted line) or 12.5% pre-immune serum (open histogram, thick black line). THP-1 cells alone (open histogram, thin black line). THP-1 cells were gated using FSC and SSC. 30,000 events were acquired using a FACSCalibur and data was analysed with Cell Quest software (Becton Dickinson). Graphs are representative of at least three independent experiments.

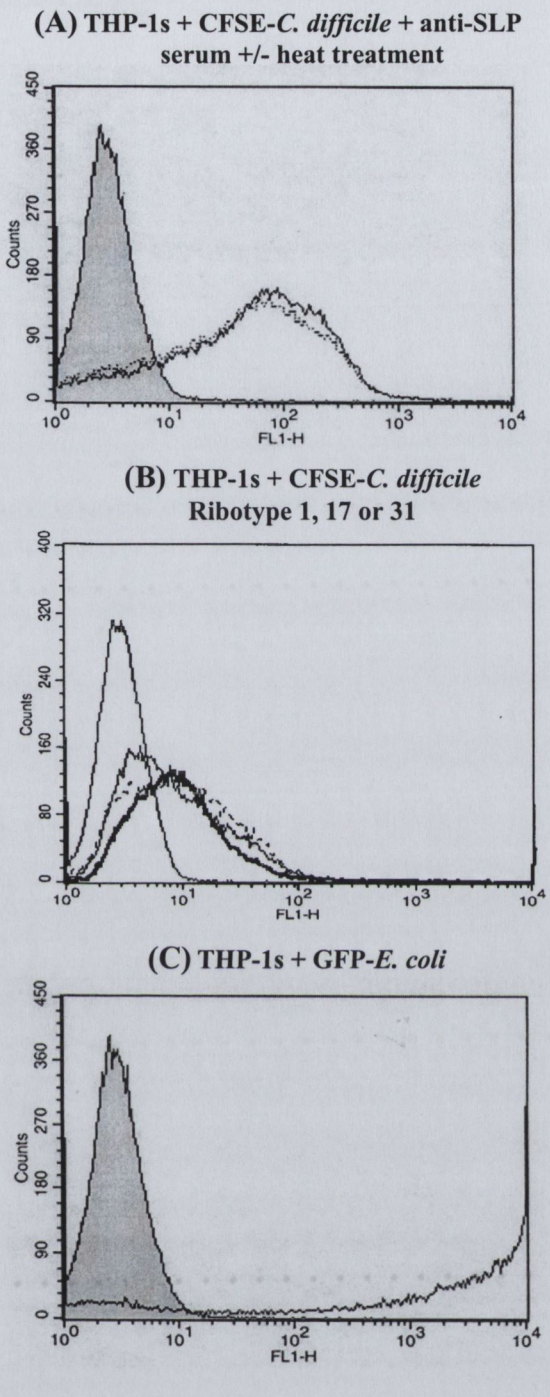



**Fig. 4.12** **Controls for phagocytosis assay.** (A) Phagocytosis of *C. difficile* by THP-1 cells in the presence of anti-SLP serum (open histogram) or heat-treated (56°C for 30 min) anti-SLP serum (open histogram, dotted line), THP-1 cells alone (grey histogram). (B) Phagocytosis of Ribotype 1 (open histogram, thick black line), Ribotype 17 (open histogram, dashed line) and Ribotype 31 (open histogram, dotted line) *C. difficile* strains, THP-1 cells alone (open histogram, thin black line). (C) Phagocytosis of *E. coli* DH5α (pKFW408) expressing a modified GFP, by THP-1 cells (open histogram). THP-1 cells alone (grey histogram). Single THP-1 cells were gated using FSC and SSC. 30,000 events were acquired using a FACSCalibur and data was analysed with Cell Quest software. Graphs are representative of at least three independent experiments.

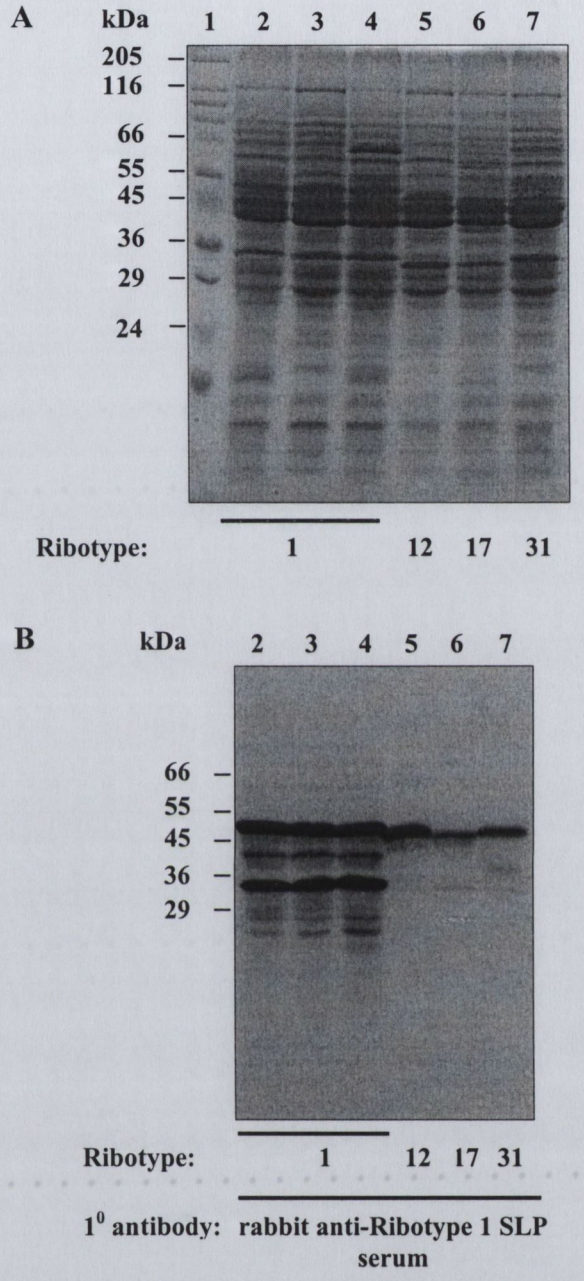



**Fig. 4.13 Western blot of *C. difficile* lysates from six clinical isolates probed with rabbit anti-Ribotype 1 SLP serum.** (A) *C. difficile* lysates (10 µl) from 6 clinical isolates run on 12% SDS-PAGE gel stained with Coomassie blue. Molecular masses of markers, in kDa, are indicated on the left of each panel. PHLS UK ID numbers are in brackets. Lanes 2, 3 and 4 = Ribotype 1 strains R13537, R12879 and R14637 respectively; Lane 5 = Ribotype 12 strain R12822; Lane 6 = Ribotype 17 strain R13702; Lane 7 = Ribotype 31 strain R13711. (B) Western blot of an identical gel probed with rabbit anti-SLP serum (1:20000) raised against a Ribotype 1 strain, R13537. The secondary antibody used was swine anti-rabbit IgG HRP. The blot was developed with ECL and exposed for 10 seconds.

from the three Ribotype 1 strains (**Figure 4.13, B,** lanes 1-3). Conversely, the antiserum did not recognise the highly variable low-MW SLPs from the Ribotype 12, 17 or 31 strains, but reacted only with the more antigenically conserved high-MW SLP **(Figure 4.13, B,** lanes 3-6**)**. This data is in agreement with the well-known heterogeneity among and poor cross-reactivity between low-MW SLPs from different *C. difficile* strains (Cerquetti *et al.*, 2000, McCoubrey and Poxton, 2001).

## 4.4 DISCUSSION

In this study, the protective capacity of rabbit anti-*C. difficile* SLP serum against CDAD was tested in a lethal hamster challenge model. The anti-SLP serum was assessed as a single parameter independently of the toxins, which have previously been shown to protect against CDAD in the hamster model of disease (Giannasca *et al.*, 1999, Lyerly *et al.*, 1991).

When challenged with $10^2$ CFU *C. difficile*, all anti-SLP treated hamsters (n = 5) died from *C. difficile* infection. Two out of three pre-immune treated and one out of two *C. difficile* only hamsters survived challenge with the same inoculum. This suggests that a $10^2$ CFU inoculum is at the lower threshold of an infective dose, potentially explaining why some of the control hamsters survived. It was also a possibility that the inoculum was in fact less than the calculated $10^2$ CFU *C. difficile*. Although every precaution was taken, it was possible that *C. difficile* was exposed to oxygen while in transit to the animal facility. As *C. difficile* is a fastidious anaerobe, there would undoubtedly have been some die off in the inoculum under these circumstances. However, it was also possible that the survival advantage of these control hamsters was due to an immune response to some *C. difficile* antigen or antigens. The hamsters might have developed antibodies to *C. difficile* antigens had they been exposed to the bacterium prior to the challenge, although this was unlikely as the hamsters were bred and housed in a specific pathogen free environment. To investigate this possibility, Western blot analysis of *C. difficile* lysates probed with serum from surviving hamsters was performed. The absence of any significant early antibody response indicates that the control hamsters did not survive due to an IgG antibody response to a *C. difficile* antigen. Given the dynamics of antibody production, it would have been more relevant to measure the IgM anti-SLP levels in relationship to the acute response. However, no anti-hamster IgM conjugated secondary antibody was available at the time of this study. It is possible that IgM antibodies were present, but were not detected with the anti-hamster IgG secondary antibody used for the Western blot. Following

challenge with a ten-fold higher dose of *C. difficile* ($10^3$ CFU), two out of five anti-SLP treated hamsters survived and all pre-immune treated (n = 3) and *C. difficile* only (n = 2) hamsters died from *C. difficile* infection.

Histological analysis of colon and caecum from the surviving hamsters revealed extensive damage of the tissues, indicating that these hamsters were indeed colonised with *C. difficile*. It is likely they did not receive a large enough bacterial dose, possibly due to the action of the anti-SLP serum, to develop clinical symptoms and had sufficient normal colonic and caecal mucosa to maintain normal function of these tissues.

Passive immunisation using anti-SLP antibodies and challenge with $10^5$ *C. difficile* was shown to significantly delay progress of *C. difficile* infection in the lethal hamster challenge model. Although all experimental and control hamsters eventually succumbed to disease, this result indicates that anti-SLP antibodies can modulate the course of *C. difficile* infection and may therefore merit closer investigation as vaccine candidates against CDAD. This identifies anti-SLP antibodies as playing a protective, albeit incomplete, role in an *in vivo* model of *C. difficile* infection for the first time. It is likely that many additional factors are involved in the attachment of *C. difficile* to the gut wall, including SLP paralogs, the adhesion cwp66 and environmental factors. The limited protective effect of passive immunisation with anti-SLP serum may partly reflect this fact.

The mechanism of action of the anti-SLP serum is most likely multifactorial. Following *C. difficile* colonisation of the gut epithelium and subsequent toxin production, the epithelial barrier is severely disrupted resulting in an influx of phagocytic cells. Upon arrival at the subepithelial space, *C. difficile* will encounter locally resident as well as newly infiltrated professional phagocytic cells. Despite the presence of such phagocytes, *C. difficile* can persist in this inflammatory exudate (Dailey *et al.*, 1987). Using an *in vitro* assay, a marked increase in phagocytosis of *C. difficile* by a human monocytic/phagocytic cell line was observed in the presence of the anti-SLP serum. These data suggest that the prolonged survival of anti-SLP treated hamsters might be explained through an increase in phagocytosis of *C. difficile* at damaged mucosal surfaces. However, as shown *in vitro,* the anti-SLP serum might also inhibit colonisation by agglutinating *C. difficile in vivo* and therefore inhibiting binding of the bacterium to the epithelium. A correlation between anti-toxin mediated agglutination of *C. difficile* and protection against death has previously been demonstrated in a hamster study (Torres *et al.*, 1995).

Hamsters are highly sensitive to the *C. difficile* toxins and the systemic action results in death (Bartlett *et al.*, 2004, Borriello *et al.*, 1987, Delmée and Avesani, 1990). In the final passive immunisation study all hamsters showed a similar time to first symptoms and eventually died, indicating that independently of treatment all were exposed to the *C. difficile* enterotoxins. Hence, the prolonged survival in the anti-SLP treated group most likely reflects exposure to lower toxin concentrations from a lower bacterial load achieved by increased phagocytosis. The recognition and interaction of the anti-SLP antibodies with SLP on intact *C. difficile* could be hindered by spatial and temporal constraints inherent to the *in vivo* setting, allowing for the presence of free bacteria. To minimise this possibility in our system, the *C. difficile* inoculum was pre-incubated with the respective antiserum prior to infection, an approach that has been successfully used elsewhere in passive immunisation for a gastrointestinal pathogen (Blanchard *et al.*, 1995).

The phagocytosis results are in agreement with Dailey *et al.* (1987) who showed that *C. difficile* requires opsonisation for significant phagocytosis to occur, and suggested the presence of an anti-phagocytic determinant on the surface of the bacterium. This phenomenon is seen in *Campylobacter fetus*, whose SLPs have been shown to protect the bacterium from phagocytosis and serum killing, while spontaneous mutants lacking the SLPs are serum and phagocytosis sensitive (Grogono-Thomas *et al.*, 2003, Blaser and Pei, 1993). It is tempting to speculate that the *C. difficile* SLPs may perform a similar role in contributing to the virulence of this pathogen. Recurrence of *C. difficile* symptoms occurs in 20% of cases and is associated with persistence of *C. difficile* in the stools, and in some instances the recurrence is due to the same strain of *C. difficile* (Surawicz and McFarland, 1999). Further studies involving disruption of the *C. difficile* S-layer would be necessary to investigate whether the SLPs play a role in such evasion of phagocytosis and consequent persistence of infection.

*C. difficile* toxins have been shown to be cytopathic for various cells *in vitro*, including polymorphonuclear cells, lymphocytes, monocytes and macophages (Johal *et al.*, 2004, Siffert *et al.*, 1993). Siffert *et al.*, (1993) demonstrated that toxin B profoundly reduced the phagocytic function of macrophages, its effect being greatest on non-opsonised rather than opsonised yeast. Previous studies have also demonstrated killing of macrophages by *C. difficile* toxins (Mahida *et al.*, 1998) and a reduction in the number of macrophages in colonic biopsies has been observed in patients with CDAD (Johal *et al.*, 2004). It was therefore a possibility that *C. difficile* toxins, being produced by the bacterium in the

phagocytosis assay, were interfering with the phagocytic ability of the THP-1 cells. Analysis of strains, which produce both toxin A and B, toxin B only or neither toxin showed this not to be the case. It is likely that the amount of toxin being produced in the system in the two-hour incubation time is insufficient to have an effect on the phagocytic ability of the THP-1 cells.

In this study, a *C. difficile* strain from the most commonly occurring ribotype, Ribotype 1, was used. Ribotype 1 accounts for over half of all the hospital isolates from England and Wales (Brazier, 1998b). Therefore, a vaccine based on the anti-SLP antiserum used in this study would be expected to be effective in at least fifty percent of cases. Preliminary Western blot analysis revealed that the anti-SLP serum reacted strongly with both the low and high-MW SLPs from three Ribotype 1 clinical isolate strains, but only with the more conserved high-MW SLP from Ribotype 12, 17 and 31 clinical isolate strains. The three Ribotype 1 isolates were from different *C. difficile* episodes, separated in time and space. Sequencing of their SLPs revealed 100% sequence identity (Dr. Déirdre Ní Eidhin, manuscript in preparation). However, given the well-known heterogeneity among SLPs from different *C. difficile* strains (Cerquetti *et al.*, 2000, McCoubrey and Poxton, 2001) an effective vaccine offering cross-protection between strains should contain multiple SLPs. However, it should be pointed out that this study was not designed to achieve cross protection between strains and was a proof of principal on which to base further studies.

In the gastrointestinal tract, the mucosal immune system has the vital role of preventing the uptake of harmful agents (Acheson and Luccioli, 2004) and is the major port of entry for pathogens, including *C. difficile*. Because *C. difficile* is predominantly a mucosal pathogen, the mucosal immune system is likely to be important in the host response to the organism. Based on this study, it seems possible that the development of a strong, specific mucosal immune response against the SLPs has the potential to result in improved long-term protection and elimination of bacterial carriage. Although the antiserum used in this study was IgG, it was delivered directly to the mucosal surface, thereby mimicking a mucosal immune response. This approach has been successfully used with IgG and IgY anti-toxin antibodies in the hamster model of CDAD (Giannasca *et al.*, 1999, Kink and Williams, 1998). Historically, success in passive immunisation has been a key element in devising successful strategies to develop vaccines to produce humoral immunity. Success with passive immunisation has led the way for active vaccination against tetanus, diphtheria,

poliomyelitis, meningococcal and *Haemophilus influenza* meningitis, pneumococcal pneumonia and hepatitis A and B (Krause, 1999).

In conclusion, these findings point to a role for SLPs in the design of effective vaccines against CDAD and that SLPs are strong candidate components of a composite vaccine against *C. difficile.* An active immunisation regimen based on the SLPs combined with toxin neutralisation, could provide both a preventative and therapeutic vaccine strategy by reducing bacterial colonisation and carriage, as well as toxin-mediated pathology. Potential preventative approaches against *C. difficile* disease using active immunisation regimens based on the SLPs are explored in subsequent chapters.

# CHAPTER V

# ACTIVE IMMUNISATION OF HAMSTERS AND MICE WITH *CLOSTRIDIUM DIFFICILE* SURFACE LAYER PROTEINS, AND *IN VITRO* EFFECTS OF SURFACE LAYER PROTEINS ON MURINE DENDRITIC CELLS

# CHAPTER V

## 5.1 INTRODUCTION

Passive administration of antibodies against the *C. difficile* SLPs prolonged survival of hamsters infected with *C. difficile* as demonstrated in Chapter IV **(Section 4.3.3, Figure 4.5 (B))**. However, passive immunisation does not result in the development of immunological memory, has no effector cells and results in short lived protection. Passive protection against *C. difficile* would therefore only be useful for prophylaxis or where the infection has been rapidly identified, and would not prevent re-infection through the recall of immunological memory. This study sought to investigate the potential of the *C. difficile* SLPs, when administered in combination with well-characterised adjuvants, to induce protective immunity in the hamster model of disease. The immune response against the SLPs was also investigated in a non-challenge BALB/c mouse model.

Active immunity against the *C. difficile* toxins has had some success in the hamster model of disease (Giannasca *et al.*, 1999, Torres *et al.*, 1995) and initial clinical trials in humans have been carried out (Kotloff *et al.*, 2001). Acambis Incorporated (Cambridge, Massachusetts, U.S.A.) are currently pursuing manufacture of a modified toxoid vaccine against *C. difficile* and are continuing additional clinical trials. However, toxoid based vaccines are unlikely to result in elimination of bacterial carriage and further investigations into alternative preventative approaches against *C. difficile* are warranted. In a toxin based active immunisation study in hamsters, Torres *et al.* (1995) observed that serum from hamsters protected against CDAD recognised proteins in the range 35 kDa, 45 kDa and >100 kDa on Western blotting against a *C. difficile* lysate. It is likely that the 35 kDa and 45 kDa antigens are the major surface proteins of *C. difficile*, suggesting that these proteins may play some role in protection against CDAD. The use of S-layer proteins as vaccine antigens is not novel, and successful vaccines against *C. fetus* (Grogono-Thomas *et al.*, 2003) and *Aeromonas salmonicida* (Lund *et al.*, 2003) have been developed based on their respective S-layers.

Several studies using human sera have reported elevated antibody levels against non-toxin antigens of *C. difficile*. Kyne *et al.*, (2000) showed that serum levels against non-toxin antigens were higher in asymptomatic carriers than in patients with CDAD, but these differences were not statistically significant. Mulligan *et al.* (1993) observed higher levels of serum IgA, IgM and polyvalent immunoglobulins against whole *C. difficile* in

asymptomatic carriers than in symptomatic patients. Elevated IgM levels against the SLPs have also been associated with a reduced risk of recurrent CDAD in humans (Drudy *et al.*, 2004). Pantosti *et al.* (1989) demonstrated that the *C. difficile* SLPs could elicit a serum IgG response, and that the low-MW SLP was the SLP most consistently recognised by individuals infected with *C. difficile*. This study did not find any association between IgG anti-SLP levels and protection from disease, but suggested that S-IgA antibodies might be more relevant to protection in *C. difficile* infection. These studies suggest that the host immune response against non-toxin antigens is an important factor in the development of CDAD.

The importance, or otherwise, of immunity to the cell surface components of *C. difficile* in infection was investigated in *in vivo* studies in hamsters using purified SLPs. Since IgM, IgA or cytokine responses could not be measured in hamsters, the associated immunological pathways could not be elucidated in this model. Rational vaccine design requires sensibly formulated combinations of antigen and adjuvant. To address this issue, BALB/c mice were immunised with the SLPs, in combination with a selection of adjuvants, in parallel to the hamsters. In mice, systemic responses as measured by serum IgG, mucosal responses as measured by serum and faecal IgA, and the direction of the immune response as measured by cytokine production from splenic T cells, could be analysed. Hence, our understanding of the antigen:adjuvant formulation which would be most likely to give a protective response against CDAD in a challenge model could be further developed. Although BALB/c mice are not established as a model for *C. difficile* infection, they have been used in toxin studies in the 1980's (Corthier *et al.*, 1989, Onderdonk *et al.*, 1980). Onderdonk *et al.* (1980) found the mortality rate of mice infected with *C. difficile* to be less than 2%, and the use of mice as an infection model has been abandoned. The absence of a *C. difficile* challenge, combined with the fact that Golden Syrian hamsters are outbred, made it difficult to draw parallels between the immune response to SLPs in hamsters and mice. However, due to the limited nature of the hamster model it was necessary to take this experimental approach to further investigate the immune response to the SLPs.

Highly purified antigenic preparations are often poorly immunogenic because they do not contain additional bacterial components, which often function as built-in adjuvants in traditional vaccines. Such vaccines, therefore, do not have a sufficient capacity of switching on the innate immune response, which is required to drive the adaptive immune

response (Audibert, 2003, McNeela and Mills, 2001). Vaccines lacking these natural immunomodulators therefore require adjuvants that will engage the innate immune system to stimulate antigen presentation and cytokine production necessary for successful immunisation. A successful adjuvant must be able to induce a high and long lasting, and vitally, biologically appropriate response, by modulation of the immune system. Since *C. difficile* is a mucosal pathogen, the development of a successful vaccine against *C. difficile* based on the SLPs would most likely require immunity that would prevent attachment and colonisation at the mucosal surfaces. Adjuvants known for their ability to induce mucosal immune responses against co-administered antigens were tested in this study. The main protective effector function elicited by mucosal immunisation is the stimulation of a local S-IgA antibody response and an associated immunological memory (Holgren *et al.*, 2003). Three well-characterised vaccine adjuvants, namely $Al(OH)_3$, Ribi adjuvant system (RAS) and CT were tested for their ability to induce protective immunity against the SLPs in the hamster model of infection. Additionally, Ribi, CT and two varieties of the mucosal adjuvant chitosan were investigated as effective adjuvants for inducing immune responses against the SLPs in BALB/c mice.

Aluminium adjuvants have been used in human vaccines since 1930 and are still the main adjuvants commercially available for clinical use. They have an extensive safety record and therefore have become the benchmark for evaluating new adjuvant formulations (Gupta, 1998). Aluminium adjuvant augments mainly humoral immunity through IL-4 by activating Th2 type cells, and is the adjuvant of choice where infections can be prevented by induction of serum antibodies (Cox and Coulter, 1997, Gupta, 1998). Aluminium hydroxide was the adjuvant of first choice in the hamster immunisation study because it can induce strong Th2 immune responses and because of its compatibility with clinical use. $Al(OH)_3$ has high surface area with a p*I* of 11, which favours adsorption of negatively charged proteins at neutral pH (Gupta, 1998). Giannasca *et al.* (1999) demonstrated that $Al_2O_3$ administered intramuscularly, both by itself and in combination with intranasally administered LT, induced protective immune responses against the *C. difficile* toxins in the hamster model of CDAD.

CT is one of the most widely used experimental mucosal adjuvants and has an impressive track record for inducing powerful mucosal immune responses against co-administered antigens (McNeela and Mills, 2001, Williams *et al.*, 1999). CT has been shown to stimulate mixed Th1/Th2 immune responses, with a strong bias towards Th2 (Gagliardi *et*

*al.*, 2000, Holmgren *et al.*, 2003). Thus, CT was tested as an effective adjuvant for induction of protective mucosal immune responses against the SLPs in hamsters. RAS is considered a less toxic alternative to Freund's adjuvant and has emerged as a safe and effective adjuvant in both pre-clinical and clinical trials (De Becker *et al.*, 2000, Evans *et al.*, 2003). MPL, a component of Ribi, has been shown to induce mixed Th1/Th2 immune responses (Balbridge *et al.*, 2000, Ulrich and Myers, 1995). While Ribi alone can augment humoral and cellular immune responses to foreign antigens, it has been found to be more effective when used in combination with other adjuvants (McNeela and Mills, 2001). Ribi (i.p.) administered with a toxoid vaccine conferred 100% protection against death in *C. difficile* infected hamsters, and when used in combination with CT (i.n.), also gave 100% protection against diarrhoea (Torres *et al.*, 1995). In this study, Ribi was tested both independently and in combination with CT for its ability to induce a protective immune response against the SLPs in hamsters. Furthermore, CT and a combination of CT and Ribi were tested in parallel in active immunisation regimens in BALB/c mice.

In addition to CT and Ribi, the mucosal adjuvant chitosan glutamate and a derivative of same, N-trimethyl chitosan chloride (TMC), were investigated in BALB/c mice as potential mucosal adjuvants for administration with the SLPs. Chitosan glutamate has previously been shown to induce systemic and mucosal responses against FHA of *Bordetella pertussis* when administered i.n. (Jabbal-Gill *et al.*, 1998). Chitosan glutamate is a weak base and a certain amount of acid is required to transform the glucosamine units into the positively charged, water-soluble form (Van der Lubben *et al.*, 2001b). Only protonated, soluble chitosan has the ability to trigger the opening of tight junctions that contributes to chitosans adjuvant ability (Kotzé *et al.*, 1998a). At neutral pH most chitosan molecules will lose their charge and precipitate from solution. Conversely, many purified protein antigens will precipitate or are poorly soluble in an acidic environment. To overcome this problem, TMC, a derivative of chitosan glutamate, has been synthesised, which is soluble at neutral and basic pH values up to pH 9 (Van der Lubben *et al.*, 2001b, Kotzé *et al.*, 1998b). TMC was especially useful in this study, as the SLPs have acidic isoelectric points (p*I*'s) (Hagiya *et al.*, 1992, Mastrantonio *et al.*, 1995, Takeoka *et al.*, 1991) and are therefore most soluble in a basic environment (approximately pH 8.0-8.5, personal experience). Although TMC has superior solubility to chitosan glutamate, it has been shown to be less effective at increasing the permeability of intestinal epithelial cell layers (Kotzé *et al.*, 1998b), and therefore may not be as effective an adjuvant as chitosan glutamate. We compared the effectiveness of chitosan glutamate and TMC as adjuvants for

inducing strong systemic and mucosal immune responses against the SLPs when administered intranasally in BALB/c mice.

Since innate immune cells, particularly DCs, direct the differentiation of naïve $CD4^+$ T cells into functionally distinct Th1, Th2 or Tr cell subtypes (McGuirk *et al.*, 2002), the influence of the SLPs on activation of DCs *in vitro* was examined. DCs play a pivotal role in antigen presentation *in vivo* and are present in several tissues including mucosal epithelia (Gagliardi *et al.*, 2000). Little information existed at the time of this study about the influence of the SLPs on the priming of an immune response. We examined the effects of SLPs on DCs, both independently and in combination with CT, Ribi and LPS. CT has previously been shown to induce Tr1/Th2 responses to co-administered antigen (Lavelle *et al.*, 2003). Current thinking suggests that immunosuppressive Tr cells can be induced by pathogens, either as an evasion mechanism to subvert protective Th1 responses or as a protective mechanism of the host to limit pathogen-induced pathology (reviewed in McGuirk and Mills, 2002). Preliminary *in vitro* experiments addressed the hypothesis that the *C. difficile* SLPs might activate DCs in a similar manner.

This study revealed that $Al(OH)_3$ was the most effective adjuvant for inducing serum IgG anti-SLP antibodies in hamsters, but this response was not protective against CDAD upon *C. difficile* challenge. Despite induction of low serum IgG anti-SLP titres in hamsters, Ribi and CT/Ribi combined conferred low levels of protection against CDAD. In BALB/c mice, CT was the most effective adjuvant for inducing both systemic and mucosal immune responses against the SLPs. Inclusion of Ribi in the prime immunisation did not improve immune responses in these mice. Neither chitosan glutamate nor TMC were effective adjuvants for inducing an immune response against the SLPs in mice. Finally, the SLPs were shown to induce IL-10, TGF-$\beta$1, IL-12p70 and TNF-$\alpha$ production from DCs, behaving in a very similar manner to LPS. A combination of the SLPs and CT enhanced production of the immunomodulatory cytokines IL-10 and TGF-$\beta$1, and abolished or reduced production of the pro-inflammatory cytokines IL-12p70 and TNF-$\alpha$, respectively. The addition of Ribi to the combination of SLPs and CT further enhanced the anti-inflammatory effect of SLPs and CT.

## 5.2 OBJECTIVES

1. To analyse the effects of various adjuvants on the immune response to *C. difficile* SLPs in hamsters and mice.
2. To analyse disease outcome of immunised hamsters in a *C. difficile* hamster challenge model.
3. To analyse the *in vitro* effects of purified *C. difficile* SLPs, both independently and in combination with various adjuvants, on cytokine production from murine DCs.

## 5.3 RESULTS

**OBJECTIVES 1 AND 2**

### 5.3.1 Serum IgG response in SLP/Al(OH)$_3$ immunised hamsters.

Serum IgG antibodies against the *C. difficile* SLPs were measured by ELISA in hamsters immunised i.p. with SLPs plus Al(OH)$_3$ adjuvant at each stage of the active immunisation regimen (Dr. Déirdre Ní Eidhin). As seen in **Figure 5.1**, hamsters immunised with the higher doses of SLPs (50 μg, 100 μg and 200 μg) exhibited strong serum IgG anti-SLP responses, with the 100 μg SLP dose giving the most consistent response. Increasing the SLP dose to 200 μg did not result in any further increase in serum IgG response when compared to the 100 μg SLP dose. The response was highly variable within groups. Interestingly, with the lower doses, particularly the 10 μg SLP dose, the serum antibody titre dropped with each subsequent immunisation. This is also seen with the 25 μg SLP dose, between the second boost and sacrificial bleed, both in the presence and absence of Al(OH)$_3$. A poor serum IgG anti-SLP response is seen in the absence of an adjuvant, demonstrating that the SLPs do not have any adjuvant effect of their own. No serum antibody response to the SLPs was seen in the Al(OH)$_3$ only control group. The absence of an early antibody response to the first vaccine dose suggests that the hamsters were immunologically naïve and had not been previously primed with *C. difficile* SLPs. Final titres are summarised in **Table 5.1**.

### 5.3.2 Post-challenge survival of SLP/Al(OH)$_3$ immunised hamsters.

Hamsters were predisposed to *C. difficile* infection with a 0.5 mg dose of clindamycin, and subsequently challenged with $10^5$ *C. difficile* 18 days after their final immunisation. The outcome of the *C. difficile* challenge in all vaccine groups is shown in **Figure 5.2 (A)**. All immunised and control hamsters developed diarrhoea and subsequently died from CDAD

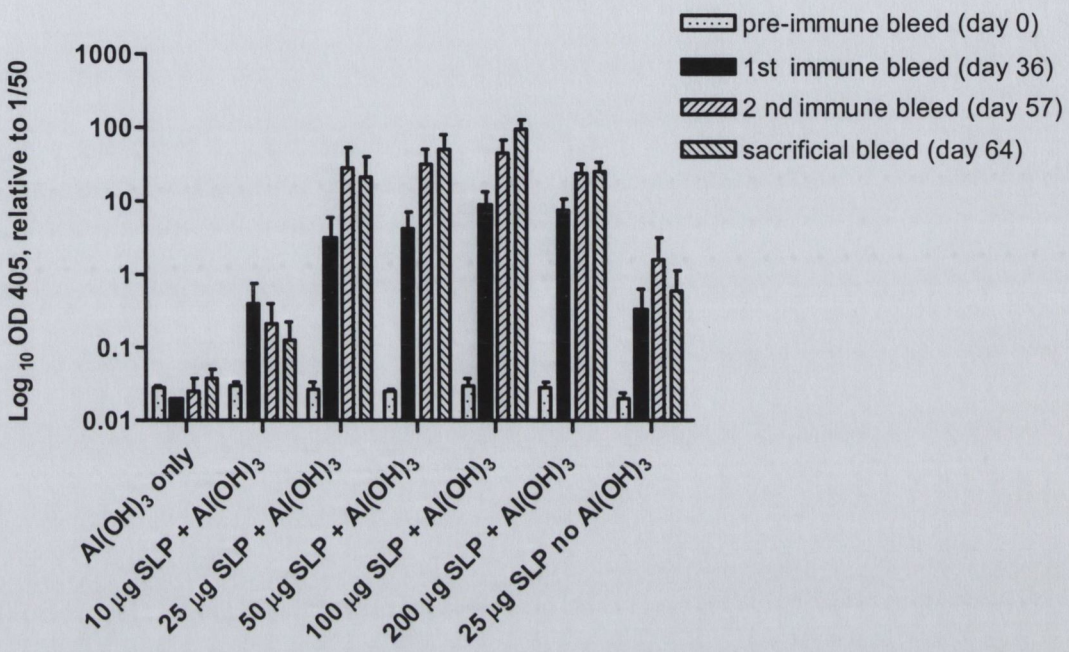



**Fig. 5.1 Serum IgG response to *C. difficile* SLPs in hamsters immunised with SLPs plus $Al(OH)_3$.** Hamsters were immunised i.p. with SLPs (10 µg, 25 µg, 50 µg, 100 µg, 200 µg) plus $Al(OH)_3$ (n = 3 per group; note: one of the 25 µg group died after 1st immune bleed). Control groups received $Al(OH)_3$ only or 25 µg SLPs only (n = 3 per group). Serum taken before the prime, after each subsequent immunisation and at sacrifice were analysed for IgG anti-SLP antibodies by ELISA. The secondary antibody used was goat anti-hamster IgG alkaline phosphatase. Titres are expressed as $\log_{10}$ of OD 405 nm and are normalised to a 1:50 dilution. Bars represent mean and SEM for each group.

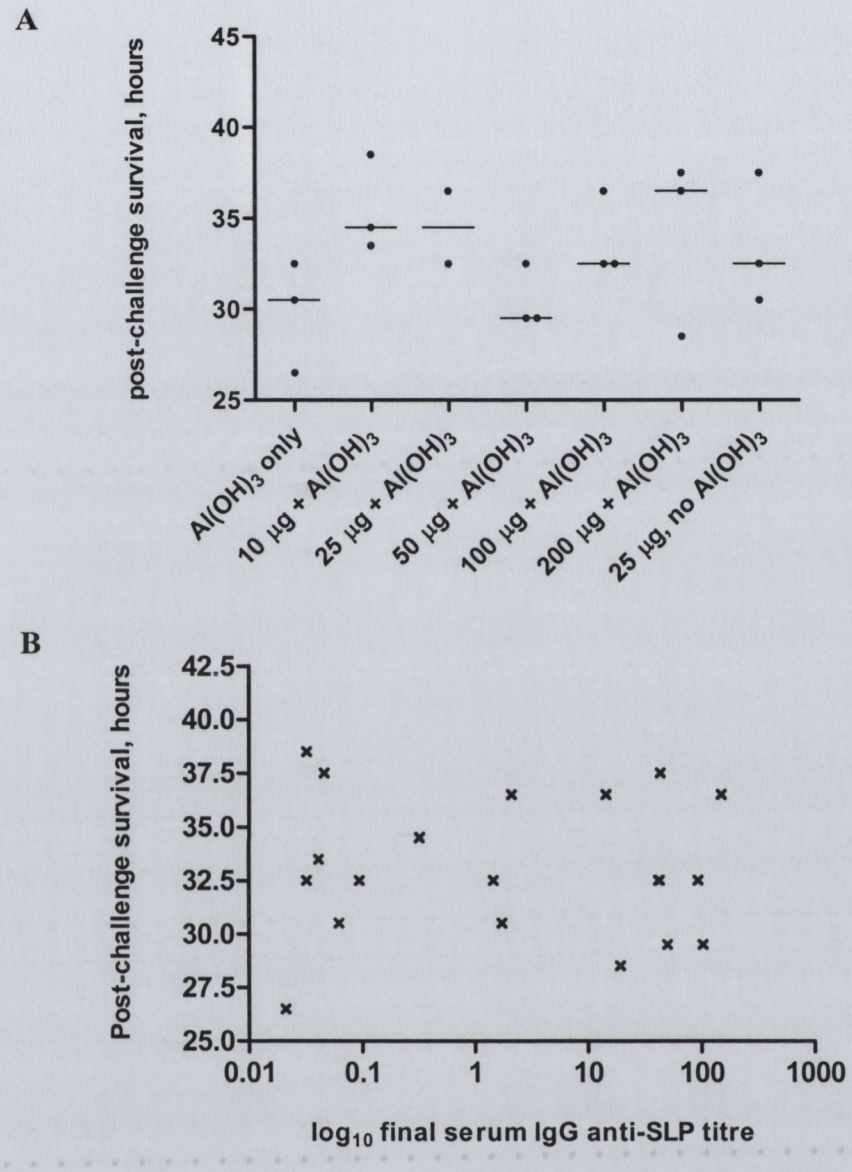



**Fig. 5.2 Post-challenge survival of SLP/Al(OH)$_3$ immunised hamsters.** Hamsters were immunised i.p. with SLPs (10 µg, 25 µg, 50 µg, 100 µg, 200 µg) plus Al(OH)$_3$ (n = 3 per group). Control groups received Al(OH)$_3$ only or 25 µg SLPs only (n = 3 per group). After their second boost, hamsters were predisposed to *C. difficile* infection with 0.5 mg clindamycin and challenged with $10^5$ *C. difficile*. (A) Post-challenge survival. Dots represent individual hamsters; bars represent median values for each group. (B) Post-challenge survival time as a function of serum IgG anti-SLP titre. Crosses represent individual hamster titres. Titres are expressed as $\log_{10}$ of OD 405 nm and are normalised to a 1:50 dilution.

within 2 days of infection (median = 32.5 h, range (26.5 h, 38.5 h)). Histological analysis of caecum and colon sections taken at sacrifice revealed the typical severe epithelial damage and inflammation seen in CDAD in the hamster model, with complete desquamation of the epithelium seen in virtually all sections (as seen in sections shown in **Chapter IV, Section 4.3.4, Figures 4.5, 4.6 and 4.7**). The median survival time of the seven groups shown in **Figure 5.2 (A)** were not significantly different were compared with a non-parametric Kruskal-Wallis test (P = 0.27). Correlation analysis was undertaken to quantify the correlation, if any, between serum IgG anti-SLP titre and post-challenge survival time. As seen in **Figure 5.2 (B)** a random scatter of points is evident, indicating no correlation between serum IgG anti-SLP titre and post-challenge survival time. Correlation analysis confirmed this observation (Spearman r = - 0.08; P = 0.74).

**TABLE 5.1 Final serum IgG anti-SLP titres[a] in hamsters immunised with SLPs/Al(OH)$_3$**

| Regimen | Mean anti-SLP titre[a] | +/- SEM |
|---|---|---|
| Al(OH)$_3$ only | 0.04 | 0.01 |
| 10 μg SLPs + Al(OH)$_3$ | 0.13 | 0.01 |
| 25 μg SLPs + Al(OH)$_3$ | 22.12 | 16.34 |
| 50 μg SLPs + Al(OH)$_3$ | 51.79 | 29.52 |
| 100 μg SLPs + Al(OH)$_3$ | 95.60 | 30.84 |
| 200 μg SLPs + Al(OH)$_3$ | 25.74 | 9.00 |
| 25 μg SLPs only | 0.63 | 0.56 |

[a]Titres are defined as OD 405 and are normalised to a 1:50 dilution.

### 5.3.3 Post-challenge weight loss of SLP/Al(OH)$_3$ immunised hamsters.

In order to look for a correlation between weight loss and protection from diarrhoea and death, hamster weight following *C. difficile* challenge was monitored. In this experiment no hamsters survived the challenge, but weight loss was analysed nonetheless and is shown in **Figure 5.3 (A)**. There was no difference in median percentage weight loss between immunisation regimens as tested by a non-parametric Kruskal-Wallis test (P = 0.36). Similarly, no correlation between percentage weight loss and post-challenge survival **(Figure 5.3, B)** was seen (Spearman r = - 0.41; P = 0.08). Since all hamsters succumbed to CDAD rapidly following challenge, differences in percentage weight loss between

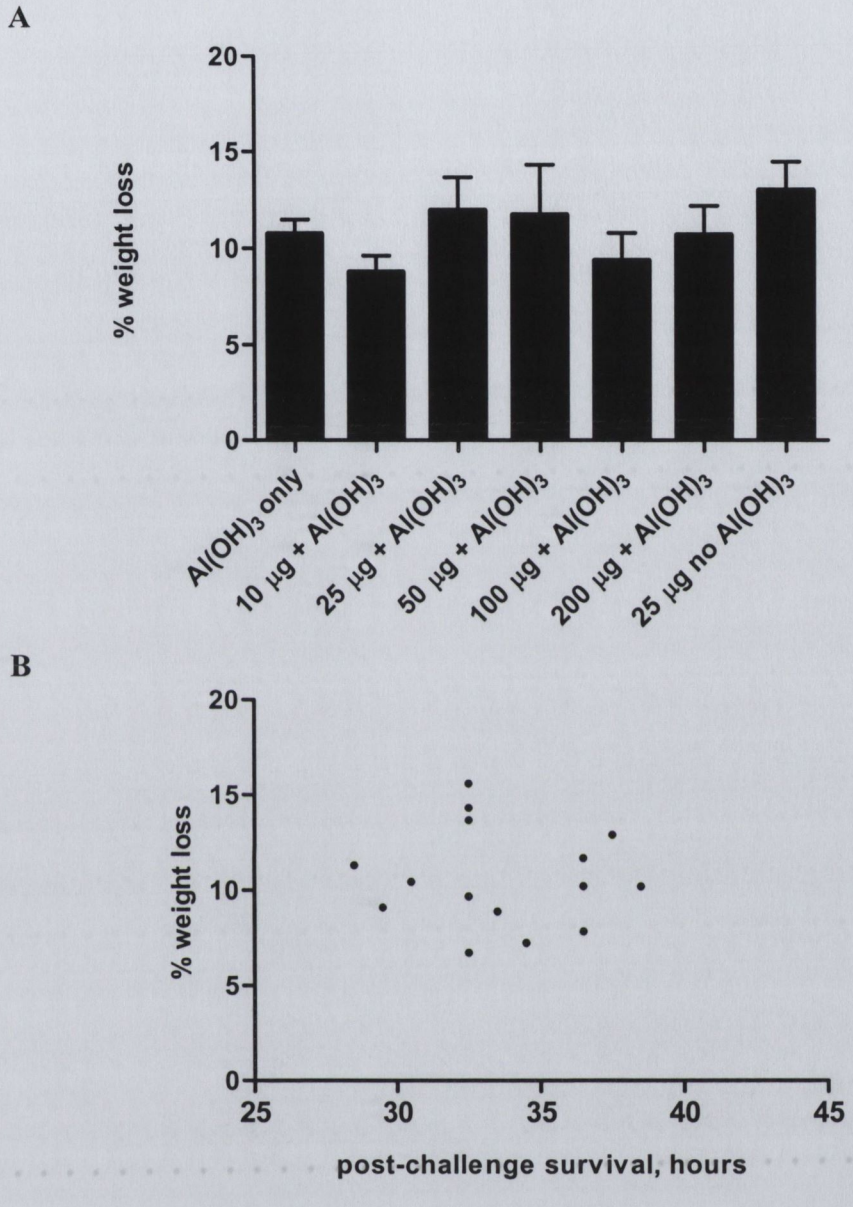



**Fig. 5.3 Post-challenge weight loss of SLP/Al(OH)$_3$ immunised hamsters.** Hamsters were weighed daily and starting weight was calculated as an average of weight at day –1 and day 0 (day 0 = time of administration of *C. difficile*). Weight at time of death was used as final weight. (A) Percentage weight loss as a function of immunisation regimen. Bars represent mean and SEM for each group. (B) Percentage weight loss as a function of post-challenge survival time.

immunisation groups or a correlation between percentage weight loss and survival would not have been expected.

### 5.3.4 The effect of *C. difficile* culture supernatant on polyclonal IgG and IgA.

High levels of circulating IgG anti-SLP antibodies were present in some of the hamsters immunised with SLPs plus $Al(OH)_3$ at the time of *C. difficile* challenge. However, these antibodies conferred no protection against CDAD. It was possible that *C. difficile* was producing a factor(s) that was breaking down the antibodies and therefore destroying their protective effect. To analyse this, the effect of concentrated *C. difficile* culture supernatant on polyclonal rabbit immunoglobulin and polyclonal human IgA was examined using Western blotting. The polyclonal rabbit immunoglobulin was purified from rabbit serum on a recombinant protein A column and eluted with glycine buffer (pH 3.0). For the purpose of this experiment it was assumed that the rabbit immunoglobulin was primarily IgG and is referred to as polyclonal rabbit IgG. In **Figure 5.4 (A)** and **(B)**, it can be seen that, within this system, the culture supernatant had no effect on the integrity of either the IgG or IgA over a time course from zero minutes to overnight. Intact immunoglobulin ($H_2L_2$, $M_r$: 146,000 – 170,000) and the mixed disulphide (HL, $M_r$: 73,000 – 85,000), as well as the major fragment of heavy chain (H, $M_r$: 51,000 – 60,000) in the case of IgG, can be seen at all time points. The intact immunoglobulin was not reduced further over time.

### 5.3.5 Serum IgG response in SLP/Ribi and SLP/Ribi/CT immunised hamsters.

Serum IgG anti-SLP and anti-CT antibodies were measured by ELISA in hamsters immunised with SLPs and Ribi (i.p.) or SLPs and Ribi/CT (i.n., i.p, respectively) at each stage of the active immunisation regimen (Dr. Déirdre Ní Eidhin). Overall, low serum IgG anti-SLP titres were obtained using these adjuvants **(Figure 5.5)**. Final titres are summarised in **Table 5.2**. Using Ribi alone, the titres did not exhibit a dose response with the SLPs. In fact, the best overall serum antibody response was seen with the lowest dose of SLPs used (25 μg). Using Ribi/CT, an overall higher mean serum IgG anti-SLP titre was when compared to using Ribi alone.

To assess whether the SLPs were modulating the serum IgG antibody response to CT, serum IgG anti-CT levels were compared in hamsters immunised with SLPs plus Ribi/CT and Ribi/CT alone **(Figure 5.6)**. Serum IgG responses to CT were low in both the SLP/Ribi/CT (1.75 +/- 0.02) and Ribi/CT only (1.06 +/- 0.18) group. When compared

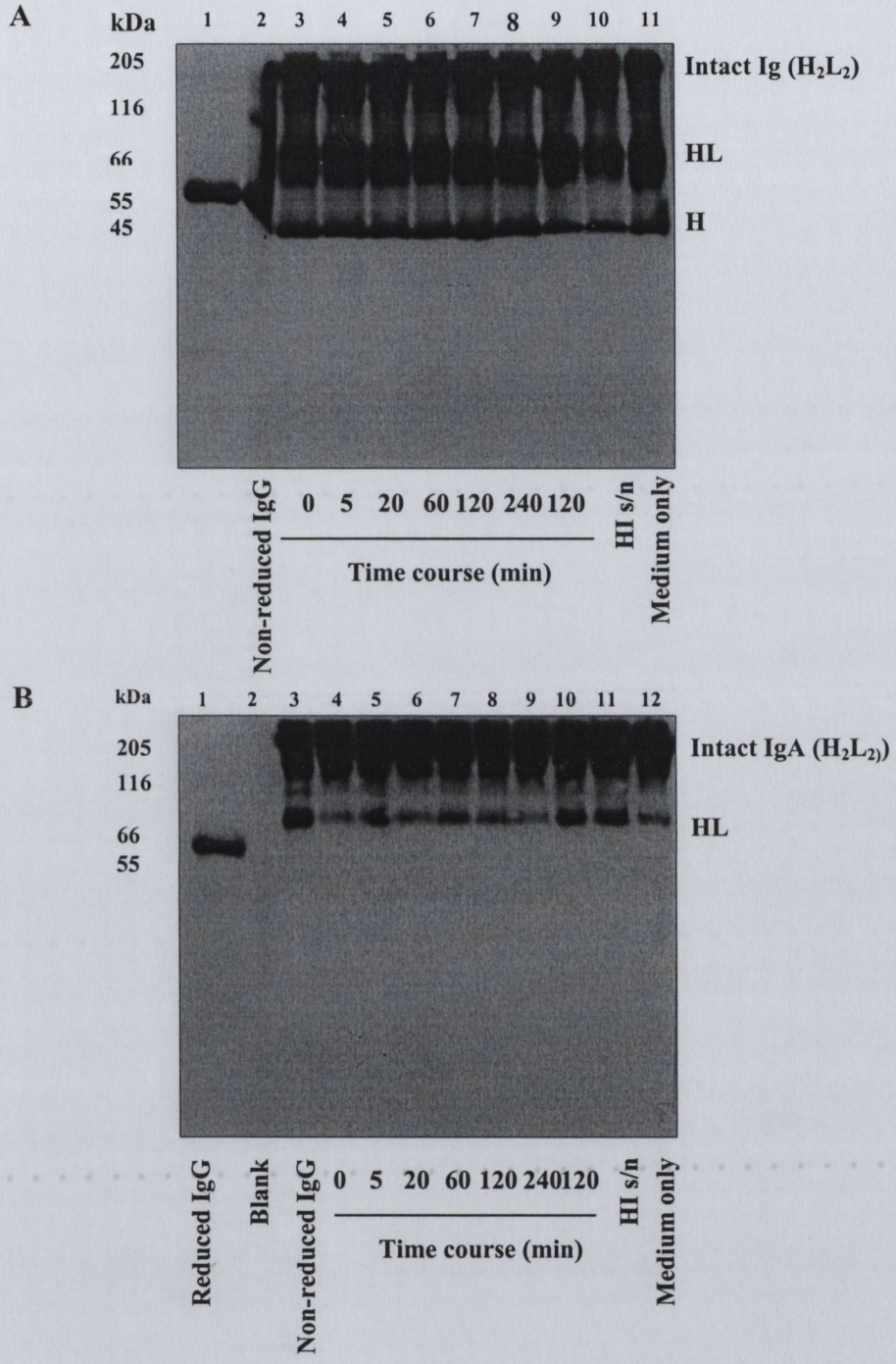



**Fig. 5.4 The effect of *C. difficile* culture supernatant on IgG and IgA.** Supernatant (s/n) from an o/n *C. difficile* culture was concentrated 5-fold and 500 µl incubated at 37°C with 100 µg of polyclonal rabbit IgG or polyclonal human IgA. Aliquots (20 µl/4µg) were taken at 0, 5, 20, 60, 120, 240 and 1200 min. Heat inactivated (HI) s/n (100°C, 15 min) and medium only served as negative controls. Samples were acetone precipitated o/n, boiled for 5 min in 20 µl 2X non-reducing sample buffer, run on 10% SDS-PAGE gels and transferred to PVDF. Proteins were detected with swine anti-rabbit IgG HRP (1:1000) or rabbit anti-human IgA HRP (1:1000). Fully reduced and non-reduced immunoglobulins served as controls. Molecular masses of markers, in kDa, are shown on the left. H = heavy chain; L = light chain.

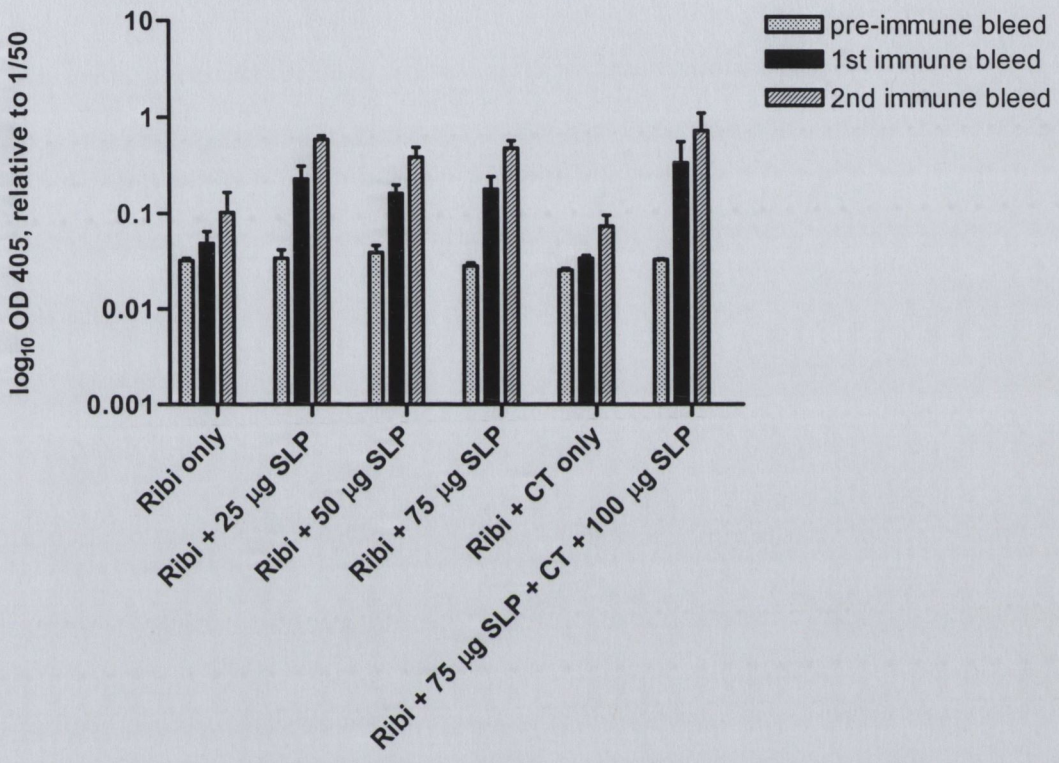



**Fig. 5.5 Serum IgG response to *C. difficile* SLPs in hamsters immunised with SLP/Ribi or SLP/Ribi/CT.** Hamsters were immunised i.p. with SLPs (25 µg, 50 µg, 75 µg) plus Ribi (n = 5 per group) or i.p. with SLPs (75 µg) plus Ribi and i.n. with SLPs (100 µg) plus CT (5 µg) (n = 3). Control groups received Ribi (n = 5) or Ribi/CT only (n = 3). Serum taken before the prime and after each subsequent immunisation were analysed for IgG anti-SLP antibodies by ELISA. The secondary antibody used was goat anti-hamster IgG alkaline phosphatase. Titres are expressed as $\log_{10}$ of 0D 405 nm and are normalised to a 1:50 dilution. Bars represent mean and SEM for each group.

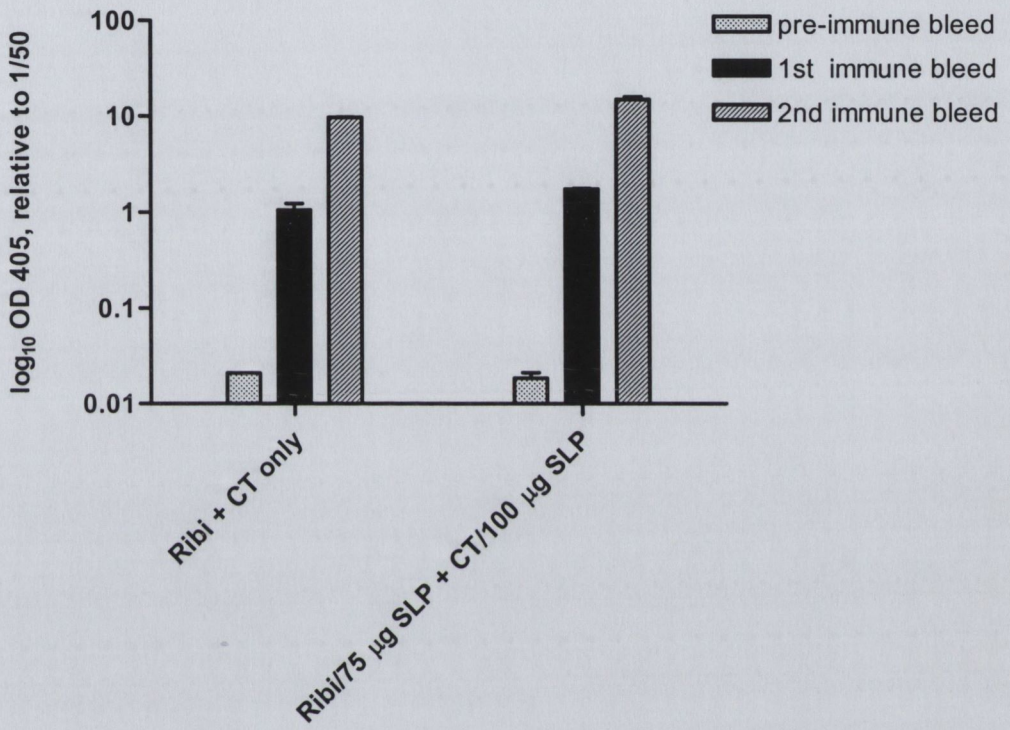



**Fig. 5.6 Serum IgG response to CT in hamsters immunised with SLP/Ribi/CT.** Hamsters were immunised i.p. with SLPs (75 μg)/Ribi and i.n. with SLPs (100 μg)/ CT (5 μg) (n = 3). Control group received Ribi/CT only (n = 3). Serum taken before the prime and after the final immunisation were analysed for anti-CT IgG antibodies by ELISA. The secondary antibody used was goat anti-hamster IgG alkaline phosphatase. Titres are expressed as $\log_{10}$ of 0D 405 nm and are normalised to a 1:50 dilution. Bars represent mean and SEM for each group.

using an unpaired two-tailed t-test no statistically significant difference in serum IgG anti-CT response between groups was seen (P = 0.06).

**TABLE 5.2 Final serum IgG anti-SLP titres[a] in hamsters immunised with SLP/Ribi or SLP/Ribi/CT**

| Regimen | Mean anti-SLP titre | +/- SEM |
|---|---|---|
| Ribi only | 0.10 | 0.06 |
| 25 μg SLPs + Ribi | 0.59 | 0.05 |
| 50 μg SLPs + Ribi | 0.39 | 0.09 |
| 75 μg SLPs + Ribi | 0.48 | 0.11 |
| Ribi/CT only | 0.07 | 0.02 |
| 75 μg SLPs + Ribi/100 μg + CT | 0.73 | 0.37 |

[a]Titres are defined as OD 405 and are normalised to a 1:50 dilution.

### 5.3.6 Post-challenge survival of SLP/Ribi and SLP/Ribi/CT immunised hamsters.

Hamsters were predisposed to *C. difficile* infection with a 2 mg dose of clindamycin, and subsequently challenged with $10^5$ *C. difficile* 13 days after their final immunisation. The outcome of the *C. difficile* challenge is shown in **Figure 5.7 (A)**. Hamsters protected from CDAD were maintained for 20 days after the *C. difficile* challenge to ensure they did not succumb to the infection.

In the groups immunised with SLPs plus Ribi, two hamsters survived the challenge, exhibiting no clinical symptoms of CDAD. Interestingly, the surviving hamsters were from the group that received the lowest dose of SLPs (25 μg). Although titres in all the groups were low, this was the group with the highest mean serum IgG anti-SLP titre. Histological analysis of caecum, colon and small intestine sections taken at sacrifice from these two surviving hamsters revealed typical desquamation of the epithelium of all three tissues in one hamster, but only of the caecum in the second hamster, which had normal colon and small intestine (data not shown).

In the group that received SLPs plus Ribi and CT, two out of three hamsters survived, again exhibiting no clinical symptoms of CDAD. Histological analysis of caecum, colon

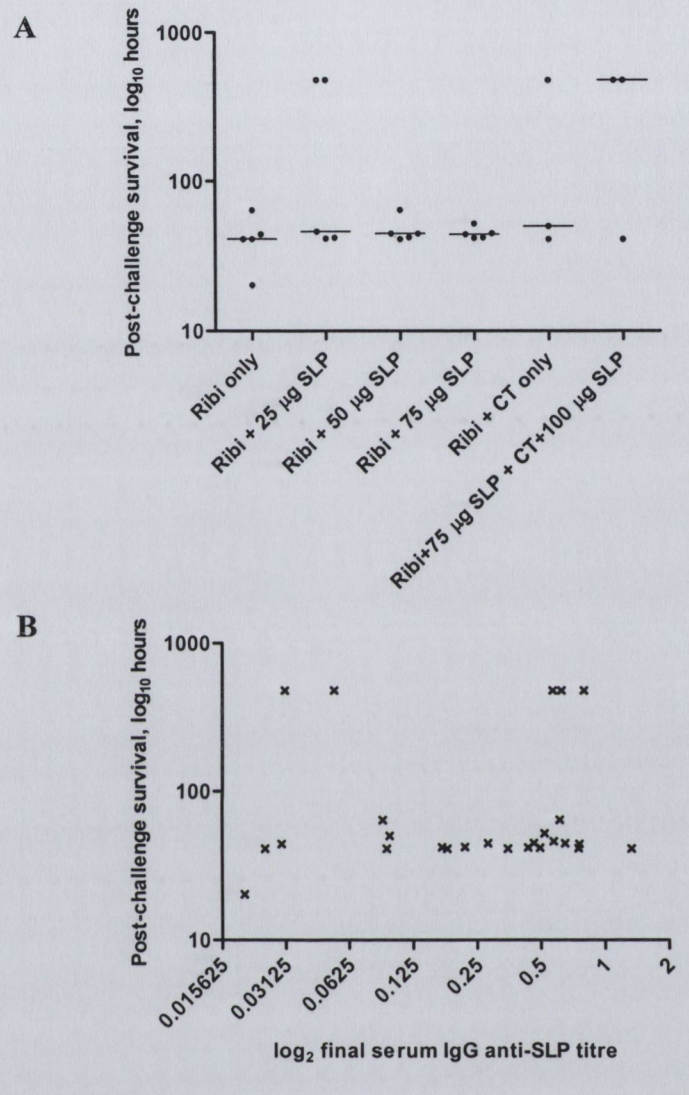



**Fig. 5.7 Post-challenge survival of hamsters immunised with SLP/Ribi and SLP/Ribi/CT.** Hamsters were immunised i.n. with SLPs (25 µg, 50 µg, 75 µg) plus Ribi (n = 5 per group) or i.p. with SLPs (75 µg) plus Ribi and i.n. with SLPs (100 µg) plus CT (5 µg). Control groups received Ribi only or Ribi and CT only (n = 5 per group). After their second boost, hamsters were predisposed to *C. difficile* infection with 0.5 mg clindamycin and challenged with $10^5$ *C. difficile*. (A) Post-challenge survival as a function of immunisation regimen. Dots represent individual hamsters; bars represent median values for each group. Time is expressed as $\log_{10}$ hours. (B) Post-challenge survival time as a function of serum IgG anti-SLP titre. Crosses represent individual hamster titres. Titres are expressed as $\log_2$ of 0D 405 nm and are normalised to a 1:50 dilution.

and small intestine sections taken from these two surviving hamsters at sacrifice, revealed typical epithelial damage of caecum, colon and small intestine in one hamster. The second hamster had damaged caecum but normal colon and small intestine (data not shown). One out of three control hamsters that received Ribi and CT only survived the challenge, exhibiting no clinical symptoms of CDAD. This hamster had severe epithelial desquamation in the caecum, colon and small intestine (data not shown).

No correlation between serum IgG anti-SLP titre and post-challenge survival time was evident from a scatter plot **(Figure 5.7, B)**. Correlation analysis confirmed this observation (Spearman r = 0.19; P = 0.36). No significant differences in median survival time between the six groups shown in **Figure 5.7 (A)** were found when compared with a non-parametric Kruskal-Wallis test (P = 0.56). However, the group that received SLPs plus Ribi and CT showed a trend towards protection. Had a more powerful study been carried out, for example by increasing group sizes, it is possible that a protective effect might have been identified.

Histological analysis of caecum, colon and small intestine from hamsters that succumbed to CDAD demonstrated severe desquamation of the epithelium in all sections. To assess any potential toxicity of the adjuvants in the hamsters, liver and kidney sections were analysed and were normal in all hamsters, indicating that Ribi or CT used in the amounts employed for these experiments did not have obvious toxic effects on these organs in hamsters.

### 5.3.7 Post-challenge weight loss of SLP/Ribi and SLP/Ribi/CT immunised hamsters.

Weight was monitored following *C. difficile* challenge and post-challenge percentage weight loss for the different immunisation regimens is shown in **Figure 5.8 (A).** Median percentage weight loss between immunisation regimens was not significantly different when analysed using a non-parametric Kruskall-Wallis test (P = 0.31). There was no correlation between percentage weight loss and post-challenge survival **(Figure 5.8, B)**, (Spearman r = - 0.14; P = 0.48). The weight of the surviving hamsters continued to fluctuate until sacrifice. This most likely reflects the intestinal epithelial damage present in these hamsters.

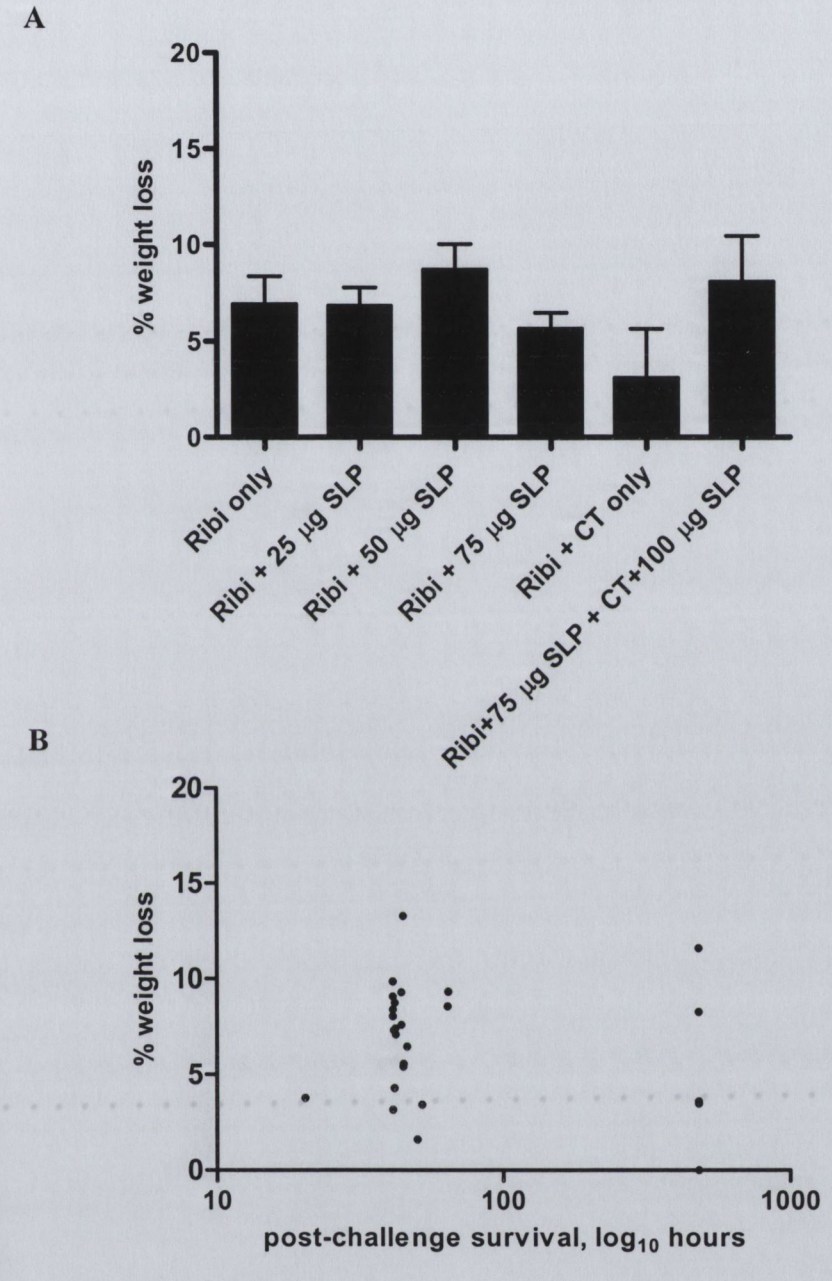



**Fig. 5.8 Post-challenge weight loss of hamsters immunised with SLP/Ribi or SLP/Ribi/CT.** Hamsters were weighed daily and starting weight was calculated as an average of weight at day –1 and day 0 (day 0 = time of administration of *C. difficile*). Weight at time of death was used as final weight. (A) Percentage weight loss versus immunisation regimen. Bars represent mean and SEM for each group. (B) Percentage weight loss as a function of post-challenge survival time. Time is expressed as $\log_{10}$ hours.

### 5.3.8 Western blot of *C. difficile* lysate probed with immune hamster sera.

Since post-challenge survival of hamsters immunised with SLPs in combination with Ribi or Ribi and CT did not correlate with serum IgG anti-SLP levels, and the fact that the antibody response to the SLPs using this immunisation regimen was generally poor, it was unlikely that protected hamsters survived due to the presence of anti-SLP antibodies. Western blotting against a crude *C. difficile* lysate was used to assess whether antibodies to other *C. difficile* antigens were present at the time of sacrifice **(Figure 5.9)**. Reactivity to a high molecular weight antigen is seen in three of the sacrificial serum samples and this most likely represents antibodies to the *C. difficile* toxins. Reactivity to a band above the 55 kDa molecular weight marker was visible in all five sacrificial serum samples, although only evident at the 1:100 dilution.

### 5.3.9 Serum IgG response in SLP/CT and SLP/Ribi/CT immunised mice.

Serum IgG antibodies against the *C. difficile* SLPs were measured by ELISA in mice immunised with SLPs plus CT (i.n.) and SLPs plus Ribi (i.p.) and CT (i.n.), at each stage of the active immunisation regimen (Dr. Déirdre Ní Eidhin). As illustrated in **Figure 5.10**, moderate serum anti-SLP IgG titres were obtained after the final boost in the group immunised with SLPs plus CT (167.58 +/- 106.70) and the group immunised with SLPs plus Ribi and CT (65.62 +/- 36.84). The inclusion of Ribi in the prime immunisation did not result in an increase in the serum IgG response to the SLPs, with no significance difference found between the two groups as tested by a two-tailed student's t-test with unequal variance ($P = 0.41$). The antibody response within the SLPs plus CT and SLPs plus Ribi/CT groups was highly variable, with ranges of (0.28, 575) and (0.99, 155.9), respectively. The control groups exhibited small increases in their IgG anti-SLP titres with subsequent immunisations, this being most pronounced in the SLP only control group. This early antibody response indicates that the mice may not have been immunologically naïve prior to their prime immunisation. It is also possible that the adjuvants may have stimulated low affinity cross-reacting antibody production in the mice.

### 5.3.10 Serum IgA response in SLP/CT and SLP/Ribi/CT immunised mice.

Serum IgA antibodies against the *C. difficile* SLPs were measured by ELISA in mice immunised with SLPs plus CT (i.n.) and SLPs plus Ribi (i.p.)/CT (i.n.), at each stage of the active immunisation regimen (Dr. Matthew McCabe) **(Figure 5.11)**. Final serum IgA anti-SLP titres were low in both the group immunised with SLPs plus CT (1.75 +/- 0.61) and the group immunised with SLPs plus Ribi/CT (2.13 +/- 1.02). The inclusion of Ribi in the

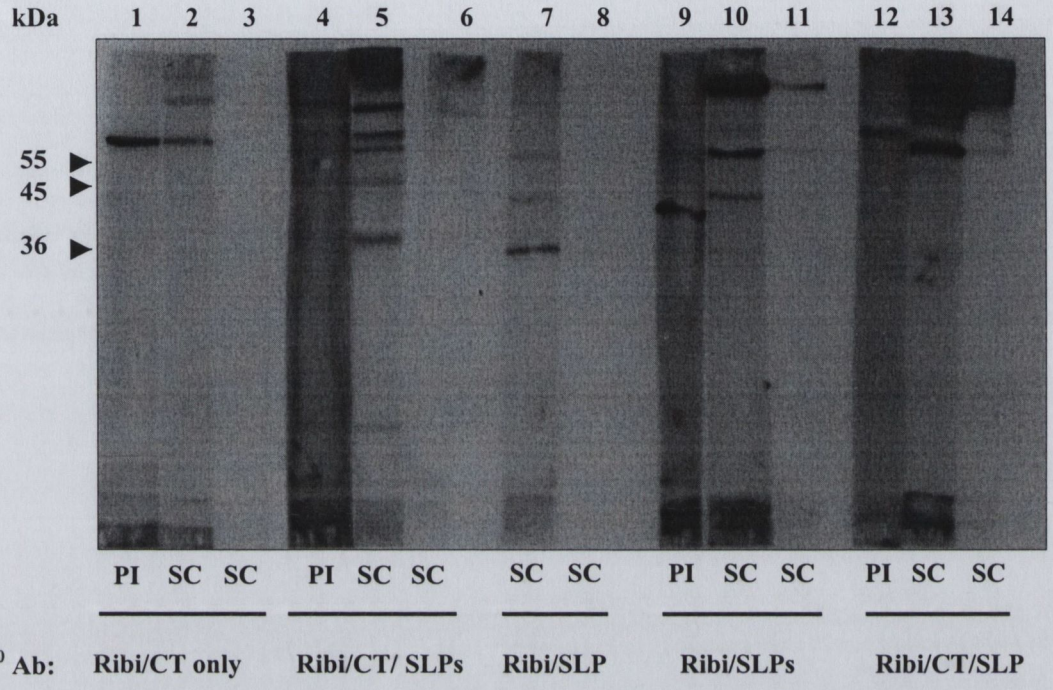



**Fig. 5.9 Western blot of *C. difficile* lysate probed with serum from surviving hamsters.** A *C. difficile* lysate (10 µl) was run on a 12% SDS-PAGE gel, transferred to PVDF membrane and probed with pre-immune (PI) or sacrificial (SC) serum from immunised hamsters protected from CDAD as follows: Ribi/CT only (lane 1 = PI (1:100), lane 2 = SC (1:100), lane 3 = SC (1:1000)); SLPs/Ribi/CT (lane 4 = PI (1:100), lane 5 = SC (1:100), lane 6 = SC (1:1000)); SLPs (25 µg)/Ribi (lane 7 = SC (1:100), lane 8 = SC (1:1000)), (note: no PI serum was available for this hamster); SLPs (25 µg)/Ribi (lane 9 = PI (1:100), lane 10 = SC (1:1000), lane 11 = SC (1:1000)); SLPs/Ribi/CT (lane 12 = PI (1:100), lane 13 = SC (1:100), lane 14 = SC (1:1000)). The secondary antibody used was goat anti-hamster IgG HRP (1:4000). Molecular masses of markers, in kDa, are shown on the left.

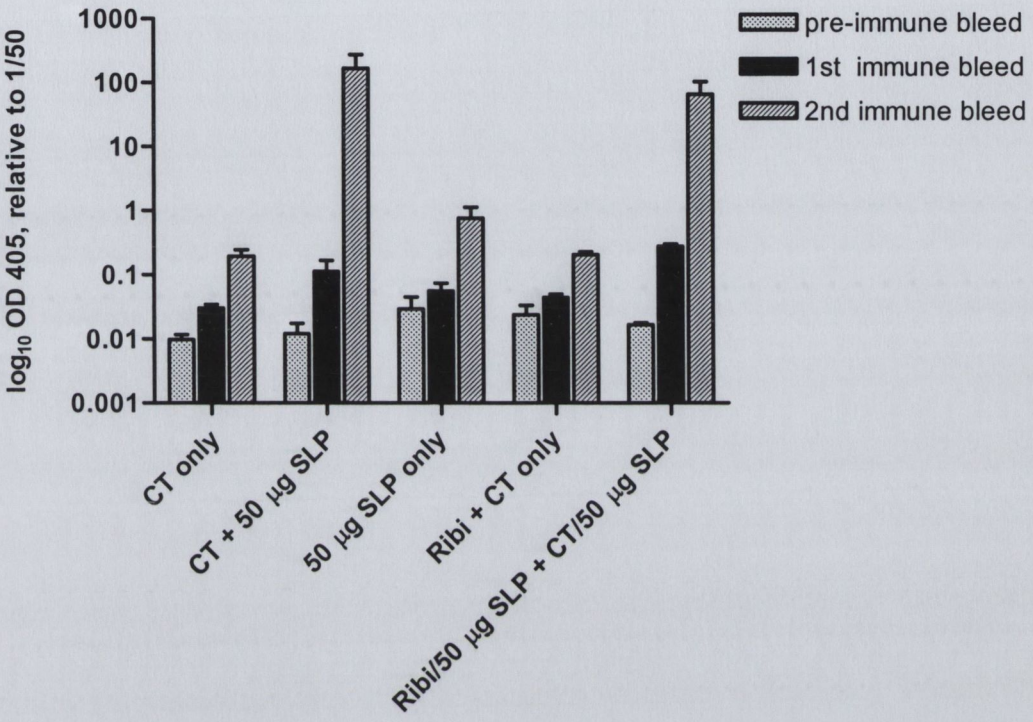



**Fig. 5.10 Serum IgG response to *C. difficile* SLPs in mice immunised with SLP/CT or SLP/Ribi/CT.** Mice were immunised i.n. with 50 µg SLPs plus CT (n = 5) or i.p. with 50 µg SLPs plus Ribi (n = 5) and simultaneously with 50 µg SLPs plus CT i.n. for their prime, and given two i.n. boosts of 50 µg SLPs plus CT. Control groups were immunised with CT alone (n = 5), 50 µg SLPs alone (n = 5) or Ribi and CT alone (n = 5). Serum taken before the prime and after each of the subsequent two immunisations were analysed for IgG anti-SLP antibodies by ELISA. The secondary antibody used was goat anti-mouse IgG alkaline phosphatase. Titres are expressed as $\log_{10}$ OD at 405 nm and are normalised to a 1:50 dilution. Bars represent mean and SEM for each group.

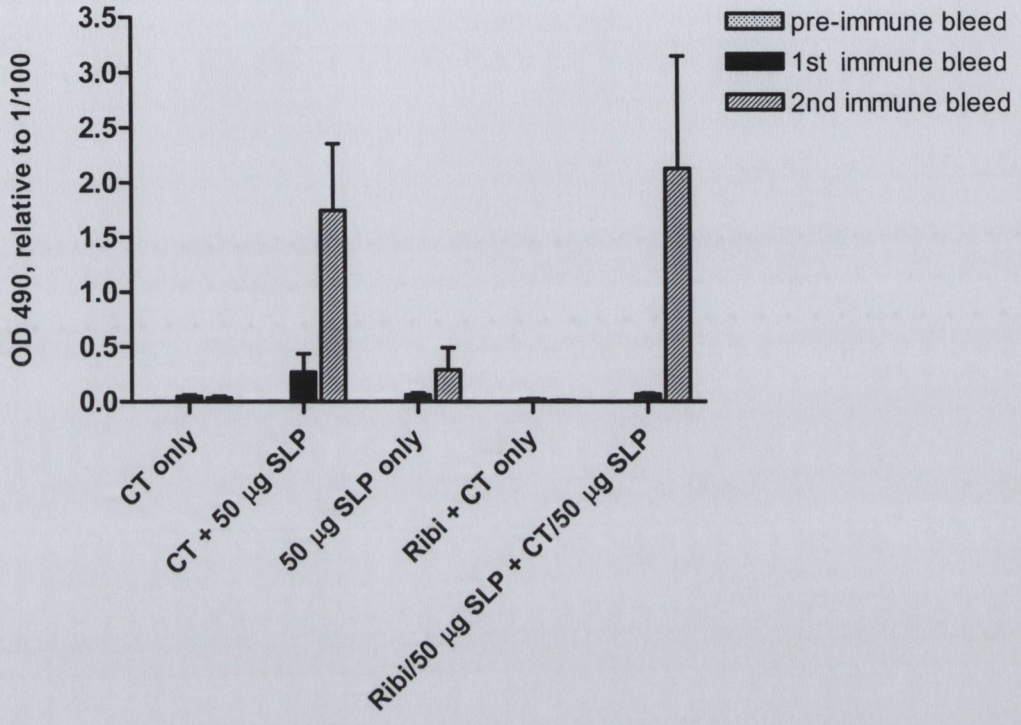



**Fig. 5.11 Serum IgA response to *C. difficile* SLPs in mice immunised with SLP/CT or SLP/Ribi/CT.** Mice were immunised i.n. with 50 µg SLPs plus CT (n = 5) or i.p. with 50 µg SLPs plus Ribi (n = 5) and simultaneously i.n. with 50 µg SLPs plus CT for their prime, and given two i.n. boosts of 50 µg SLPs plus CT. Control groups were immunised with CT alone (n = 5), 50 µg SLPs alone (n = 5) or Ribi and CT alone (n = 5). Serum samples taken before the prime and after each of the subsequent two immunisations were analysed for anti-SLP IgA antibodies by ELISA. The secondary antibody used was goat anti-mouse IgA. Titres are expressed as OD at 490 nm and are normalised to a 1:100 dilution. Bars represent mean and SEM for each group.

prime immunisation resulted in an increase in the mean serum IgA anti-SLP titre when compared to the SLP plus CT group, although this increase was not significantly different. Small increases in serum IgA anti-SLP titres was observed in the SLP only control group.

### 5.3.11 Faecal IgA response in SLP/CT and SLP/Ribi/CT immunised mice.

Faecal IgA antibodies against the *C. difficile* SLPs were measured by ELISA in mice immunised with SLPs plus CT (i.n.) and SLPs plus Ribi (i.p.) and CT (i.n.), at each stage of the active immunisation regimen (Dr. Matthew McCabe) **(Figure 5.12)**. Increases in IgA anti-SLP levels in the CT only, SLP only and Ribi/CT control groups were observed with subsequent immunisations. This might represent an enhancement of cross-reacting antibodies in the gut by CT or CT may have boosted some natural response to a component of the normal gut flora. Overall, final faecal IgA anti-SLP titres were very low in both the group immunised with SLPs and CT (0.46 +/- 0.14) and the group immunised with SLPs plus Ribi/CT (0.34 +/- 0.23). Inclusion of Ribi in the prime immunisation did not improve the faecal anti-SLP IgA titre significantly.

### 5.3.12 Serum IgG response to CT in SLP/CT and SLP/Ribi/CT immunised mice.

Due to the relatively low anti-SLP titres raised in mice, the possibility that SLPs decreased general antibody response to bystander antigen was considered. Serum IgG anti-CT titres in all groups that received CT, either on its own or in combination with the SLPs and/or Ribi, was measured by ELISA (Dr. Matthew McCabe). As illustrated in **Figure 5.13**, high anti-CT titres were obtained in all groups immunised with CT regardless of the presence of the SLPs. No difference in anti-CT titres was observed between groups, suggesting that the SLPs did not modulate the serum IgG response to CT.

### 5.3.13 Serum IgG response in SLP/chitosan glutamate and SLP/TMC immunised mice.

To assess, and compare, the efficacy of chitosan glutamate and TMC at inducing a serum antibody response against the SLPs, serum IgG anti-SLP titres were measured by ELISA in mice immunised i.n. with SLPs plus either chitosan glutamate or TMC, at each stage of the active immunisation regimen (Dr. Déirdre Ní Eidhin). As illustrated in **Figure 5.14**, final IgG anti-SLP titres were low in both the chitosan glutamate (7.76 +/- 4.34) and the TMC group (12.25 +/- 11.80). The responses within groups were very variable, with ranges of (0.95, 18.72) and (0.19, 59.43), respectively. Although TMC gave a higher mean anti-SLP titre, this was largely due to one mouse, which had an anti-SLP titre of 59.43. Only one out

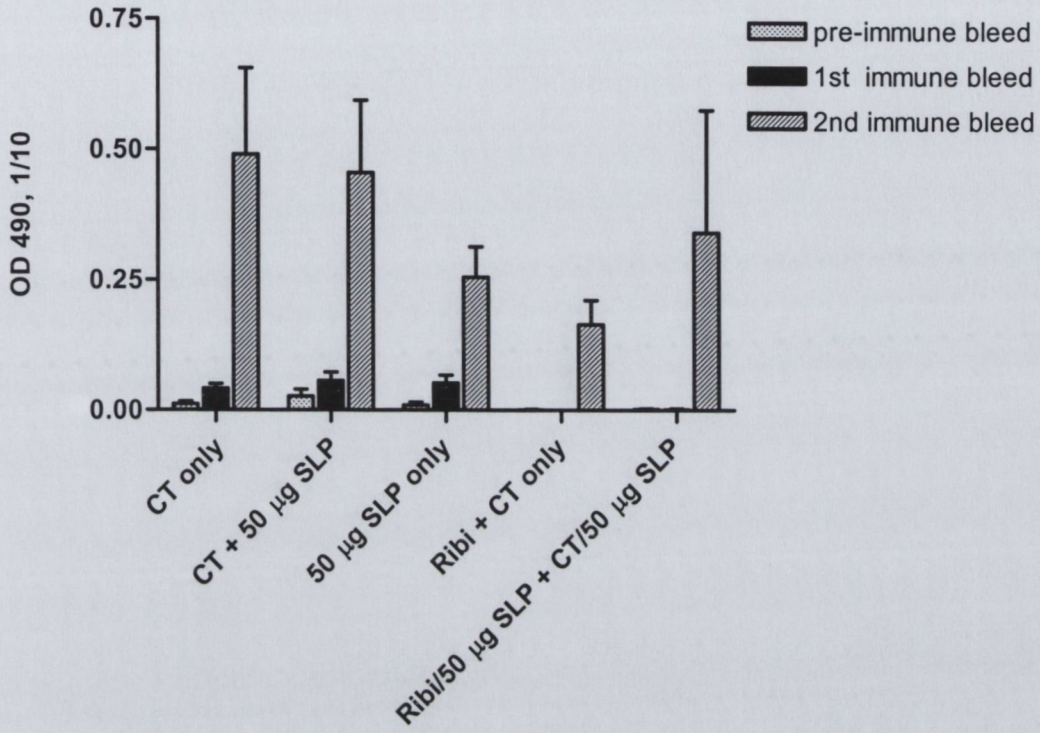



**Fig. 5.12 Faecal IgA response to *C. difficile* SLPs in mice immunised with SLP/CT or SLP/Ribi/CT.** Mice were immunised i.n. with 50 µg SLPs plus CT (n = 5) or i.p. with 50 µg SLPs plus Ribi (n = 5) and simultaneously i.n. with 50 µg SLPs plus CT for their prime, and given two i.n. boosts of 50 µg SLPs plus CT. Control groups were immunised with CT alone (n = 5), 50 µg SLPs alone (n = 5) and Ribi and CT alone (n = 5). Faeces (diluted 1:10) taken before the prime and after each of the subsequent two immunisations were analysed for IgA anti-SLP antibodies by ELISA. The secondary antibody used was goat anti-mouse IgA. Titres are expressed as OD at 490 nm. Bars represent mean and SEM for each group.

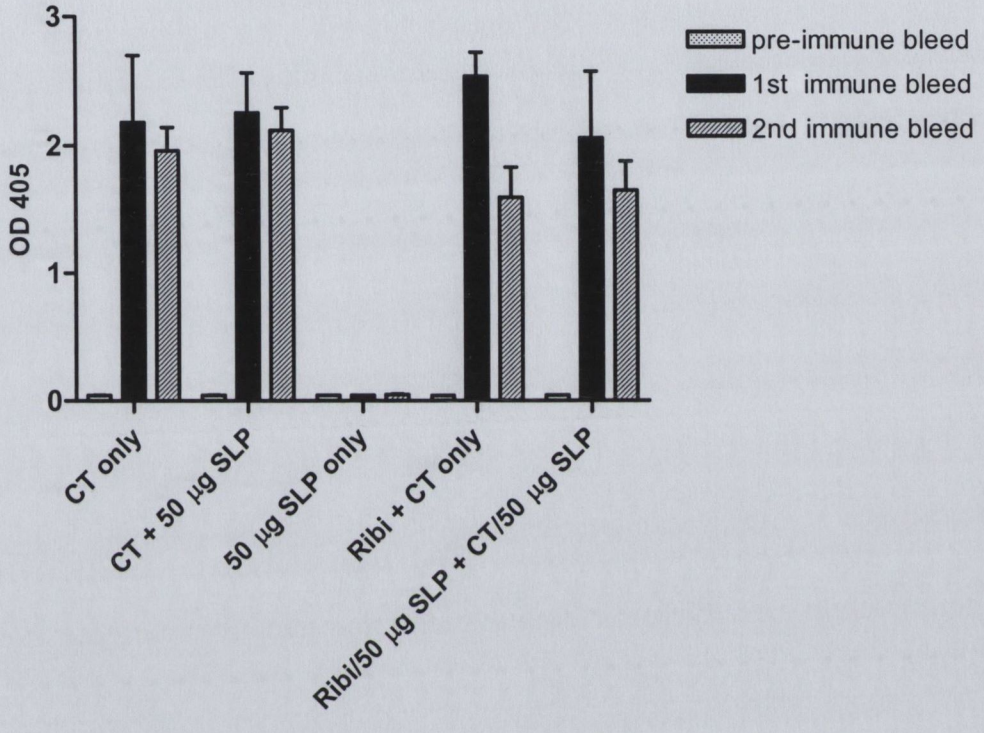



**Fig. 5.13 Serum IgG response to CT in mice immunised with SLP/CT or SLP/Ribi/CT.** Serum taken before the prime and after each of the subsequent two immunisations were analysed for IgG anti-CT antibodies by ELISA. Pre-immune and first immune sera were diluted 1:100, final immune sera was diluted 1:1000. The secondary antibody used was biotinylated goat anti-hamster IgG. Titres are expressed as 0D at 405 nm. Bars represent mean and SEM for each group.

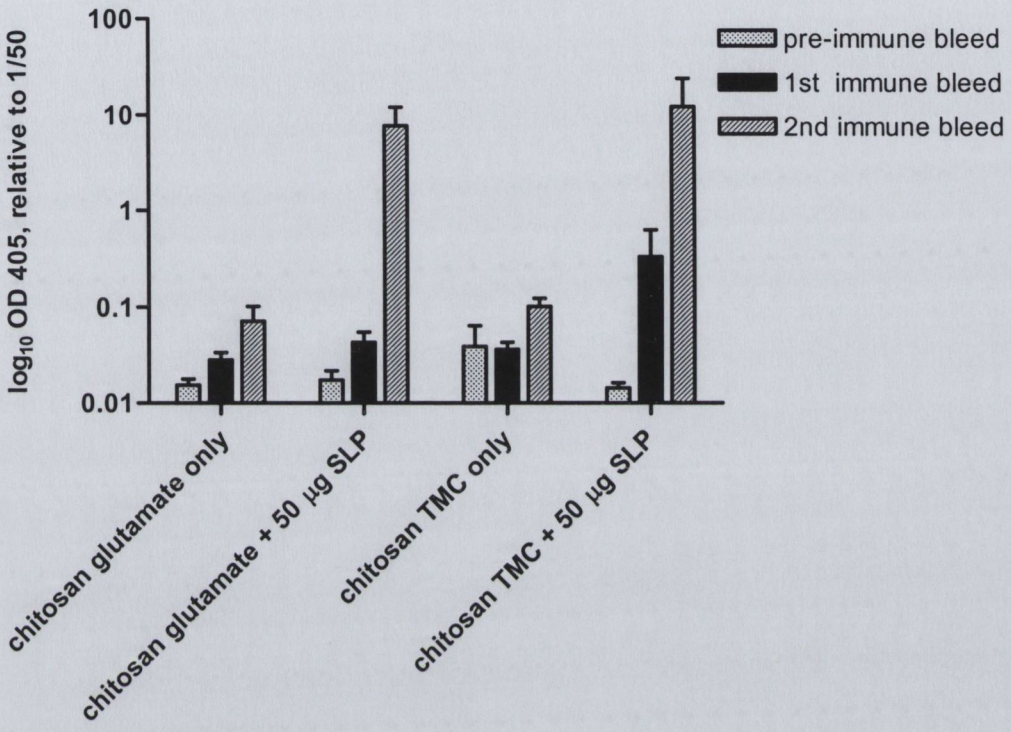



**Fig. 5.14 Serum IgG response to *C. difficile* SLPs in mice immunised with SLP/chitosan glutamate or SLP/TMC.** Mice were immunised i.n. with 50 µg SLPs plus chitosan glutamate (n = 5) or TMC (n = 5). Control groups were immunised with chitosan glutamate (n = 5) or TMC (n = 5) alone. Serum samples taken before the prime and after each of the subsequent two immunisations were analysed for IgG anti-SLP antibodies by ELISA. The secondary antibody used was goat anti-mouse IgG alkaline phosphatase. Titres are expressed as $\log_{10}$ OD at 405 nm and are normalised to a 1:50 dilution. Bars represent mean and SEM for each group.

of five mice in the SLP plus TMC group and two out of five mice in the SLP plus chitosan glutamate group had high anti-SLP titres (59.43, 17.98 and 18.72, respectively). All the other mice in these groups had anti-SLP titres of less than 2. A small increase in the IgG anti-SLP titre in the chitosan glutamate only control group was observed. Similarly, a small increase in the IgG anti-SLP titre in the chitosan glutamate only control group was observed, but only after the final and not after the prime immunisation.

### 5.3.14 Serum IgA response in SLP/chitosan glutamate and SLP/TMC immunised mice.

Serum IgA antibodies against the *C. difficile* SLPs were measured by ELISA in mice immunised i.n. with SLPs plus either chitosan glutamate or TMC, at each stage of the active immunisation regimen (Dr. Matthew McCabe) **(Figure 5.15)**. Final serum IgA anti-SLP titres were low in both groups (0.72 +/- 0.38 and 1.12 +/- 1.10, respectively). The responses within groups were very variable, with ranges of (0.01, 1.77) and (0.05, 5.56), respectively. This variation was particularly pronounced in the TMC group. Although the mean anti-SLP titre was higher in the TMC group compared to the chitosan glutamate group, this was again largely due to one mouse, which had a titre of 5.56. This was the same mouse that had a high serum IgG anti-SLP titre outlined in **Section 5.3.13.**

### 5.3.15 Faecal IgA response in SLP/chitosan glutamate and SLP/TMC immunised mice.

To assess, and compare, the efficacy of chitosan glutamate and TMC at inducing a mucosal immune response against the SLPs, faecal IgA anti-SLP titres were measured by ELISA in mice immunised i.n. with SLPs plus either chitosan glutamate or TMC, at each stage of the active immunisation regimen (Dr. Matthew McCabe) **(Figure 5.16)**. Final faecal IgA anti-SLP titres obtained in both groups were low (1.13 +/- 0.05 and 0.20 +/- 0.17, respectively). Small increases in IgA anti-SLP titres in the chitosan glutamate and TMC only control groups were observed (0.05 +/- 0.01 and 0.06 +/- 0.02, respectively) and may represent cross-reacting antibody production stimulated by the adjuvants alone.

### 5.3.16 Phagocytosis of *C. difficile* by PMA differentiated THP-1 cells in the presence of immune hamster and mouse serum.

Where hamster and mouse serum was available, the ability of the serum to promote phagocytosis of *C. difficile* by PMA differentiated THP-1 cells was assessed *in vitro*. Graphs are representative of single experiments only, as serum was unavailable to perform

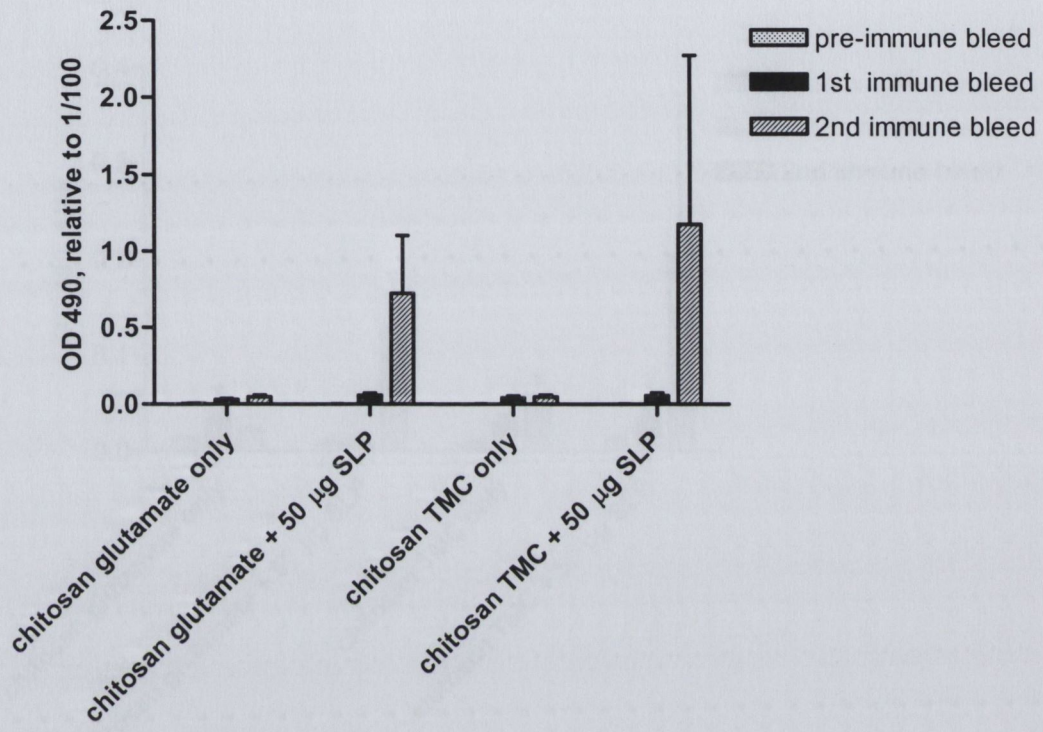



**Fig. 5.15 Serum IgA response to the *C. difficile* SLPs in mice immunised with SLP/chitosan glutamate or SLP/TMC.** Mice were immunised i.n. with 50 µg SLPs plus chitosan glutamate (n = 5) or TMC (n = 5). Control groups were immunised with chitosan glutamate (n = 5) or TMC alone. Serum samples taken before the prime and after each of the subsequent two immunisations were analysed for IgA anti-SLP antibodies by ELISA. The secondary antibody used was goat anti-mouse IgA. Titres are expressed as OD at 490 nm and are normalised to a 1:100 dilution. Bars represent mean and SEM for each group.

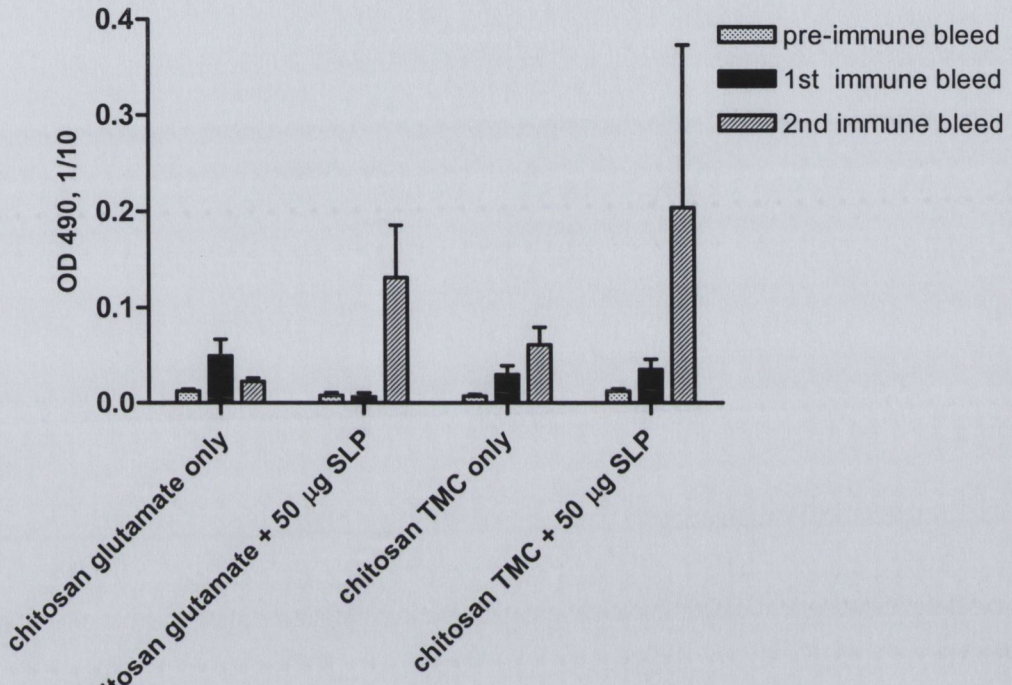



**Fig. 5.16 Faecal IgA response to *C. difficile* SLPs in mice immunised with SLP/chitosan glutamate or SLP/TMC.** Mice were immunised i.n. with 50 µg SLPs plus either chitosan glutamate (n = 5) or TMC (n = 5). Control groups received chitosan glutamate (n = 5) or TMC (n = 5) alone. Faecal samples taken before the prime and after each of the subsequent two immunisations were analysed for IgA anti-SLP antibodies by ELISA. The secondary antibody used was goat anti-mouse IgA. Titres are expressed as OD at 490 nm and are normalised to a 1:100 dilution. Bars represent mean and SEM for each group.

repeats. Controls shown in **Figure 5.17** illustrate that *C. difficile* was poorly phagocytosed by the THP-1 cells in the absence of an opsonin and rabbit anti-SLP serum increased the levels of phagocytosis dramatically. Relatively high titres of anti-SLP antibodies were raised in hamsters using SLPs in combination with $Al(OH)_3$ **(Section 5.3.1, Figure 5.1)**. As seen in **Figure 5.18**, immune serum from $Al(OH)_3$ immunised hamsters did not increase phagocyotosis of *C. difficile* by PMA differentiated THP-1 cells. Similarly, no increase in phagocytosis was seen when serum from hamsters immunised with SLPs plus Ribi or SLPs plus Ribi and CT was assessed in the same system **(Figures 5.19, 5.20 and 5.21)**.

Some of the mice immunised with SLPs in combination with CT and Ribi/CT had high anti-SLP titres. As illustrated in **Figure 5.22**, immune sera from a mouse immunised with SLPs plus Ribi/CT, which had a serum IgG anti-SLP titre of 0.24, as well as a Ribi/CT only control mouse, did not increase phagocytosis. Furthermore, immune serum from a mouse immunised with SLPs plus Ribi/CT, which had a substantial serum IgG anti-SLP titre of 155.9, did not increase phagocytosis. However, as illustrated in **Figure 5.23**, immune serum from a mouse immunised with SLPs plus CT, which had a serum IgG anti-SLP titre of 575, resulted in a substantial increase in phagocytosis of *C. difficile*, with 55.75% of THP-1 cells ingesting *C. difficile*. This was comparable to the level of *C. difficile* phagocytosis observed with rabbit anti-SLP serum (78.84%), which had been shown to prolong survival of hamsters infected with *C. difficile* **(Chapter IV, Section 4.3.3, Figures 4.3 and 4.4)**.

### 5.3.17 IgG response to SLPs in SLP/CT immunised hamsters.

The data collected to this point in the study indicated that CT adjuvant was the most likely adjuvant tested to induce protective immunity to the SLPs in the hamster model. Specifically, an immunisation regimen of SLPs plus Ribi/CT gave a trend towards protection in hamsters, with two out of three of the immunised hamsters surviving the *C. difficile* challenge **(Section 5.3.6, Figure 5.7, A)**. Furthermore, an immunisation regimen of SLPs and CT induced the best antibody response to the SLPs in mice. The inclusion of Ribi in this regimen did not offer any benefit with regard to antibody titre **(Sections 5.3.9 - 5.3.11, Figures 5.10, 5.11 and 5.12)**. Therefore, CT as an adjuvant on its own was tested for its ability to induce protective immunity against the SLPs in hamsters. Two different regimens were tested, one in which the hamsters received a prime immunisation and two boosts, and a second in which hamsters received a prime immunisation and three boosts.

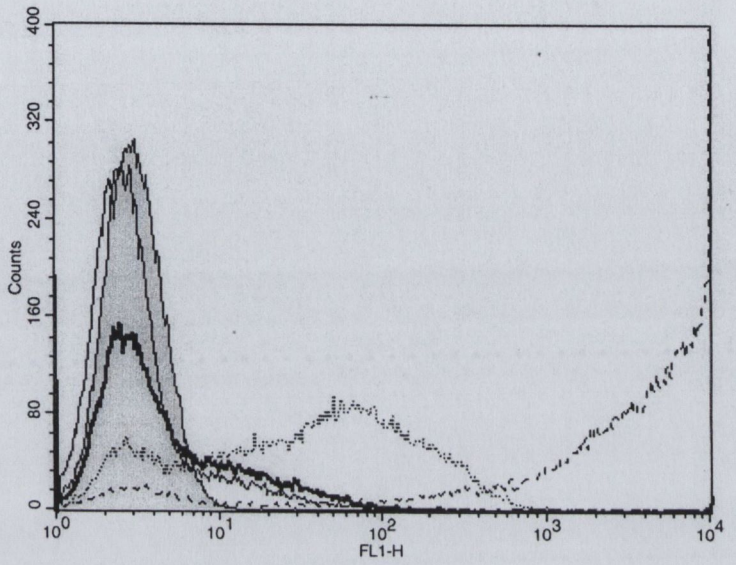
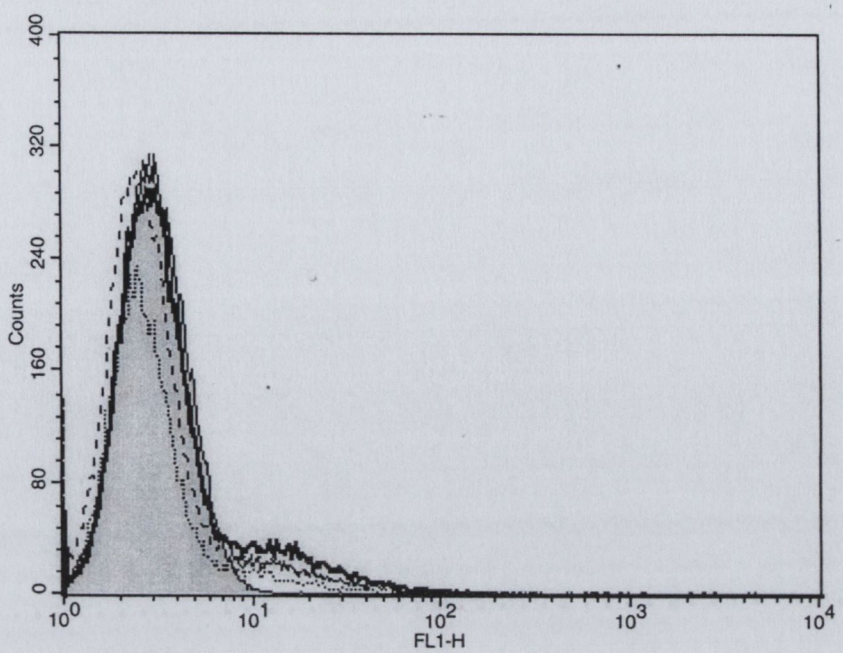
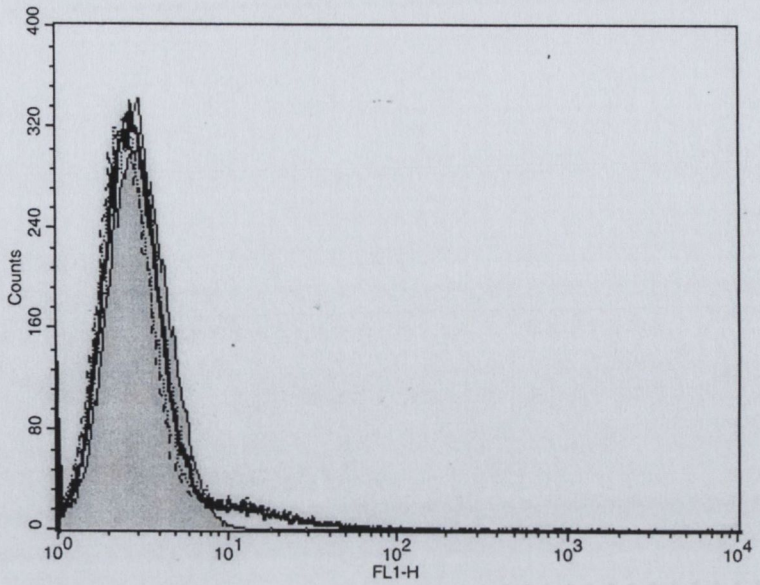



**Fig. 5.17** ***C. difficile*** **phagocytosis controls.** PMA differentiated THP-1 cells were incubated for 2 h with *C. difficile* (MOI of 400) in the presence or absence of rabbit anti-SLP serum or rabbit pre-immune serum, or with GFP *E. coli* DH5α (pKFW408). THP-1 cells alone (grey histogram); *C. difficile* only (open histogram, thick black line); *C. difficile* plus rabbit anti-SLP serum (12.5%) (open histogram, dotted line); *C. difficile* plus rabbit pre-immune serum (12.5%) (open histogram, thin black line); THP-1 cells plus GFP *E. coli* DH5α (pKFW408) (open histogram, dashed line). Single THP-1 cells were gated using FSC and SSC. Generally, 30,000 events were acquired using a FACSCalibur and data was analysed with Cell Quest software (Becton Dickinson). Controls shown were included in every phagocytosis experiment.



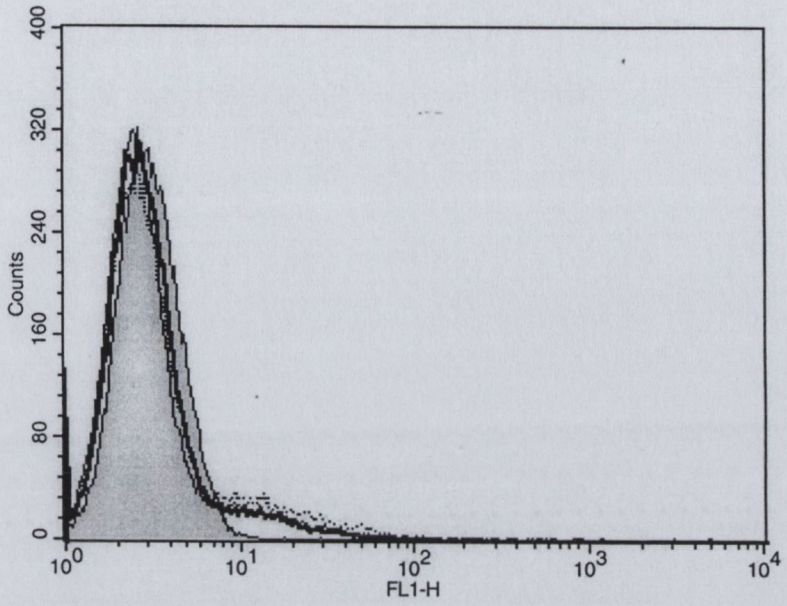

| Hamster immunisation regimen | Serum IgG anti-SLP titre, 0D 405 normalised to 1:50 | Effect of serum on *in vitro* phagocytosis of *C. difficile* | Hamster outcome after *C. difficile* challenge |
|---|---|---|---|
| $Al(OH)_3$ only (thin line ———) | 0.05 | No increase | Diarrhoea and death |
| 25 μg SLP + $Al(OH)_3$ (thick line ▬▬▬) | 56.08 | No increase | Diarrhoea and death |
| 50 μg SLP + $Al(OH)_3$ (dotted line ·····) | 0.71 | No increase | Diarrhoea and death |
| 200 μg SLP + $Al(OH)_3$ (dashed line - - - - - - -) | 14.60 | No increase | Diarrhoea and death |

**Fig. 5.18 FACS analysis of *C. difficile* phagocytosis by THP-1 cells with serum from $Al(OH)_3$ immunised hamsters.** PMA differentiated THP-1 cells were incubated for 2 h with *C. difficile* (MOI of 400) plus various sera (8%) from hamsters immunised with SLPs plus $Al(OH)_3$. THP-1 cells alone (grey histogram); serum from $Al(OH)_3$ only hamster (open histogram, thin black line); serum from SLP (25 μg)/$Al(OH)_3$ hamster (open histogram, thick black line); serum from SLP (50 μg)/$Al(OH)_3$ hamster (open histogram, dotted line); serum from SLP (200 μg)/$Al(OH)_3$ hamster (open histogram, dashed line). Single THP-1 cells were gated using FSC and SSC. Generally, 30,000 events were acquired using a FACSCalibur and data was analysed with Cell Quest software (Becton Dickinson).



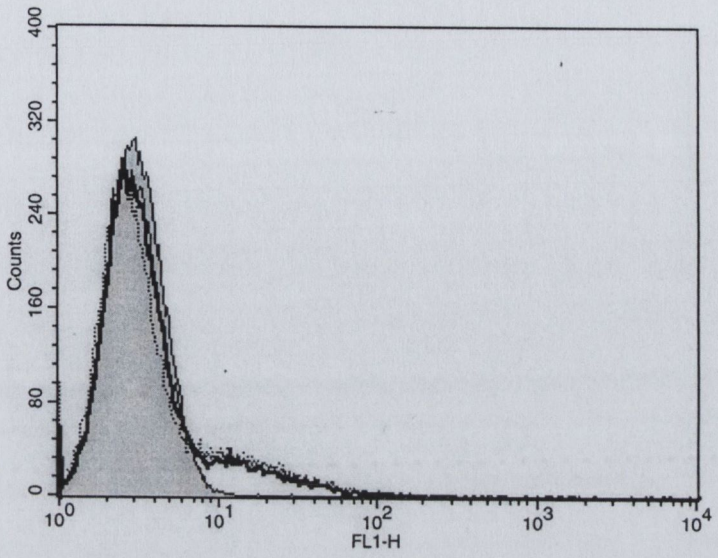

| Hamster immunisation regimen | Serum IgG anti-SLP titre, 0D 405 normalised to 1:50 | Effect of serum on *in vitro* phagocytosis of *C. difficile* | Hamster outcome after *C. difficile* challenge |
|---|---|---|---|
| Ribi only (thin line ——— ) | 0.03 | No increase | Diarrhoea and death |
| 25 µg SLP + Ribi (thick line ——— ) | 0.62 | No increase | No diarrhoea, survived |
| 75 µg SLP + Ribi (dotted line ····· ) | 0.44 | No increase | Diarrhoea and death |
| 25 µg SLP + Ribi (dashed line - - - - - - - ) | 0.76 | No increase | Diarrhoea and death |
| 25 µg SLP + Ribi (dotted/dashed - · - · - · - ) | 0.17 | No increase | Diarrhoea and death |

**Fig. 5.19 FACS analysis of *C. difficile* phagocytosis by THP-1 cells with serum from Ribi immunised hamsters.** PMA differentiated THP-1 cells were incubated for 2 h with *C. difficile* (MOI of 400) plus various sera (10%) from hamsters immunised with SLPs plus Ribi. THP-1 cells alone (grey histogram); serum from Ribi only hamster (open histogram, thin black line); serum from SLP (25 µg)/Ribi hamster (open histogram, thick black line); serum from SLP (25 µg)/Ribi hamster (open histogram, dotted line); serum from SLP (75 µg)/Ribi hamster (open histogram, dashed line); serum from SLP (25 µg)/Ribi hamster (open histogram, dotted and dashed line). Single THP-1 cells were gated using FSC and SSC. Generally, 30,000 events were acquired using a FACSCalibur and data was analysed with Cell Quest software (Becton Dickinson).



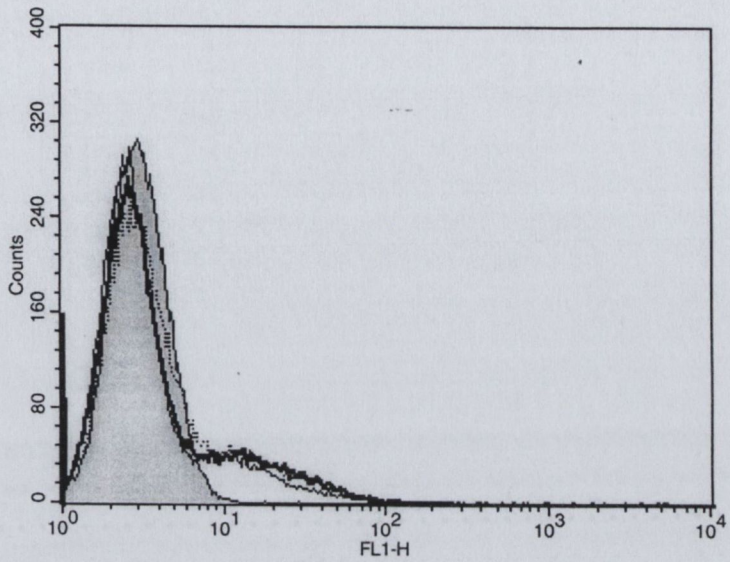

| Hamster immunisation regimen | Serum IgG anti-SLP titre, 0D 405 normalised to 1:50 | Effect of serum on *in vitro* phagocytosis of *C. difficile* | Hamster outcome after *C. difficile* challenge |
|---|---|---|---|
| Ribi/CT only (thin line ———) | 0.09 | No increase | Diarrhoea and death |
| Ribi/CT only (thick line ———) | 0.10 | No increase | Diarrhoea and death |
| Ribi/CT only (dotted line ·····) | 0.03 | No increase | No diarrhoea, survived |

**Fig. 5.20 FACS analysis of *C. difficile* phagocytosis by THP-1 cells with serum from Ribi/CT control hamsters.** PMA differentiated THP-1 cells were incubated for 2 h with *C. difficile* (MOI of 400) plus various sera (8%) from hamsters immunised with Ribi and CT only. THP-1 cells alone (grey histogram); serum from Ribi/CT only hamster (open histogram, thin black line); serum from Ribi/CT only hamster (open histogram, thick black line); serum from surviving Ribi/CT only hamster (open histogram, thin dotted line). Single THP-1 cells were gated using FSC and SSC. Generally, 30,000 events were acquired using a FACSCalibur and data was analysed with Cell Quest software (Becton Dickinson).



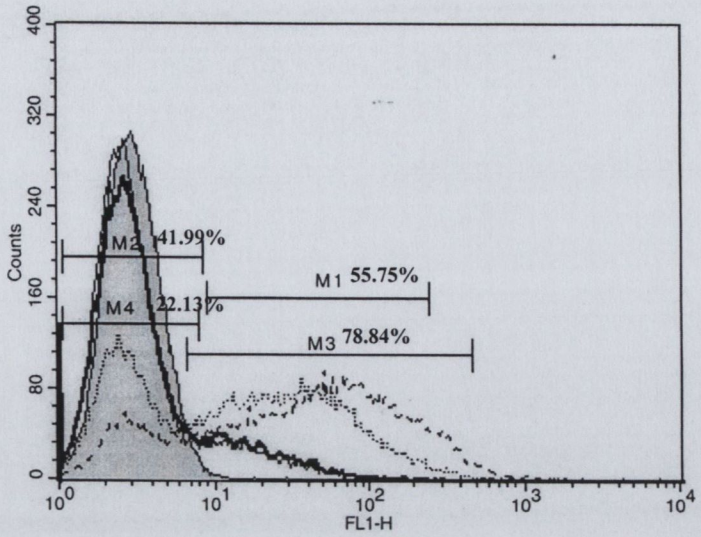

| Hamster immunisation regimen | Serum IgG anti-SLP titre, 0D 405 normalised to 1:50 | Effect of serum on *in vitro* phagocytosis of *C. difficile* | Hamster outcome after *C. difficile* challenge |
|---|---|---|---|
| 75 μg SLP + Ribi 100 μg SLP + CT (thin line ——— ) | 0.80 | No increase | No Diarrhoea, survived |
| 75 μg SLP + Ribi 100 μg SLP + CT (thick line ——— ) | 1.33 | No increase | Diarrhoea and death |
| 75 μg SLP + Ribi 100 μg SLP + CT (dotted line ····· ) | 0.05 | No increase | No Diarrhoea, survived |

**Fig. 5.21 FACS analysis of *C. difficile* phagocytosis by THP-1 cells with serum from Ribi/CT immunised hamsters.** PMA differentiated THP-1 cells were incubated for 2 h with *C. difficile* (MOI of 400) plus various sera from hamsters immunised with SLP (75 μg)/Ribi and SLP (100 μg)/CT. THP-1 cells alone (grey histogram); serum (8%) from SLP/Ribi/CT surviving hamster (open histogram, thin black line); serum (6%) from SLP/Ribi/CT hamster (open histogram, thick black line); serum (4%) from SLP/Ribi/CT surviving hamster (open histogram, dotted line). Single THP-1 cells were gated using FSC and SSC. Generally, 30,000 events were acquired using a FACSCalibur and data was analysed with Cell Quest software (Becton Dickinson).




| Mouse immunisation regimen | Serum IgG anti-SLP titre, 0D 405 normalised to 1:50 | Effect of serum on *in vitro* phagocytosis of *C. difficile* |
|---|---|---|
| Ribi/CT only (thin line———) | 0.22 | No increase |
| 50 μg SLP + Ribi/CT (thick line———) | 0.24 | No increase |
| 50 μg SLP + Ribi/CT (dotted line·····) | 155.9 | No increase |

**Fig. 5.22 FACS analysis of *C. difficile* phagocytosis by THP-1 cells with serum from Ribi/CT immunised mice.** PMA differentiated THP-1 cells were incubated for 2 h with *C. difficile* (MOI of 400) plus various sera (10%) from mice immunised with SLPs plus Ribi/CT. THP-1 cells alone (grey histogram); serum from Ribi/CT only mouse (open histogram, thin black line); serum after the first boost from SLP (50 μg)/Ribi/CT mouse (open histogram, thick black line); serum after the second boost from SLP (50 μg)/Ribi/CT mouse (open histogram, dotted line); Single THP-1 cells were gated using FSC and SSC. Generally, 30,000 events were acquired using a FACSCalibur and data was analysed with Cell Quest software (Becton Dickinson).




| Mouse immunisation regimen | Serum IgG anti-SLP titre, 0D 405 normalised to 1:50 | Effect of serum on *in vitro* phagocytosis of *C. difficile* |
|---|---|---|
| CT only (thin line ——— ) | 0.05 | No increase |
| 50 µg SLP + CT (thick line ——— ) | 0.03 | No increase |
| 50 µg SLP + CT (dotted line ····· ) | 575 | 55.75% THP-1 cells phagocytosed *C. difficile* |

**Fig. 5.23 FACS analysis of *C. difficile* phagocytosis by THP-1 cells with serum from CT immunised mice.** PMA differentiated THP-1 cells were incubated for 2 h with *C. difficile* (MOI of 400) plus various sera (10%) from mice immunised with SLPs plus CT. THP-1 cells alone (grey histogram); serum from CT only mouse (open histogram, thin black line); serum after the first boost from SLP (50 µg)/CT mouse (open histogram, thick black line); serum after the second boost from SLP (50 µg)/CT mouse (open histogram, dotted line); rabbit anti-SLP serum (12.5%) served as a positive control (open histogram, dashed line). Single THP-1 cells were gated using FSC and SSC. Generally, 30,000 events were acquired using a FACSCalibur and data was analysed with Cell Quest software (Becton Dickinson).

Serum IgG anti-SLP antibodies in hamsters immunised i.n. with SLPs plus CT, and serum IgG anti-CT antibodies in hamsters immunised i.n. with SLPs plus CT and CT only, were measured by ELISA before the prime and after the final immunisation (Dr. Déirdre Ní Eidhin). Low serum IgG anti-SLP titres (0.28 +/- 0.05) were generated in the hamsters that received three doses (**Figure 5.24).** However, administration of an additional fourth dose of SLPs plus CT resulted in a mean 16-fold increase in the serum IgG anti-SLP response (4.48 +/- 2.30) compared to when three doses were given. The antibody response was highly variable within the group that received four doses, with a range of (0.41, 12.77). Antibody titres were still relatively low following administration of the fourth dose and no statistically significant difference was found in the anti-SLP titre between the groups receiving three versus four doses, as tested by a two-tailed student's t-test with unequal variance ($P = 0.13$).

Given the very low anti-SLP titres generated in the hamsters, we again considered the possibility that the SLPs were modulating the serum IgG antibody response to CT. To assess this possibility, serum IgG anti-CT titres were compared in the groups that received SLPs plus CT and CT only **(Figure 5.25)**. When three doses were administered, there was no significant difference in anti-CT titres between the groups immunised with SLPs plus CT (16.79 +/- 1.05) compared to CT only (12.0 +/- 2.4) ($P = 0.09$). However, in the groups that received four doses, significantly higher anti-CT titres were evident in the group immunised with SLPs plus CT (76.70 +/- 11.9) compared to CT only (16.00 +/- 1.8) (***, $P < 0.001$).

Faecal IgG anti-SLP antibody levels were measured by ELISA (Dr. Déirdre Ní Eidhin) in a selection of nine hamsters during the immunisations. No faecal IgG anti-SLP antibodies were detectable by this method.

### 5.3.18 Post-challenge survival of SLP/CT immunised hamsters.

Hamsters were predisposed to *C. difficile* infection with a 2 mg dose of clindamycin, and subsequently challenged with $10^5$ *C. difficile* 10 days after their final immunisation. The outcome of the *C. difficile* challenge is shown in **Figure 5.26**. No protection against CDAD was evident and all hamsters died from CDAD within two days of infection (median = 42.2 h, range (38 h, 48.6 h)). No significant differences in median survival times between the five groups shown in **Figure 5.26 (A)** were found when the groups compared with a non-parametric Kruskal-Wallis test ($P = 0.32$). No correlation between serum IgG

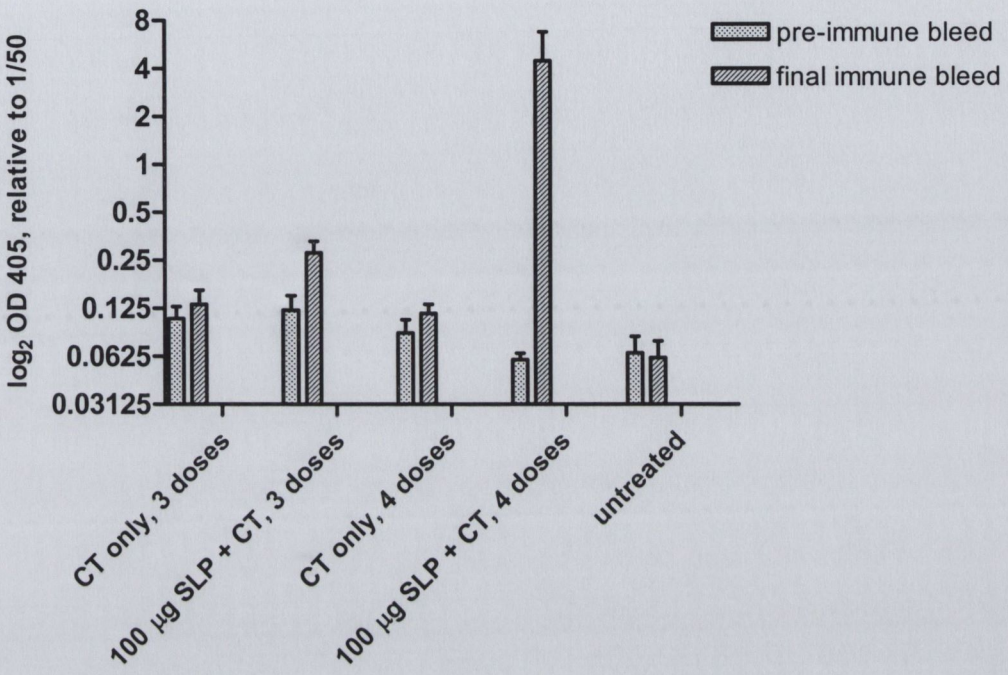



**Fig. 5.24 Serum IgG response to *C. difficile* SLPs in hamsters immunised with SLP/CT.** Hamsters were immunised i.n. with three or four doses of SLPs (100 µg) plus CT (5 µg) (n = 6 per group). Control groups received three or four doses of CT only (n = 6 per group). Serum taken before the prime and after the final immunisation were analysed for IgG anti-SLP antibodies by ELISA. The secondary antibody used was goat anti-hamster IgG alkaline phosphatase. Titres are expressed as $\log_2$ 0D 405 nm and are normalised to a 1:50 dilution. Bars represent mean and SEM for each group.

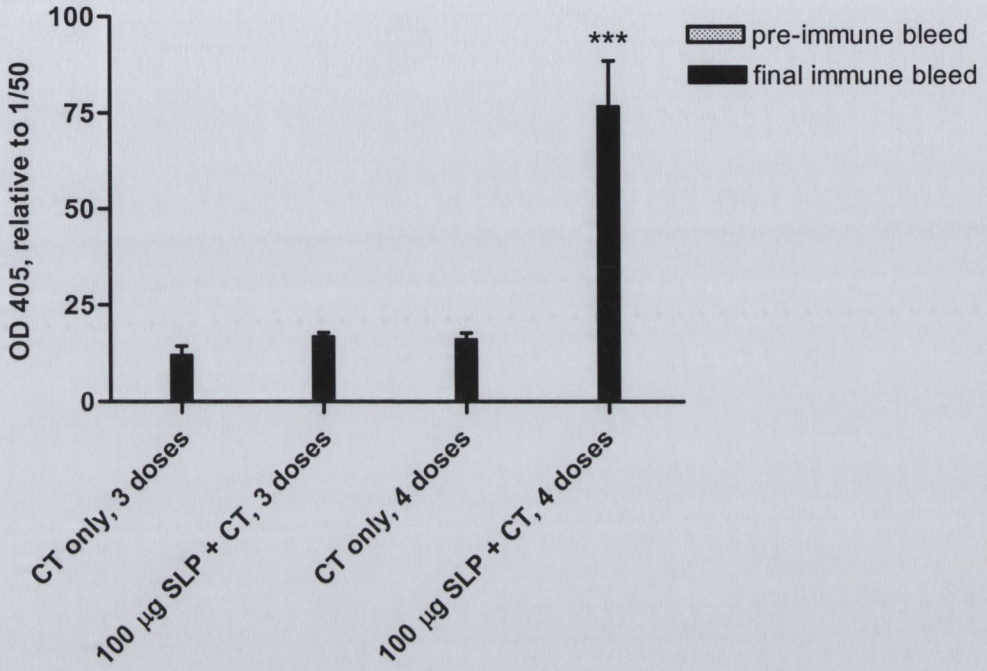



**Fig. 5.25 Serum IgG response to CT in SLP/CT immunised hamsters.** Hamsters were immunised i.n. with either three or four doses of SLPs (100 µg) plus CT (5 µg) (n = 6 per group). Control groups received three or four doses of CT only (n = 6 per group). Serum taken before the prime and after the final immunisation were analysed for anti-SLP IgG antibodies by ELISA. The secondary antibody used was goat anti-hamster IgG alkaline phosphatase. Titres are expressed as 0D 405 nm and are normalised to a 1:50 dilution. Bars represent mean and SEM for each group; ***, $P < 0.001$, four doses of SLP/CT versus three doses of SLP/CT compared by student's t-test.

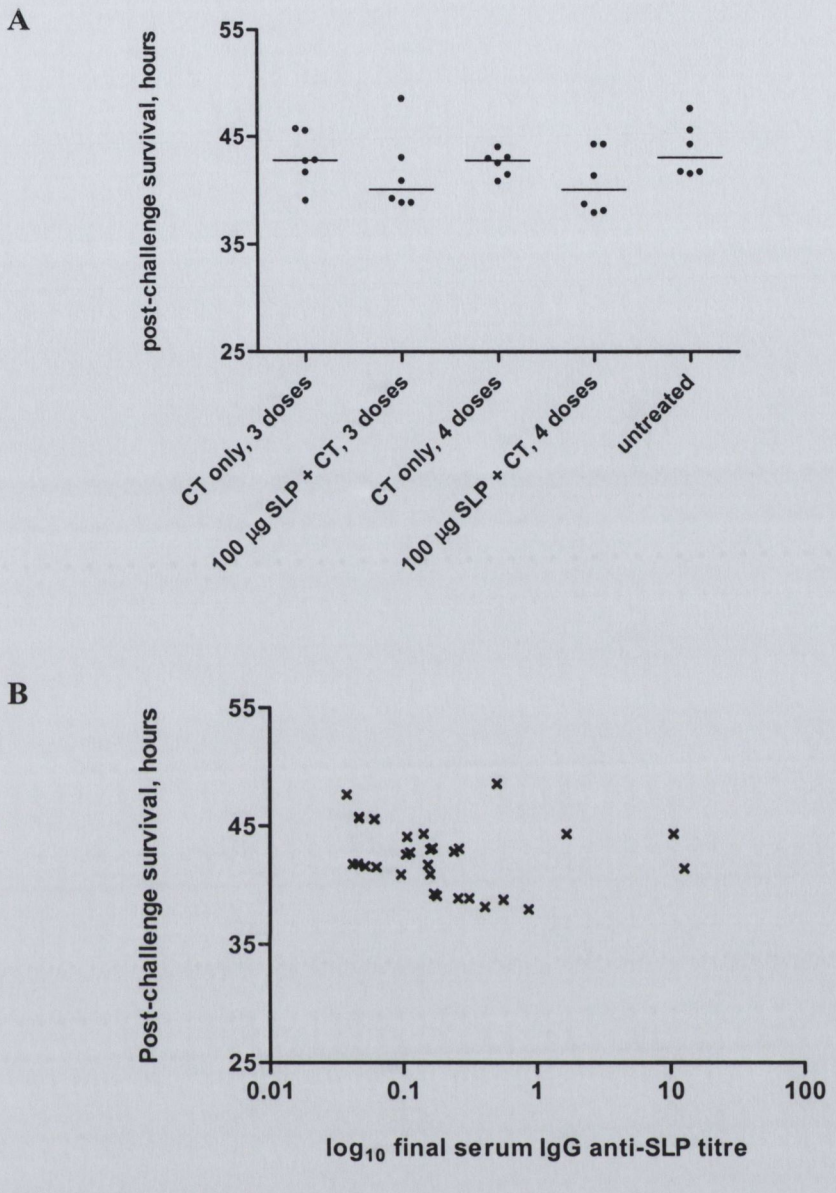



**Fig. 5.26** **Post-challenge survival of SLP/CT immunised hamsters.** Hamsters were immunised i.n. with either three or four doses of SLPs (100 µg) plus CT (5 µg) (n = 6 per group). Control groups received either three or four doses of CT only (5 µg) (n = 6 per group). After their final boost, hamsters were predisposed to *C. difficile* infection with 2 mg clindamycin and challenged with $10^5$ *C. difficile*. (A) Post-challenge survival versus immunisation regimen. Dots represent individual hamsters; bars represent median values for each group. (B) Post-challenge survival time versus serum IgG anti-SLP titre. Titres are expressed as $\log_{10}$ 0D 405 nm and are normalised to a 1:50 dilution. Crosses represent individual hamster titres.

anti-SLP titre and post-challenge survival time was evident **(Figure 5.26, B)** (Spearman r = - 0.33; P = 0.07).

### 5.3.19 Post-challenge weight loss of SLP/CT immunised hamsters.

Hamster weight loss following *C. difficile* challenge was monitored and post-challenge percentage weight loss is shown in **Figure 5.27 (A)**. There was no difference in median percentage weight loss between groups as tested by a non-parametric Kruskall-Wallis test (P = 0.45). Similarly, there was no correlation between percentage weight loss and post-challenge survival **(Figure 5.27, B)** (Spearman r = - 0.11; P = 0.57).

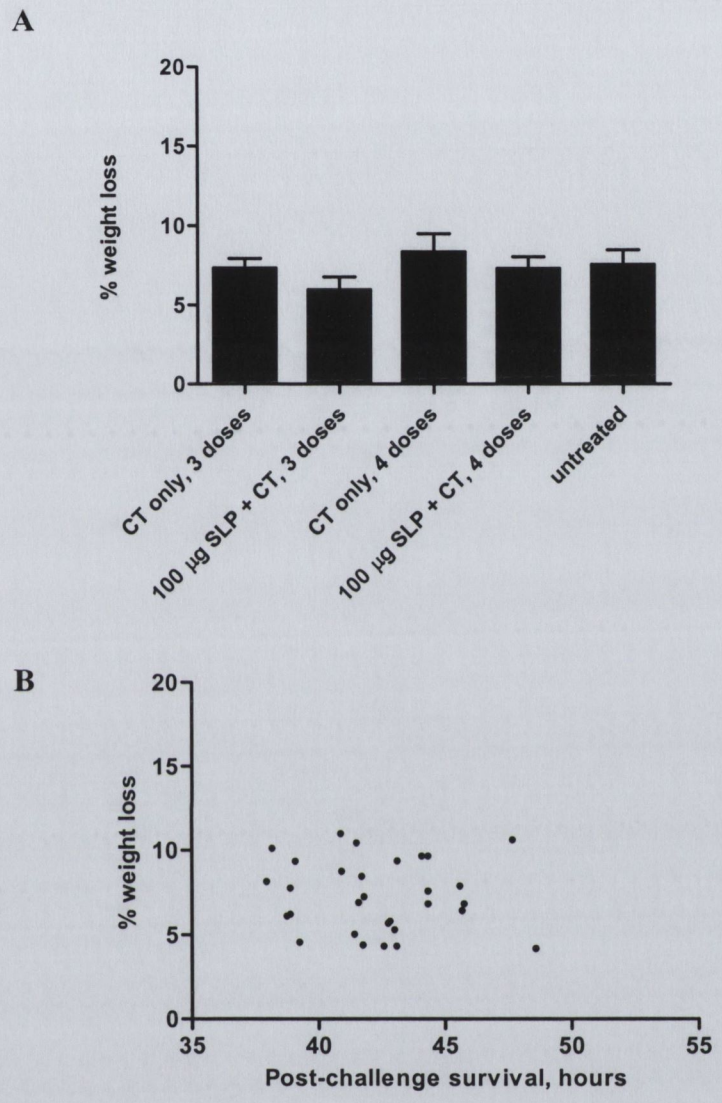



**Fig. 5.27 Post-challenge weight loss of SLP/CT immunised hamsters.** Hamsters were weighed daily and starting weight was calculated as an average of weight at day –1 and day 0 (day 0 = time of administration of *C. difficile*). Weight at time of death was used as final weight. (A) Percentage weight loss versus immunisation regimen. Bars represent mean and SEM for each group. (B) Percentage weight loss versus post-challenge survival time.

**OBJECTIVE 3**

### 5.3.20 Levels of cytokine production from splenic cells isolated from SLP immunised mice re-stimulated *in vitro* with SLPs.

Cytokine production from splenic cells isolated from SLP immunised mice and re-stimulated *in vitro* with SLPs was used to determine the type of immune response induced by the SLPs. Culture supernatants were assayed for IL-2, IL-4, IL-5, IL-10, IFN-$\gamma$ and TGF-$\beta$1 by ELISA **(Figure 5.28)**. Only the groups immunised with SLPs and CT and SLPs and CT/Ribi produced significant amounts of IL-2. Low levels of IFN-$\gamma$ and IL-10 were also detected in the group immunised with SLPs and CT. However, IL-10 (44.2 pg/ml) was detected in only one of the five mice in this group. The only cytokine detected in the chitosan glutamate and TMC immunised mice was IL-2, with very low levels detected. No IL-10 or IFN-$\gamma$ was detected in the chitosan glutamate or TMC groups. IL-4, IL-5 and TGF-$\beta$1 were undetectable in any of the supernatants, despite the fact that assays for IL-4 and IL-5 were optimised to give sensitivity as low as 4-8 pg/ml. The TGF-$\beta$1 assay was not as sensitive due to the interference of endogenous TGF-$\beta$1 in the medium.

### 5.3.21 Purified *C. difficile* SLPs, and SLPs in combination with CT, have a dose dependent effect on cytokine production from murine DCs.

The profile of cytokine production from SLP re-stimulated murine splenic T cells **(Section 5.3.20, Figure 5.28)** suggested the induction of immunosuppressive T cell responses in SLP immunised mice. Furthermore, the post-challenge survival of hamsters was not demonstrably correlated to humoral antibody response; lending further support to the possibility of the involvement of T cell mediated immunity. To investigate this possibility, immature DCs isolated from the femurs and tibiae of BALB/c mice were stimulated *in vitro* with the SLPs, both on their own and in combination with CT. Cytokines associated with the induction of a regulatory immune response, namely IL-10 and TGF-$\beta$1, and cytokines associated with the induction of a Th1 immune response, namely IL-12p70 and TNF-$\alpha$, were then measured in the DC culture supernatants by ELISA **(Figure 5.29)**.

Purified SLPs induced low levels of IL-10 at the highest concentration used (40 $\mu$g/ml). CT on its own failed to induce any IL-10, which is in agreement with previous studies (Gagliardi *et al.*, 2000, Lavelle *et al.*, 2003). The combination of SLPs and CT acted synergistically to induce large amounts of IL-10 **(Figure 5.29, A)**. Purified SLPs were

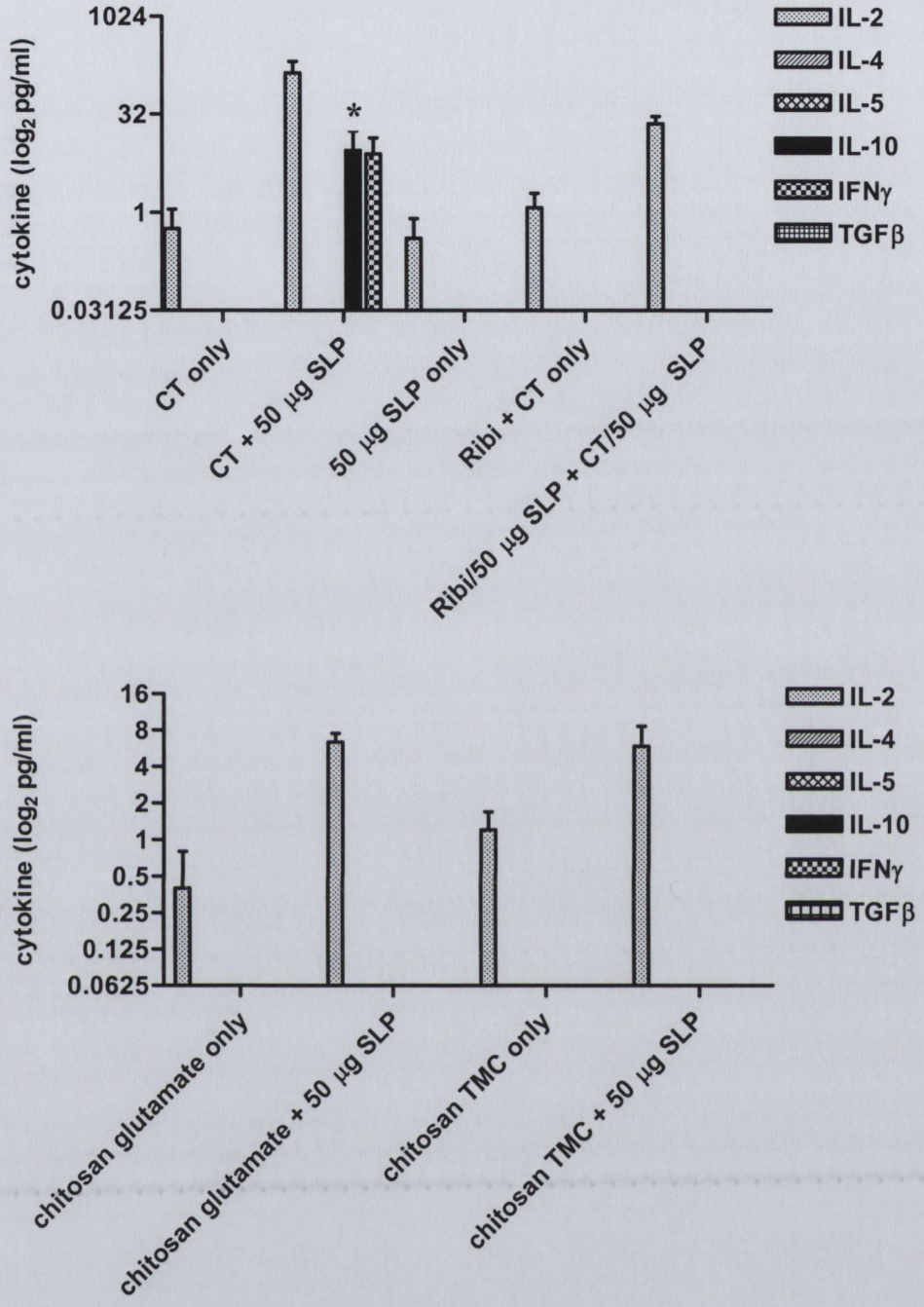



**Fig. 5.28 Cytokine production from murine splenic cells isolated from SLP immunised mice re-stimulated *in vitro* with SLPs.** Murine splenic cells (1 x $10^6$) were incubated with 20 μg/ml SLPs and supernatants collected after 24 h for IL-2 detection and after 72 h for IL-4, IL-5, IL-10, IFN-γ and TGF-β1 detection, and stored at - 20°C until required. Cytokines in the supernatants were detected using paired antibodies. Bars represent mean and SEM for each group. * Data shown for IL-10 is mean of five mice, but IL-10 detected in one of five only.

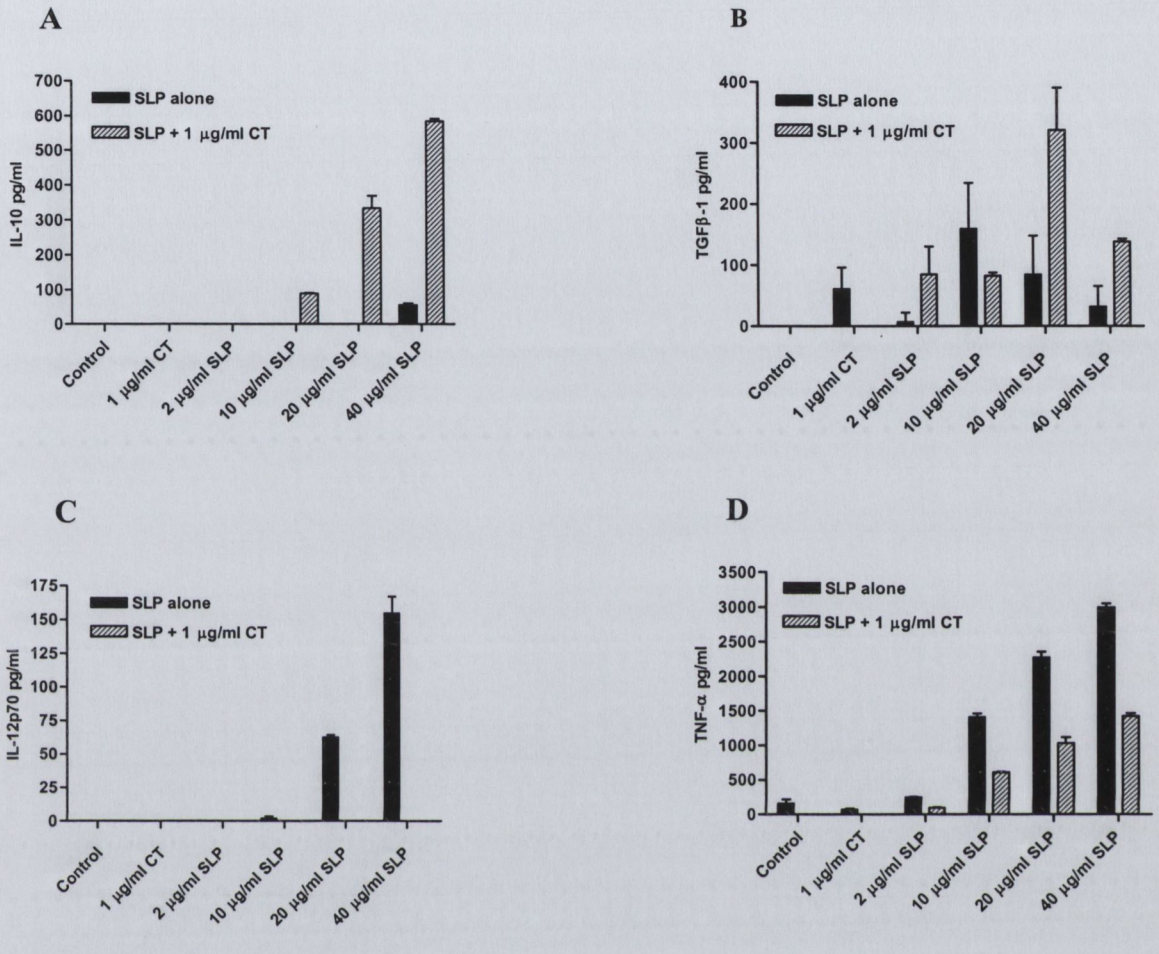



**Fig. 5.29 IL-10, IL-12p70, TGF-β1 and TNF-α production from murine DCs stimulated *in vitro* with SLPs alone or SLP/CT.** Bone marrow-derived immature DCs (1 x $10^6$) isolated from the femurs and tibiae of BALB/c mice were stimulated with SLPs (1 μg/ml, 2 μg/ml, 10 μg/ml, 20 μg/ml and 40 μg/ml) in the presence or absence of CT (1 μg/ml). Supernatants were collected after 24 h and analysed for the presence of IL-10, IL12p70, TGF-β1 and TNF-α using paired antibodies. Graph shown is representative of three independent experiments. Bars represent mean and SEM for triplicate cultures.

capable of inducing TGF-β1, with most TGF-β1 induced with 10 μg/ml SLPs. The amounts of TGF-β1 induced by the SLPs decreased when 20 μg/ml and 40 μg/ml were used. CT alone was capable of inducing small amounts of TGF-β1. When combined with CT, 20 μg/ml and 40 μg/ml SLPs resulted in an increase in the amounts of TGF-β1 induced **(Figure 5.29, B)**.

Purified SLPs also induced production of the pro-inflammatory cytokines IL-12p70 (at 20 μg/ml and 40 μg/ml) and TNF-α (at 2 μg/ml, 10 μg/ml, 20 μg/ml and 40 μg/ml) in a dose dependent manner **(Figure 5.29, C** and **D**, respectively). CT on its own failed to induce either IL-12p70 or TNF-α. The combination of SLPs and CT resulted in complete abolition of IL-12p70 production, as well as a large reduction in TNF-α production. Culture supernatants were also analysed for the Th2 inducing cytokine IL-4 and no IL-4 was found.

These findings suggest that the SLPs are Th1 inducing proteins, and that CT can modulate the response to the SLPs towards the induction of a regulatory response by enhancing production of IL-10 and TGF-β1, and by inhibiting or reducing production of IL-12p70 and TNF-α, thus inhibiting the activation of Th1-inducing DCs.

### 5.3.22 Effects of purified *C. difficile* SLPs, in combination with CT, Ribi and LPS, on cytokine production from murine DCs.

A combination of SLPs plus CT and SLPs plus Ribi/CT resulted in production of cytokines associated with a regulatory response from murine T cells **(Section 5.3.20)**. Furthermore, CT apparently modulated the effects of SLP-induced cytokine production from murine DCs **(Section 5.3.21)**. Therefore, we considered the possibility that Ribi might also modulate SLP-induced cytokine production in a similar manner to CT. Furthermore, we considered the possibility that the SLPs themselves might have immunomodulatory effects on LPS, a Th1-inducing molecule. To assess these possibilities, the effects of the SLPs in combination with CT, Ribi and LPS were investigated by measuring the amounts of IL-10, TGF-β1, IL-12p70 and TNF-α in murine DC culture supernatants. A 20 μg/ml SLP dose was chosen, as this gave the most consistent results in the dose response experiment outlined in **Section 5.3.21**.

**Figure 5.30** illustrates the effects of the SLPs, CT, Ribi and LPS, and various combinations of these, on IL-10 production from DCs. SLPs and CT on their own induced

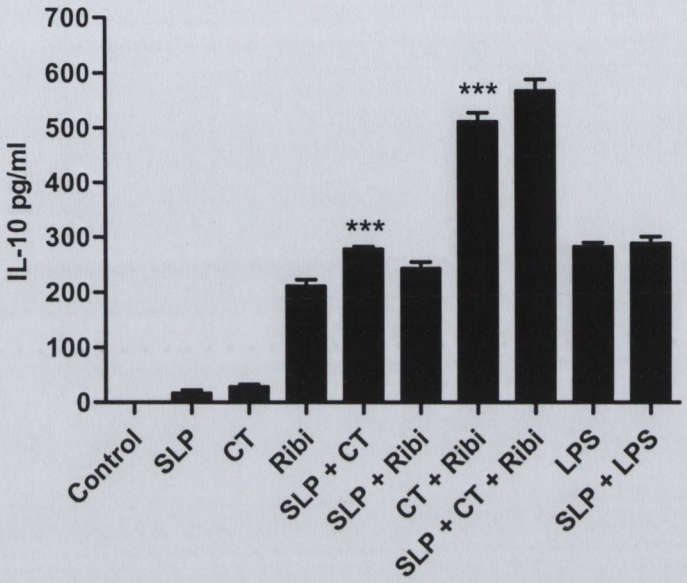



**Fig. 5.30 IL-10 production from murine DCs stimulated *in vitro* with SLPs, CT, Ribi, LPS and combinations of these.** Bone marrow-derived immature DCs (1 x $10^6$) isolated from the femurs and tibiae of BALB/c mice were stimulated with 20 μg/ml SLPs, 5 μg/ml CT, 5 μg/ml Ribi, 100 ng/ml LPS and combinations of these. Supernatants were collected after 24 h and analysed for the presence of IL-10 using paired antibodies. Bars represent mean and SEM for triplicate cultures. Graph shown is representative of three independent experiments. Cytokine concentrations were compared by one-way ANOVA. Where differences were found, the Tukey multiple comparisons test was used to identify differences between individual groups. ***, $P < 0.001$, SLPs + CT vs SLPs or CT alone; CT + Ribi vs CT or Ribi alone.

low amounts of IL-10, as previously described in **Figure 5.29 (A)**. A combination of the SLPs and CT resulted in a dramatic increase in the amount of IL-10 produced compared to either SLPs or CT alone (***, $P < 0.001$). This effect was clearly synergistic, as the amount of IL-10 induced was significantly higher than the total amounts of the IL-10 induced by the SLPs or CT individually. Ribi on its own induced similar amounts of IL-10 to the SLPs/CT combination. Addition of the SLPs to Ribi did not increase IL-10 production further. However, Ribi-induced IL-10 was significantly enhanced by the addition of CT (***, $P < 0.001$). This effect was also synergistic, as the amount of IL-10 induced was significantly higher than the total amounts of the IL-10 induced by CT or Ribi individually. When the SLPs were added to the CT/Ribi combination no further increase in IL-10 production was evident. LPS induced moderate amounts of IL-10, and the addition of the SLPs to LPS did not alter the amounts of IL-10 produced.

The effect of the SLPs, CT, Ribi and LPS, and various combinations of these, on production of the immunomodulatory cytokine TGF-$\beta$1 from DCs is illustrated in **Figure 5.31**. The SLPs induced moderate amounts of TGF-$\beta$1, similar to LPS, and no synergy between the SLPs and LPS was evident. CT on its own also induced TGF-$\beta$1. SLP-induced TGF-$\beta$1 was enhanced by the addition of CT (*, $P < 0.05$), although this effect appeared to be additive. Ribi on its own also induced TGF-$\beta$1 and when combined with the SLPs a reduction in TGF-$\beta$1 production was evident. A combination of CT and Ribi increased TGF-$\beta$1 production above that seen with either CT or Ribi individually (*, $P < 0.05$), although this effect again appeared to be additive. No change was seen in TGF-$\beta$1 production on addition of the SLPs to this CT/Ribi combination.

**Figure 5.32** illustrates the effects of the SLPs, CT, Ribi and LPS, and various combinations of these, on production of the pro-inflammatory cytokine IL-12p70 from DCs. The SLPs alone induced low levels of IL-12p70, supporting the hypothesis that the SLPs are Th1 inducing proteins. CT alone failed to induce IL-12p70, as previously demonstrated in **Figure 5.29 (C)**. A combination of the SLPs and CT resulted in complete loss of SLP-induced IL-12p70 (***, $P < 0.001$), indicating that CT was modulating the response to the SLPs. Ribi induced large amounts of IL-12p70, as previously shown in human monocyte derived DCs by Ismaili *et al.* (2002). When the SLPs were combined with Ribi a drop in IL-12p70 production was seen, although this was not statistically significant. On addition of CT to Ribi, there was complete loss of Ribi-induced IL-12p70 production (***, $P < 0.001$). Addition of the SLPs to the CT/Ribi combination did alter IL-

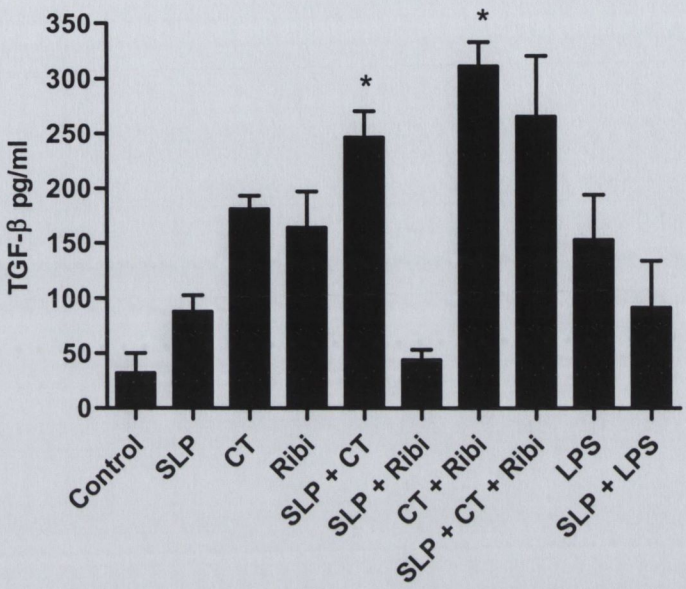



**Fig. 5.31 TGF-β1 production from murine DCs stimulated *in vitro* with SLPs, CT, Ribi, LPS and combinations of these.** Bone marrow-derived immature DCs (1 x $10^6$) isolated from the femurs and tibiae of BALB/c mice were stimulated with 20 μg/ml SLPs, 5 μg/ml CT, 5 μg/ml Ribi, 100 ng/ml LPS and combinations of these. Supernatants were collected after 24 h and analysed for the presence of TGF-β1 using paired antibodies. Bars represent mean and SEM for triplicate cultures. Graph shown is representative of three independent experiments. Cytokine concentrations were compared by one-way ANOVA. Where differences were found, the Tukey multiple comparisons test was used to identify differences between individual groups. *, $P < 0.05$, SLPs + CT vs SLPs alone; Ribi + CT vs Ribi alone.

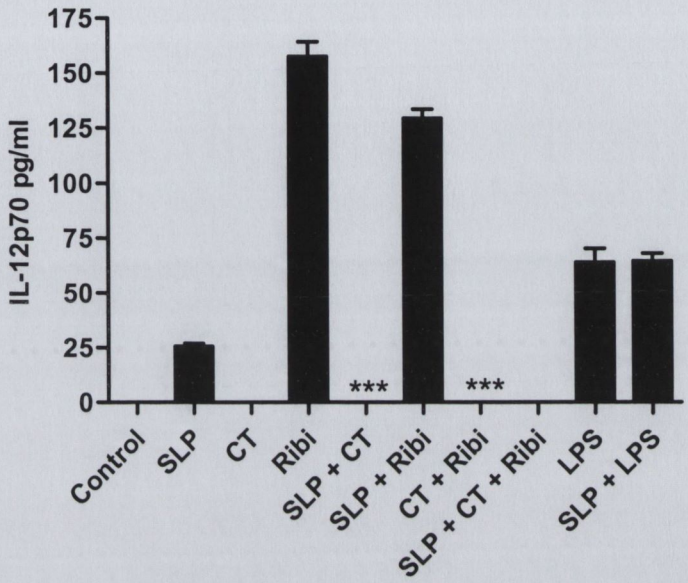



**Fig. 5.32 IL-12p70 production from murine DCs stimulated *in vitro* with SLPs, CT, Ribi, LPS and combinations of these.** Bone marrow-derived immature DCs (1 x $10^6$) isolated from the femurs and tibiae of BALB/c mice were stimulated with 20 μg/ml SLPs, 5 μg/ml CT, 5 μg/ml Ribi, 100 ng/ml LPS and combinations of these. Supernatants were collected after 24 h and analysed for the presence of IL-12p70 using paired antibodies. Bars represent mean and SEM for triplicate cultures. Graph shown is representative of three independent experiments. Cytokine concentrations were compared by one-way ANOVA. Where differences were found, the Tukey multiple comparisons test was used to identify differences between individual groups. ***, $P < 0.001$, SLPs + CT vs SLPs alone; CT + Ribi vs Ribi alone.

12p70 production. These data indicate that CT can reduce both SLP- and Ribi-induced IL-12p70 from DCs. LPS induced moderate amounts of IL-12p70, and addition of the SLPs to LPS had no effect on the amount of IL-12p70 produced.

**Figure 5.33** illustrates the effects of the SLPs, CT, Ribi and LPS, and various combinations of these, on TNF-α production from DCs. SLPs on their own induced large amounts of pro-inflammatory TNF-α, similar to LPS. There was no synergy between the SLPs and LPS with regard to TNF-α production. CT induced small amounts of TNF-α, as previously seen in **Figure 5.29 (D)**. A combination of the SLPs and CT resulted in a significant reduction in TNF-α production (***, $P < 0.001$), lending further support to the hypothesis that CT was modulating the response to the SLPs. Ribi on its own induced large amounts of TNF-α. Addition of the SLPs to Ribi did not alter TNF-α production. Addition of CT to Ribi significantly reduced TNF-α production (***, $P < 0.001$). Addition of the SLPs to this combination did not modulate TNF-α production.

## 5.4 DISCUSSION

This work was chiefly concerned with inducing a strong active immune response to the *C. difficile* SLPs with a view to protecting hamsters from CDAD. When the SLPs were administered i.p. with $Al(OH)_3$ adjuvant, high titre IgG anti-SLP antibodies were raised in hamsters. The antibody response exhibited a dose response from 10 μg to 100 μg SLPs, with saturation reached at 100 μg. A large degree of variability in antibody titre within groups was evident, but this might be expected given the small group sizes and fact that Golden Syrian hamsters are out-bred. However, other studies have also found a large degree of variability in antibody response to the SLPs in different patients infected with *C. difficile* (Drudy *et al.*, 2004, Pantosti *et al.*, 1989, Dr. Déirdre Ní Eidhin, unpublished observations). This apparent variability in response to the SLPs may represent differences in the intestinal flora or some genetic differences in individuals.

An interesting observation was a definite drop in antibody response with subsequent immunisations, when the lower doses of SLPs were administered (10 μg SLPs plus $Al(OH)_3$, 25 μg SLPs plus $Al(OH)_3$, 25 μg SLPs no $Al(OH)_3$) **(Figure 5.1)**. This is especially obvious with the 10 μg dose, and may represent some kind of inhibition of antibody response by the SLPs. Why the same inhibition was not seen with the higher doses of the SLPs is not clear. However, a decrease in anti-SLP antibody titres in patients infected with *C. difficile* was also observed in our laboratory when sequential human sera

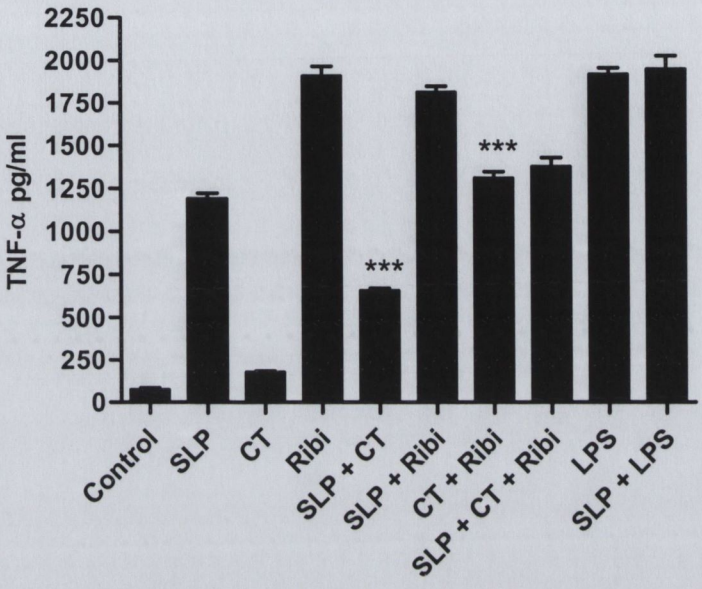



**Fig. 5.33 TNF-α production from murine DCs stimulated *in vitro* with SLPs, CT, Ribi, LPS and combinations of these.** Bone marrow-derived immature DCs (1 x $10^6$ cells/ml) isolated from the femurs and tibiae of BALB/c mice were stimulated with 20 μg/ml SLPs, 5 μg/ml CT, 5 μg/ml Ribi, 100 ng/ml LPS and combinations of these. Supernatants were collected after 24 h and analysed for the presence of TNF-α using paired antibodies. Bars represent mean and SEM for triplicate cultures. Graph shown is representative of three independent experiments. Cytokine concentrations were compared by one-way ANOVA. Where differences were found, the Tukey multiple comparisons test was used to identify differences between individual groups. ***, $P < 0.001$, SLPs + CT vs SLPs alone; Ribi + CT vs Ribi alone.

from *C. difficile* infected patients was analysed by ELISA (Dr. Déirdre Ní Eidhin, unpublished observations).

Following *C. difficile* challenge, all SLP plus $Al(OH)_3$ immunised and non-immunised hamsters succumbed to CDAD and post-challenge survival time was not correlated in any way to serum IgG anti-SLP levels. Therefore, although it was possible to generate high titre serum IgG anti-SLP antibodies with $Al(OH)_3$, it was evident that this was not an appropriate immune response in terms of protection from CDAD in hamsters. It is possible that the anti-SLP antibodies were not present at the gut mucosa where *C. difficile* colonises and establishes infection. In theory, it would be possible for these serum antibodies to gain access to the gut lumen as a consequence of toxin-induced inflammation of the mucosa. However, once the actions of the toxins take effect the hamsters die rapidly (Borriello *et al.*, 1987), and hence any protective effect of the antibodies might not be obvious in the absence of a toxin neutralising factor. It was not possible to assess if a cellular immune response was taking place in the hamsters due to the absence of commercial hamster reagents; however, aluminium adjuvants are not efficient at inducing cell-mediated immune responses (Gupta, 1998).

Some bacteria produce proteolytic enzymes, termed immunoglobulin proteases, which cleave immunoglobulins and therefore destroy their protective properties. Examples of such bacteria are *Neisseria gonorrhoeae*, *Neisseria meningitidis* and *Streptococcus pneumoniae* (Batten *et al.*, 2003, Hauck and Meyer, 1997). These proteolytic enzymes are important virulence factors in these organisms. For example, one of the key features that distinguish pathogenic from non-pathogenic *Neisseria* is the secretion of an IgA1-specific serine protease (Hauck and Meyer, 1997). The absence of any protective effect of the circulating anti-SLP antibodies in the $Al(OH)_3$ immunised hamsters might have been explained by the possibility that *C. difficile* was producing such an immunogloblin protease. When analysed by Western blotting, *C. difficile* culture supernatant had no effect on the integrity of IgG or IgA, indicating that a mechanism such as that outlined above was not at play. It should be noted that this method only took secreted bacterial factors into account and does not rule out the possibility of a more complex bacterial inhibition of antibody function.

It was clear from the $Al(OH)_3$ active immunisation experiment that circulating IgG anti-SLP antibodies were not protective in the hamster model of CDAD. It has been well

established that protection against many enteric pathogens is more correlated with the presence of immunoglobulins in local secretions than with that of serum immunoglobulins (Acheson and Luccoli, 2004, Mulligan *et al.*, 1993). S-IgA, in particular, can block adhesion of pathogens to epithelial surfaces, thus preventing colonisation. Hence, adjuvants that were known for their ability to induce mixed or primarily mucosal immune responses against co-administered antigens, namely Ribi and CT, were assessed for their capacity to induce protective responses against the SLPs in hamsters.

Very low serum IgG anti-SLP titres were raised in hamsters with Ribi, and when used in combination with CT no significant increase in serum response to the SLPs was evident. Ribi has been extensively assessed as an alternative to FA, with conflicting results. Tiong *et al.* (1993) and Kenney *et al.* (1989) observed intermediate and low antibody responses, respectively, after Ribi injection. Leenaars *et al.* (1998) also found Ribi to be ineffective at inducing a serum IgG antibody response against a weakly immunogenic antigen, (SP215, a synthetic peptide of 21 amino acids) in mice, when compared to FCA, Specol, TiterMax or Montanide. However, Leenaars *et al.* found that despite the low serum antibody responses after Ribi injection, significantly higher numbers of activated T cells and cytokine producing cells were present in spleen sections of these mice. These findings, and our study, conflict with the findings of Geerlings *et al.* (1989) and Lipman *et al.* (1992), who observed comparable serum antibody responses after injection with FCA and Ribi. Due to the absence of a commercially available hamster anti-IgA, an assay for hamster IgA based on mouse anti-IgA conjugate was investigated (Dr. Matthew McCabe). However, the mouse conjugate failed to adequately recognise the hamster IgA and therefore serum, faecal or salivary IgA could not be measured. Despite the absence of a strong serum IgG anti-SLP response and the lack of information regarding the IgA anti-SLP response, these hamsters were nonetheless challenged with *C. difficile*.

Following *C. difficile* challenge, two out of five hamsters immunised i.p. with 25 μg SLPs and Ribi were completely protected from disease, exhibiting no clinical symptoms of infection, with the exception of slight weight loss. Additionally, two out of three hamsters immunised with SLPs and a combination of CT (i.n.) and Ribi (i.p.) were also completely protected from disease, exhibiting no clinical symptoms of infection, again with the exception of slight weight loss. A single adjuvant only control hamster, which received CT (i.n.) and Ribi (i.p.), was also protected from disease, with no clinical symptoms of infection except slight weight loss. The fact that an adjuvant only control hamster survived

the *C. difficile* challenge made it impossible to say with certainty that the surviving hamsters immunised with SLPs plus Ribi or SLPs plus Ribi and CT were protected due to an immune response to the SLPs, and not by some other independent adjuvant mediated mechanism. A recent study demonstrated that repeated oral pre-treatment of rats with CT inhibited *C. difficile* toxin A induced fluid secretion and cytotoxicity (Lönnroth *et al.*, 2003). The authors of this study speculated that as both CT and toxin A mediate their actions via the enteric nervous system, these nerves were probably mediating the observed protection. All protected hamsters were colonised with *C. difficile* as demonstrated by the limited but typical epithelial damage observed on histological analysis of caecum, colon and small intestine sections. There was no correlation between serum IgG anti-SLP levels and survival, and in fact the surviving hamsters had titres spread over the range of titres achieved after immunisation **(Figure 5.7, B)**. On Western blotting, three of the total five surviving hamsters had developed serum antibodies to a high molecular weight antigen, most likely the toxins, and all five had antibodies to an antigen just above the 55 kDa molecular weight marker **(Figure 5.9)**. It is possible that these antibodies contributed to the protection from disease seen in these hamsters, but in the absence of serum from all hamsters in the experiment it is impossible to conclude this for certain.

The fact that the surviving hamsters immunised with SLPs and Ribi were those that received the lowest dose of antigen (25 μg), and the fact that there was no correlation between antibody response and survival, suggests that the serum IgG anti-SLP antibodies were not responsible for the protection from disease seen in these hamsters. Existing work shows that MPL (a component of Ribi) can induce DCs to mature and migrate to T cell areas of lymphoid organs (De Becker *et al.*, 2000). Furthermore, macrophages stimulated with MPL have heightened bactericidal activity including enhanced phagocytosis and the production of reactive oxygen intermediates that help control microbial infections (Evans *et al.*, 2003). In a recent active immunisation study where RAS was used, Ribi by itself conferred protection to 12.5% of mice challenged with *Neospora caninum*, compared to 75% of mice that had been immunised with Ribi and the antigen being tested (Cannas *et al.*, 2003). Additionally, there is *in vivo* data demonstrating that mice pre-treated with MPL are non-specifically protected from bacterial and viral challenge (Evans *et al.*, 2003, Ulrich and Myers, 1995). In our study, two out of fifteen (13.3%) hamsters immunised with SLPs and Ribi survived the *C. difficile* challenge. It is possible that activation of the innate immune response by Ribi mediated non-specific protection in the surviving hamsters. Torres *et al.* (1995) also included a Ribi only control group in their toxoid based hamster

immunisation study. All Ribi only control hamsters died from CDAD in this latter study; however, the Ribi was administered subcutaneously to the control hamsters, whereas Ribi was administered i.p. to experimental hamsters, making comparisons between their experimental and control groups, and our control group, difficult. Furthermore, Torres *et al.* did not include a CT/Ribi only control group, even though this combination of adjuvants was used in one of the toxoid immunisation regimens tested.

In mice, the highest serum IgG responses against the SLPs were obtained in the group immunised i.n. with SLPs and CT. However, these titres were moderate at best and were much lower than anti-toxin titres obtained by Torres *et al.* (1995) who also used CT (i.n.). Ribi is known to induce both systemic and mucosal responses (Balbridge *et al.*, 2000) and was included in the prime immunisation in order to boost these responses. However, inclusion of Ribi did not improve the serum IgG anti-SLP titre, and in fact the mean titre was lower in the group that received SLPs plus Ribi and CT compared to the group that received SLP plus CT only. Serum and faecal IgA anti-SLP titres were moderate, with no evidence that inclusion of Ribi offered any additional benefit. The faecal IgA anti-SLP titres were comparable to or greater than anti-toxin titres obtained in hamsters in a study by Giannasca *et al.* (1999), in which high levels of protection against CDAD were observed. It is important to note that the IgA samples were analysed singly and therefore a certain amount of error would have been present.

Neither chitosan glutamate nor TMC were effective adjuvants for inducing systemic or mucosal responses against the SLPs in mice. Very low serum and mucosal anti-SLP titres and cytokine responses were obtained in these groups. There are several reasons why this might have been the case. First, due to the requirement of chitosan glutamate be in an acidic environment in order to be water soluble (pH 4 - 6), and since the SLPs have reported p*I*'s ranging from 3.3 to 6.3 (Hagiya *et al.*, 1992, Mastrantonio *et al.*, 1995, Takeoka *et al.*, 1991), it is likely that the SLPs were poorly soluble in this low pH environment. Second, the degree of quaternisation of TMC plays an essential role in its drug absorption-enhancing properties. The degree of quaternisation determines the number of positive charges available on the molecule for interactions with negatively charged sites on the epithelial membrane, and thereby influences its ability to open tight junctions of intestinal mucosae (Hamman *et al.*, 2003). At pH 7.2 a TMC derivative with a 60% degree of quaternisation has been shown to enhance mannitol transport across Caco-2 cells more than a TMC derivative with 40% quaternisation. TMC with the lowest degree of

quaternisation, 12.3%, has been shown to be ineffective at enhancing mannitol transport at neutral pH (Kotzé *et al.*, 1998b). The TMC used in our study had a degree of quaternisation of 21.9%. It is possible that TMC with a higher degree of quaternisation might have been more effective. Third, Baudner *et al.* (2003) demonstrated that i.n. administration of an antigen with a combination of chitosan and a mutant of LT (LTK63) resulted in induction of immune responses of at least the same level as subcutaneous immunisation. It is possible that such a combination of chitosan and LT or CT may result in improved immune responses to the SLPs and should be considered for any future studies.

An increase in the anti-SLP titres of adjuvant only control animals was evident to varying degrees among different groups. This indicates that the mice may have been exposed to *C. difficile* prior to their prime immunisation. The increase is exaggerated in some of the graphs presented due to the fact that the y-axes are on logarithmic scales. However, elevated titres are not evident in the control groups to the same extent as in the experimental groups, particularly after the final boost **(Figures 5.5, 5.10 and 5.14)**, which would have been expected if the animals had been previously exposed to *C. difficile*. Previous *C. difficile* immunisation studies have presented final experimental titres only, and it is possible that control titres went up in these studies also. Although every precaution was taken, since *C. difficile* is a spore former and is extremely difficult to contain, it is possible that *C. difficile* present in the environment was responsible for priming these hamsters. However, concerns over the prior exposure of the hamsters to *C. difficile* become less critical in light of the fact that only one *C. difficile* strain was used throughout the animal experiments, so if contamination occurred, it was by the infecting strain.

Opsonic activity of antibodies has been correlated with protection for other bacterial infections such as *Streptococcus pneumoniae* (Johnson *et al.*, 1999) and *Porphyromonas gingivalis* (Page, 2000, Wilton *et al.*, 1993). In **Chapter IV (Section 4.3.3)** rabbit anti-SLP antibodies that prolonged survival of hamsters challenged with *C. difficile* were also shown to increase phagocytosis of the bacterium. Serum from hamsters immunised with SLPs, regardless of the adjuvant used, serum anti-SLP titre or survival status of the individual hamster, did not increase phagocytosis of *C. difficile* in the same system. The fact that hamster serum anti-SLP antibodies do not appear to have opsonic activity against *C. difficile* indicates that these antibodies do not facilitate the major immune functions of

complement activation and phagocytosis normally associated with serum IgG. Conversely, using the same method, high titre mouse anti-SLP serum was shown to increase phagocytosis of *C. difficile*. However, this serum had an anti-SLP titre of 575 as compared to the highest hamster serum tested, which had an anti-SLP titre of only 56.08. It appeared that a high serum anti-SLP titre was necessary to increase phagocytosis, as mouse serum with a substantial serum IgG anti-SLP titre of 155.9 failed to increase phagocytosis. It is possible that a large amount of anti-SLP antibodies are required to neutralise the high abundance SLPs on the surface of the bacterium. The low anti-SLP titres achieved in hamsters may account for the failure of their serum to increase phagocytosis of *C. difficile* and possibly the lack of protection from CDAD observed in the challenge experiments.

A trend towards protection against CDAD was observed in the hamsters immunised with SLPs and a combination of CT and Ribi. Additionally, CT resulted in the best systemic and mucosal immune response in mice, while inclusion of Ribi in the immunisation regimens did not improve either the systemic or mucosal responses. These data suggested that CT alone might offer improved protection from disease, but it was not possible to assess this possibility in the mouse model. Therefore, CT alone was the adjuvant of choice for a subsequent larger immunisation and challenge experiment in hamsters. Generally, low serum IgG anti-SLP titres were obtained in hamsters immunised with SLPs and CT. Even after a third booster immunisation, although the serum IgG anti-SLP titre did increase, no significant increase was observed. There was again no correlation between serum IgG anti-SLP titres and post-challenge survival time in these hamsters, and in fact a negative correlation between the two was apparent **(Figure 5.26 (B))** although not statistically significant (Spearman $r = -0.33$; $P = 0.07$). Since a Ribi/CT adjuvant control hamster had survived the *C. difficile* challenge in the previous experiment, to eliminate the possibility that CT itself was conferring some protection on the hamsters, untreated and CT only control groups were included in this experiment. CT alone did not protect any hamsters from the effects of the *C. difficile* toxins. This suggests that the Ribi/CT control hamster from the previous challenge experiment did not survive due to the effects of CT alone, and its survival may have been due to the actions of either Ribi or a combination of Ribi and CT.

Due to the failure to generate significant anti-SLP titres in either hamsters or mice immunised with SLPs and CT, we assessed the possibility that the SLPs were modulating the immune response to the CT itself. In a preliminary analysis to investigate this

possibility, serum IgG anti-CT levels were measured in hamsters and mice immunised with SLPs, SLPs plus CT, SLPs plus CT and Ribi, CT, and Ribi/CT. The presence of the SLPs resulted in higher serum IgG anti-CT titres in hamsters, but had no effect on the titres in mice. This indicates that the SLPs were not inhibiting the serum antibody response to CT and suggests that the SLPs and CT might have been working synergistically to stimulate the immune system in hamsters in some way.

The failure to induce either significant serum IgG antibody responses or protective immune responses against the SLPs in the hamster model raises important questions regarding the SLPs and the model itself. It should be re-emphasised at this point that the hamster model of *C. difficile* infection is a severe model with an acute lethal outcome in the absence of any protection (Bartlett, 2004, Delmée and Avesani, 1990). An important consideration in vaccine development and immunological memory is the incubation period of the pathogen. If a pathogen becomes disease causing before memory cell response occurs, the host must maintain high levels of immunoglobulin titres rather than relying on effective responses by memory cells. In the hamster model used in this study, the first symptoms of *C. difficile* infection generally appeared within 24 to 48 hours of clindamycin administration and *C. difficile* challenge. The rapid action of the toxins may not give the adaptive immune system the time required to mount a protective response. To counteract this fact, hamsters in this study were challenged shortly after their final immunisation, when theoretically their antibody titres should have been at their highest. However, the anti-SLP antibodies present were apparently not able to prevent *C. difficile* colonisation and subsequent toxin production. Active immunisation should resolve infection before disease develops, and it is possible that the hamster model is too sensitive to the toxins, and is therefore not an ideal model for testing a vaccine which does not have toxin neutralising properties. It is also possible that there is something unique about the way hamsters generate antibodies that results in these antibodies being non-protective. However, Torres *et al.* (1995) and Giannasca *et al.* (1999) obtained high anti-toxin titres and protected hamsters from CDAD in their studies. When administered with powerful vaccine adjuvants such as Ribi and CT, there was a failure to generate significant antibody responses to the SLPs in either hamsters or mice. It seemed plausible therefore, that the SLPs, or the SLPs in combination with some of the adjuvants tested, may have been modulating the immune response in hamsters and mice.

The differentiation of T cells towards a Th1, Th2 or Tr phenotype based on their profile of cytokine production is very relevant in the investigation of regulation of immune responses. In this study, low levels of cytokine production were observed from SLP re-stimulated splenic cells. The highest levels of cytokines were observed in the mice immunised with SLPs and CT. The production of IFN-$\gamma$ and IL-2 in this group, and the lack of IL-4 or IL-5, indicates that the immune response was polarised towards a Th1 type response. Antigen-specific Th1 responses have been shown to mediate protective immunity against a range of pathogens, including *B. pertussis* (Ryan *et al.*, 1997) and *Cryptococcus neoformans* (Wormley *et al.*, 2005). No IL-4, IL-5 or TGF-$\beta$1 were observed in the supernatants from any of the SLP immunised groups. Interestingly, IL-10 was detected in duplicate supernatants from a single mouse in the group immunised with SLPs and CT. Originally, IL-10 was identified as a cytokine-synthesis-inhibiting factor, particularly with regard to the suppression of IFN-$\gamma$ production by Th1 cells (Fiorentino *et al.*, 1989, reviewed in Pestka *et al.*, 2004). IL-10 production might indicate a regulatory response in these mice, but since IL-10 was detected in only one mouse this finding is difficult to interpret. If this was the case, TGF-$\beta$1 production might also have been expected. However, there is a population of regulatory cells that exhibit potent immnosuppressive abilities both *in vitro* and *in vivo* that are not dependent on secreted TGF-$\beta$1 (Chen and Wahl, 2003, Zheng *et al.*, 2004). These $CD4^+CD25^+$ T cells, comprising approximately 5-10% of the peripheral T cell pool, are a population of regulatory cells that mediate immunosuppression via cell surface presentation of TGF-$\beta$ to TGF-$\beta$ receptor on target cells by cell-cell interaction, and not by secretion of suppressive cytokines (Nakamura *et al.*, 2001). Another possibility was that the T cells were anergic. IL-10 modulated DCs have been shown to induce anergic T cells, which are characterised by inhibited antigen-specific proliferation, a profoundly reduced production of IL-2 and IFN-$\gamma$ (as well as down-regulated expression of CD25). This may explain the pattern of cytokine production obtained from the re-stimulated T cells. However, anergic T cells do not produce immunomodulatory cytokines such as IL-10 and TGF-$\beta$1 (Jonuleit *et al.*, 2001). Further active immunisation studies would be necessary to fully elucidate the phenotype of the T cells obtained in the SLP immunised mice.

The decision for lymphocytes to differentiate into Th1 or Th2 cells occurs early in the immune response, and the ability of a pathogen to stimulate cytokine production by cells of the innate, non-adaptive immune system plays an important part in shaping the subsequent adaptive response. We demonstrated *in vitro* that purified *C. difficile* SLPs induce

production of the immunomodulatory cytokines IL-10 and TGF-β1, as well as the pro-inflammatory cytokines IL-12p70 and TNF-α, from immature murine DCs. This pattern of cytokine production is very similar to that seen with LPS, and is typical of a Th1 inducing protein. There was no evidence of synergy between the SLPs and LPS. SLP-induced IL-10 and TGF-β1 was enhanced by the addition of CT, whereas this combination suppressed IL-12p70 and TNF-α production. In fact, complete abrogation of IL-12p70 production was seen with SLPs in combination with CT. The production of IL-12 by innate cells, and activation of DC maturation, is key in directing naïve T cells to differentiate into Th1 cells, and IL-12p70 is an important marker for DC maturation and can be used as a method for selecting Th1-inducing adjuvants (Ismaili *et al.*, 2002). Analagous to the role of IL-12p70 in inducing a Th1 response, IL-10 is a critical cytokine for the induction of Tr1 cells. Anti-inflammatory effects of CT have previously been reported, in particular the down-regulation of IL-12p70 production by human DCs (Braun *et al.*, 1999). In addition, CT has previously been shown to modulate the immune response to LPS (Lavelle *et al.*, 2003). In this case, CT in combination with LPS suppressed production of Th1 cytokines and enhanced production of regulatory cytokines by DCs. The consequences of up-regulation of IL-10 and TGF-β1 and down-regulation of IL-12p70 and TNF-α, is potentially the development of a regulatory immune response. In this study, Ribi induced a very similar pattern of cytokines to LPS in DCs. Furthermore, in combination with CT, Ribi increased even further the production of the regulatory cytokines and suppression of the Th1 inducing cytokines.

In light of the DC data, it is interesting to note that the common factor in all hamsters that survived *C. difficile* challenge following immunisation was the inclusion of Ribi, with the best survival seen in the hamsters immunised with SLPs plus a combination of Ribi and CT. It is possible that *in vivo* the induction of a regulatory immune response limited the infection-induced immunopathology normally associated with CDAD, thus protecting these hamsters. Alternatively, it has to be considered that the induction of regulatory cells may not be desirable in immunisation against an infectious pathogen such as *C. difficile*, and may result in bacterial persistence by suppressing protective Th1 responses. Pathogen evasion through modulation of cytokine production by cells of the innate immune system has been described for several bacteria, many of which cause chronic or persistent infection in humans, including *B. pertussis*, *M. tuberculosis* and *V. cholerae* (reviewed in McGuirk and Mills, 2002). In some cases, immunomodulatory molecules have been identified, such as FHA from *B. pertussis*, which induces Tr cells via IL-10 production

from DCs (McGuirk *et al.*, 2002). This latter hypothesis potentially explains the overall poor survival of hamsters immunised with the SLPs. It may also partly account for the high relapse rate among *C. difficile* patients, which has been explained to date by re-infection or germination of residual spores (Kelly and LaMont, 1998, Poutanen and Simor, 2004, Tabaqchali and Jumaa, 1995).

In conclusion, it proved difficult to induce protective immunity against the *C. difficile* SLPs in the hamster model of infection, regardless of the adjuvant used. High-titre anti-SLP antibodies were not protective in this model. Ribi and a combination of Ribi and CT adjuvants conferred some non-specific protection from CDAD in hamsters. CT was shown to modulate the response to the SLPs in murine DCs, inducing production of cytokines associated with a regulatory immune response. Futhermore, Ribi enhanced this effect. Further investigation into the immunomodulatory properties of the SLPs, both independently and in combination with various adjuvants, is merited.

# CHAPTER VI

# GENERAL DISCUSSION

# GENERAL DISCUSSION

## 6.1 Rationale for an SLP-based vaccine against *C. difficile*

Since the original description of *C. difficile* as a harmless commensal organism in 1935, this pathogen has become one of the commonest causes of AAD and the most frequent cause of PMC in hospitalised patients (Hall and O'Toole, 1935, Poutanen and Simor, 2004). This is primarily due to the introduction and continued widespread use of broad-spectrum antibiotics since the 1960s. A compelling case for the development of novel prophylactic and therapeutic agents against *C. difficile* is provided by the high incidence of morbidity and mortality associated with the organism. The huge financial burden placed on modern healthcare resources by *C. difficile* infection (Kyne *et al.*, 2002, Wilcox *et al.*, 1996) further highlights the need to develop novel strategies. Furthermore, the incidence of *C. difficile* infection has become more widespread in recent years and there is evidence to suggest that more transmissible and more virulent strains of *C. difficile* are becoming endemic in western hospitals (Archibald *et al.*, 2004, Morris *et al.*, 2002). Recent outbreaks in Canada (Pépin *et al.*, 2004), in particular, highlight both the financial and human impact of *C. difficile* infection. Although infection control measures, such as restrictive antibiotic policies, have been shown to be effective in reducing the number of *C. difficile* cases (Ludlam *et al.*, 1999, Worsley, 1998), the spore-forming ability of *C. difficile* allows it to persist for months or years on environmental surfaces, healthcare workers and colonised new admissions. This huge potential for infection makes *C. difficile* inherently difficult to control in a hospital setting. Combined with the fact that antibiotic use is on the increase (Oldfield, 2004), it is likely that *C. difficile* will remain problematic in healthcare facilities until an effective prophylactic agent is available for clinical use.

This study was chiefly concerned with investigating the potential of the *C. difficile* SLPs as vaccine candidates against *C. difficile*. Although a toxin-based vaccine is currently under development, this does not obviate the need for novel preventative strategies against the organism. In particular, it is important to bear in mind that only a minority of vaccines in clinical trials will eventually result in a vaccine licensed for use in humans. Furthermore, a toxin-based vaccine is unlikely to result in elimination of bacterial carriage and therefore the reservoir of infection in hospitals will persist, and carriage and transmission will continue to be a threat. We hypothesised that a vaccine based on the SLPs could prevent adhesion of the

bacterium, and therefore subsequent colonisation and toxin-mediated disease. Given the success to date of the toxoid vaccine, a combination vaccine incorporating both the SLPs and the toxins should also be considered for any future studies. This type of composite vaccine may be effective at eliminating or reducing colonisation and neutralising the toxins, thus preventing toxin mediated pathology as well as eliminating the reservoir of infection.

This study used the SLPs purified from a Ribotype 1 clinical isolate strain, which was found to be the most common ribotype in St. James's hospital, Dublin, as well as in the U.K. (Brazier, 1998) **(Table 1.1)**. However, to be effective, a vaccine directed at the SLPs must take account of the high degree of inter-strain antigenic variation occurring in these proteins. Future approaches should include the SLPs from several clinically prevalent ribotypes. In Ireland, Ribotype 1 and Ribotype 12 strains should be considered in particular, as these strains were found to be the two most common isolates in St. James's hospital, Dublin. It is reasonable to assume that the pattern of *C. difficile* isolates in St. James's hospital is representative of that in other hospitals within Ireland. The *slpA* gene sequences from the Ribotype 46 and 92 strains isolated in St. James's hospital are identical to one another, and only a single nucleotide different to that of Ribotype 12 (Dr. Déirdre Ní Eidhin, manuscript in preparation). Therefore, a vaccine based on the SLPs from Ribotype 12 might also offer protection against Ribotypes 46 and 92. Furthermore, a composite vaccine targeting the SLPs of Ribotype 1 and 12 strains would be expected to offer protection against the majority of *C. difficile* infections in Ireland.

## 6.2 Passive, but not active, immunisation with SLPs delays CDAD progression in a hamster model of infection.

Passively administered high-titre rabbit anti-SLP antibodies were found to delay progression of CDAD in a lethal hamster challenge model. Given this finding, the possibility that active immunisation with the SLPs would offer improved protection from disease in the same model was considered. Induction of musocal immunity, S-IgA in particular, is known to be effective at preventing adhesion of bacteria to epithelial surfaces. We hypothesised that induction of anti-SLP antibodies, in particular S-IgA, at colonic mucosal surfaces would result in elimination of *C. difficile* and subsequent protection from disease. Active immunisation with the SLPs in combination with a range of adjuvants and routes of delivery, resulted, for the most part, in low serum IgG anti-SLP antibody responses in hamsters. Where high titre antibodies were generated, with $Al(OH)_3$ adjuvant, the antibodies were not protective against

disease. Low levels of protection from disease were observed with Ribi adjuvant and a combination of CT/Ribi adjuvants. However, no correlation between serum IgG antibody response and protection from disease was evident, suggesting that this protection was non-specific. However, since hamster IgA responses could not be measured, it is possible that protection was mediated by an IgA anti-SLP response. Overall, it proved difficult to induce strong serum IgG responses against the SLPs in hamsters and levels of protection against CDAD were low and most likely non-specific.

There are several possible explanations for the low antibody responses and poor protection from CDAD observed in hamsters. First, it is possible that the hamster anti-SLP antibodies produced had low affinity for their target epitopes on the bacterial surface and therefore did not bind and eliminate the bacteria efficiently. Rapid neutralisation of *C. difficile* at the colonic mucosa would be necessary to prevent colonisation and therefore subsequent toxin production and the inevitable lethal effect of the toxins. It is possible that a different adjuvant(s) may have resulted in immunological presentation of the antigen in a more conformationally relevant manner, which may have resulted in the production of higher affinity anti-SLP antibodies. Interestingly, hamster anti-SLP antibodies were unable to promote opsonophagocyosis of *C. difficile* by PMA-differentiated THP-1 monocytes *in vitro*. Conversely, rabbit anti-SLP antibodies were very effective at promoting opsonophagocytosis of the bacterium. This may reflect the relatively low titres of antibody achieved in hamsters compared to rabbit. It also has to be considered that antibody generation may be in some way defective in hamsters. Hamsters are exquisitely sensitive to *C. difficile* infection and the basis for this is not clear. The sensitivity of hamsters to infection may be partly due to their inability to mount an effective humoral immune response to *C. difficile*.

Second, the hamster model of disease may be too severe a model to test the protective effects of a vaccine based on the SLPs, particularly in the absence of a toxin neutralising factor. A hamster model based on transmission of infection by spores may be more appropriate to test the protective effects of an immune response to the SLPs. A modified hamster model could include active immunisation, subsequent inoculation with *C. difficile* spores followed by clindamycin administration some weeks later. By disrupting the normal gut flora post-*C. difficile* exposure, as opposed to pre-exposure, the severity of the model would be reduced. This environment would allow the adaptive immune system time to mount an effective

memory response. Therefore, the protective effects of SLP-specific immunity could be tested in a setting less affected by the toxins. This model may be more physiologically relevant for two reasons. First, transmission of *C. difficile* in hospitals is generally thought to be via spores present in the environment. Second, the rapid lethal effects of *C. difficile* infection in hamsters do not parallel the infection in humans, where severe disease and death results in only a small minority of cases.

### 6.3 Immunological basis of the poor immune response to the SLPs

Since it proved difficult to induce strong immune responses against the SLPs in either hamsters or mice the possibility that the SLPs were suppressing the immune response was considered. DCs are pivotal APCs in the immune system and it is their activation and elaboration of cytokines that drives the subsequent adaptive immune response. Thus, the effect of the SLPs on murine DC cytokine secretion patterns, and therefore the subsequent Th cell response, was analysed *in vitro*. The SLPs were found to have immunomodulatory properties. Specifically, they induced secretion of both pro-inflammatory (IL-12 p70 and TNF-$\alpha$) and anti-inflammatory (IL-10 and TGF-$\beta$1) cytokines from DCs. This pattern of cytokine secretion is representative of a Th1 type immune response and is similar to that seen with LPS. Addition of CT adjuvant inhibited or significantly reduced production of the SLP-induced IL-12p70 and TNF-$\alpha$, while concomitantly up-regulating production of the immunosuppressive cytokines IL-10 and TGF-$\beta$1. Given the pivotal role of IL-12p70 in inducing Th1 responses, this represents a suppression of the SLP-induced Th1 type immune response. Furthermore, Ribi adjuvant, also found to be a Th1 inducing compound, was modulated in an almost identical fashion to the SLPs when combined with CT. This pattern of cytokine secretion from DCs strongly suggests the induction of an immunosuppressive regulatory immune response and inhibition of a potentially protective Th1 immune response *in vivo*. This type of mechanism has been observed for *B. pertussis*, and is thought to represent a bacterial evasion strategy used to subvert protective Th1 responses and therefore persist in the host (McGuirk *et al.*, 2002). It is tempting to speculate that the *C. difficile* toxins might work synergistically with the SLPs in the same way as CT to induce this type of immunosuppressive response in infected individuals. Although there is no data to suggest this, CT and *C. difficile* toxin A have several features in common, including the fact that both toxins attach to glycosphingolipid receptors and exert enzymatic activities on epithelial cells. Furthermore, it is known that the enteric nervous system communicates with the gut and plays an important role in the diarrhoea

induced by both toxins (Lönnroth *et al.*, 2003). The inhibition of a protective Th1 response and induction of an immunosuppressive regulatory response might partly explain the chronic infection often seen in individuals infected with *C. difficile*.

Th1 type immune responses have been found to be dysregulated in a number of inflammatory and autoimmune disorders, including inflammatory bowel disease, Crohns disease, rheumatoid arthritis and sepsis. Induction of high levels of IL-10 and TGF-$\beta$ has been shown to inhibit Th1 immune responses associated with inflammatory diseases. Therefore, the ability to suppress Th1 type immune responses, as displayed by the CT/SLP and CT/Ribi combinations, has important therapeutic potential for the treatment of such disorders. Further studies will be required to fully characterise the Tr cells induced by these combinations, including phenotypic characterisation of the cells. Additionally, the anti-inflammatory effects of the CT/SLP and CT/Ribi combinations could easily be assessed *in vivo* in an LPS-induced mouse model of septic shock. Septic shock is induced by Gram negative bacterial LPS and results from an overproduction of inflammatory cytokines such as TNF-$\alpha$ and IL-1 (Marzocco *et al.*, 2004). Shock is induced through a complex inflammatory cascade triggered by these cytokines (Hirata *et al.*, 2001). TNF-$\alpha$ and IL-1 are elevated in the sera of patients with septic shock and most studies indicate an association between TNF-$\alpha$ levels, severity of shock and mortality levels (Matsumoto *et al.*, 2004). Furthermore, cytokine knockout mice models could be used to define the roles of the particular cytokines in the immunosuppressive effects of the CT/SLP and CT/Ribi combinations.

It is not known if the *C. difficile* SLPs interact with PRRs on the surface of macrophages and DCs. PRRs include collectins, CD14, TLRs, mannose-binding protein, complement receptors, C-reactive protein and Fc receptors, among others (reviewed in Ryan *et al.*, 2001). The importance of the TLRs in inducing Th1 type immune responses has already been established. Specifically, the TLR4 ligands LPS, CpG DNA, poly(IC), and TLR7 ligands, induce IL-12p70 and IFN-$\alpha$ from DCs and thus stimulate Th1 responses (reviewed in Pulendran, 2005). Binding of microbial products to TLRs transduces signals through a common adaptor, MYD88 (myeloid differentiation factor 88) and NF$\kappa$B, and leads to the transcription of several immune response genes including IL-1, IL-12 and TNF-$\alpha$. This enhances the ability of APCs to present antigen and to stimulate T cell activation, resulting in increased adaptive immune responses (Ryan *et al.*, 2001). At present there is no data demonstrating which, if any, TLRs

the SLPs interact with. The identification of putative cellular receptors combined with elucidation of the signalling pathways downstream of such receptors, has the potential to reveal the mechanism by which the SLPs prime the host immune response to *C. difficile*.

### 6.4 Future SLP-based subunit vaccines against *C. difficile*

Modern vaccines target very specific molecules or pieces of molecules. The production of vaccines in their most compact form, including peptide and DNA vaccines, is considered to be the safest and most rational method of developing vaccines into the future. This study assessed both the native low- and high-MW SLPs as potentially protective antigens in a vaccine against *C. difficile*. An important aspect of modern vaccine development is the identification of immunodominant regions of target proteins and this should be incorporated into any future studies on the prophylactic or therapeutic potential of the SLPs. Antigen-specific polyclonal splenic T cells from immunised animals could be used to epitope map the most efficacious vaccine antigens using overlapping synthetic protein fragments. This task might be facilitated by the fact that most of the antigenic and sequence diversity is found in the low-MW SLP, obviating the need to create peptides spanning the high-MW SLP. Additionally, immune serum from hamsters and mice immunised with the native SLPs could be used to identify the most immunogenic peptides.

Furthermore, DNA vaccines could be designed using a representative Ribotype 1 or Ribotype 12 strain. Synthetic DNA from the selected strain could be cloned into eukaryotic expression vectors, which replicate in *E. coli*, and the construct produced in quantities required for *in vivo* immunisation studies. The same DNA sequence could also be cloned in an *E. coli* expression vector and the recombinant protein used to boost immunity of immunised animals. The performance of protein and DNA based vaccines could then be compared. Additionally, chimeric genes encoding immunodominant epitopes from the most common ribotypes could be created by splicing together the immunogenic regions of the native genes. DNA constructs and the corresponding chimeric recombinant protein could then be used to immunise animals. The chimeric gene could also form the basis for a DNA vaccine. A more radical approach to the design of a chimeric vaccine could be based on immunodominant T cell epitopes, and a chimeric DNA vaccine constructed using DNA encoding 2-3 of the 20-mer synthetic peptides found to evoke a strong immune response. These approaches may ultimately lead to the development of a safe and effective SLP-based subunit vaccine against *C. difficile*.

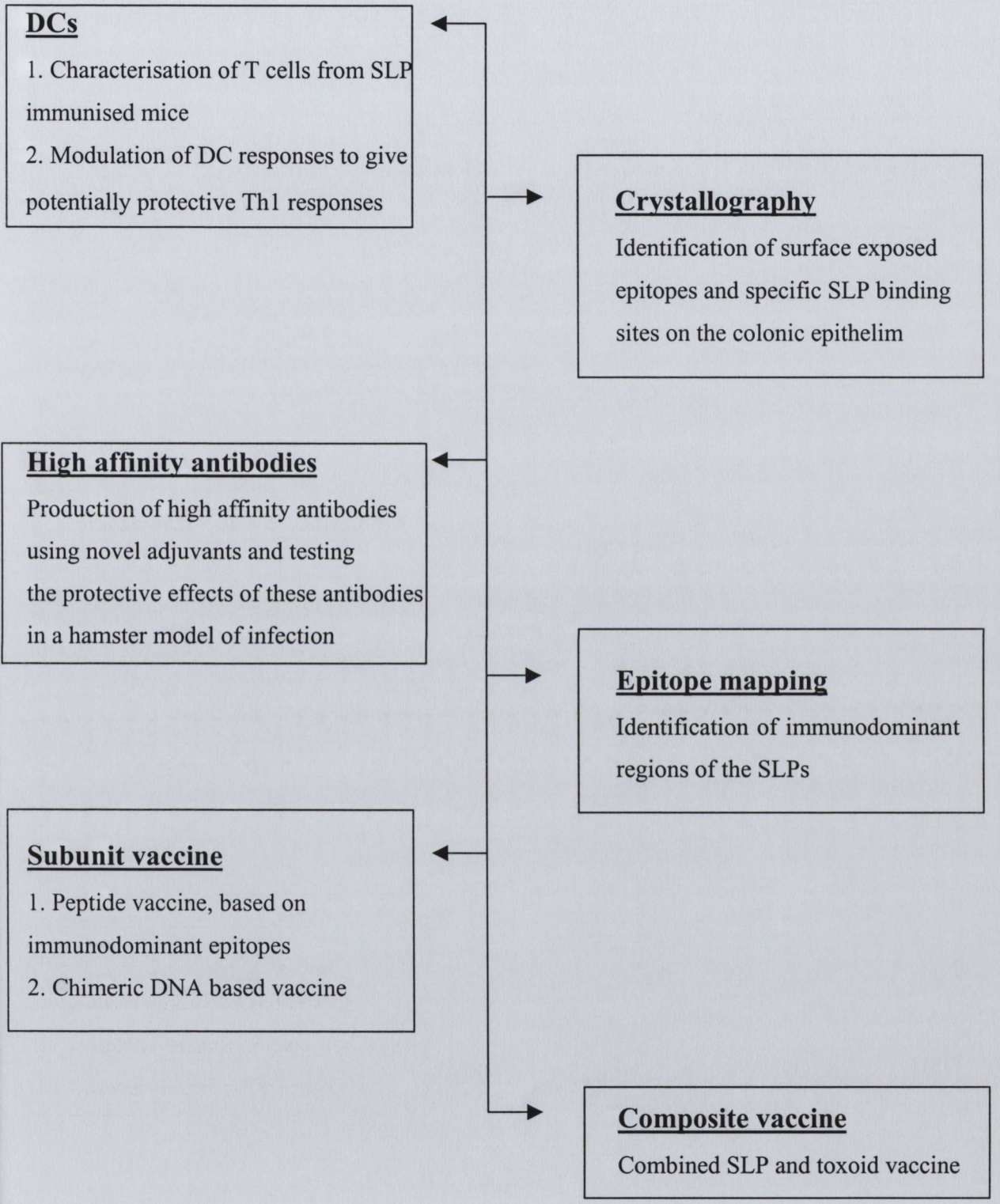
## 6.5 Potential applications of a resolved 3D structure of the SLPs

Initial crystallography studies of the recombinant low-MW SLP have identified preliminary crystallisation conditions for this protein. Crystallisation efforts with the combined native SLPs have not yielded crystals of sufficient quality for x-ray diffraction to date, however, collaborative efforts will continue to this end. Elucidation of the 3D structure of the SLPs presents exciting possibilities for examining the role of these proteins in the pathogenesis of *C. difficile*. First, it will assist in the identification of the specific binding targets of the SLPs on colonic epithelial cells. This would allow for the development of drugs to target this binding site(s) and therefore prevent colonisation and subsequent disease. Furthermore, co-crystallisation of the native low- and high-MW SLPs would give detailed insight into the interaction of the SLPs with each other, and possibly with other adhesins and SLP homologues, on the surface of the bacterium. The high-MW SLP has been shown to bind to human and mouse intestinal epithelial cells, a property that the low-MW SLP does not appear to possess. Therefore, the arrangement of the SLPs on the surface of the bacterium must allow for the high-MW SLP to be at least partially exposed on the outer surface. Identification of the specific epitope(s) exposed on the surface would allow more specific targeting of the binding sites of this adhesin. The low-MW SLP is the more immunogenic of the two and has been shown to be surface exposed (Cerquetti *et al.*, 1992). It is likely that the SLPs do not form two distinct S-layers, one on top of each other, on the surface of *C. difficile*, but rather interact with each other such that both SLPs are exposed on the surface. Finally, a resolved structure may help explain the biological reason(s) for the high degree of inter-strain variability exhibited by the SLPs. A resolved 3D structure of the SLPs from one *C. difficile* strain would allow for prediction of SLP structures from other strains. This might allow for prediction of common shapes that might help identify structures that appear on the surface of the bacterium that may be related to the virulence of particular strains.

## 6.6 Genetic manipulation of *C. difficile*

The inability to transform *C. difficile* with DNA has severely limited research into its virulence and it is this lack of effective genetic tools and transfer systems that has resulted in *C. difficile* remaining poorly characterised. Until recently, the only method of introducing DNA was based on the transfer of conjugative transposons, such as Tn*916*, from *B. subtillis* donors. However, transfer frequencies were low and in some strains the transposon had limited utility as a mutagen as it always inserted into a single site within the genome

## SUMMARY OF FUTURE WORK



(Mullaney *et al.*, 1994). Tn*916* based vectors have also been used to introduce antisense RNA encoding constructs into *C. difficile*, allowing examination of the effect of down-regulating specific genes (Roberts *et al.*, 2003). Specifically, antisense RNA for the adhesin encoding gene *cwp66* has been introduced into *C. difficile*. In this case, the recombinant conjugative transposons entered the genome at different sites. This gives rise to the possibility, that in this *C. difficile* strain at least, it may be possible to use Tn*916* as an insertional mutagen. Conceivably, any DNA could be inserted into this vector and incorporated into the *C. difficile* genome. Shuttle vectors have also been conjugated from *E. coli* donors to a toxigenic *C. difficile* strain (Herbert *et al.*, 2003). A reliable and efficient delivery system for *C. difficile* that will express foreign DNA will ultimately result in the determination of putative virulence factors of the organism. Such a strategy will definitively answer the questions regarding the role(s) of the SLPs in *C. difficile* infection and the host immune response to the organism.

## 6.7 Concluding remarks

*C. difficile* infection remains a serious problem in healthcare facilities and the development of a vaccine remains a realistic preventative strategy into the foreseeable future. The SLPs of *C. difficile* are metabolically expensive, major antigens of this pathogen and their specific roles in the pathogenesis of infection remain to be determined. However, the SLPs putatively function in bacterial colonisation and may have a role in immune evasion of the bacterium. It is clear, therefore, that these proteins represent good targets for development of a vaccine against *C. difficile*. In the future they may form part of a composite vaccine with the toxins or with other as yet uncharacterised antigens of *C. difficile*. Potential immuosuppressive properties of the SLPs may not make them ideal as vaccine candidates. However, the emergence of new adjuvants into the future may circumvent this issue and therefore the SLPs still represent promising targets for vaccine development against *C. difficile*.

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# APPENDICES

# Appendix A

## Reagents and Buffers

### Cell Culture Media

**Complete RPMI-1640 Medium**

| | |
|---|---|
| RPMI 1640 | 500 ml |
| Heat inactivated Foetal Calf Serum (FCS) | 50 ml |
| Penicillin/streptomycin/L-glutamine Culture Cocktail (Gives a final concentration of 2 mM L-glutamine, 100 $\mu$g/ml penicillin and 100 U/ml streptomycin) | 10 ml |

**Hanks Balanced Salts Solution**

| | |
|---|---|
| HBSS | 500 ml |
| N-(2-Hydroxyethyl)piperazine-N'-2-ethanesulfonic acid, (HEPES) (1 M) | 10 ml |

**Cell Culture Cryopreservative Medium**

| | |
|---|---|
| FCS | 9.0 ml |
| Dimethylsulfoxide | 1.0 ml |

**10X Phosphate Buffered Saline (PBS)**

| | |
|---|---|
| $Na_2HPO_4.2H_2O$ (8 mM) | 14.24 g |
| $KH_2PO_4$ (1.5 mM) | 2.04 g |
| NaCl (137 mM) | 80.0 g |
| KCl (2.7 mM) | 2.0 g |

Make up to 1 L final volume. Dilute to 1X PBS and pH to 7.4

**25X Protease Inhibitor Cocktail**

One tablet dissolved in 2ml distilled water.

Store 200µl Aliquots in -20°C.

**Mouse Spleen Lysing Buffer (10X)**

| | |
|---|---|
| $NH_4Cl$ | 8.29 g |
| $KHCO_3$ | 1.0 g |
| $Na_2EDTA$ | 37.2 mg |

Make up to 100 ml with distilled water, filter sterilise, make up to 1X with sterile water as required.

## *C. difficile* Culture Media

**NOTE: Sterilise all media by autoclaving**

**Brain Heart Infusion (BHI) Medium**

| | |
|---|---|
| BHI powder | 37 g |
| Distilled water | 1000 ml |

**Brain Heart Infusion (BHI)/0.5 % Thioglycolate**

| | |
|---|---|
| Sodium thioglycolate | 2.5 g |
| BHI media | 500 ml |

**Brain Heart Infusion (BHI) Agar**

| | |
|---|---|
| BHI powder | 37 g |
| Agar | 15 g |
| Distilled water | 1000 ml |

**Columbia Blood Agar *C. difficile***

| | |
|---|---|
| Columbia agar base | 3.9 g |
| Sterile horse blood | 7 ml |
| Clindamycin HCl | 50μg/ml |
| Distilled water | 93 ml |

Boil Columbia agar base in the water to dissolve, cool to 50°C and add horse blood with swirling.

**Fastidious Anaerobe Broth (FAB)**

| | |
|---|---|
| FAB powder | 29.7 g |
| Distilled water | 1 L |

**Cooked Meat Broth for *C. difficile* Stocks**

1 level teaspoon Cooked Meat into bijoux bottle

10 ml FAB medium

Heat to boiling to eliminate air.

## Polyacrylamide Gel Electrophoresis (PAGE) Reagents

**2X Reducing Sample Buffer**

| | |
|---|---|
| Tris HCl 1.0 M pH 6.8 | 0.5 ml |
| Glycerol | 0.8 ml |
| Sodium dodecyl sulphate (SDS) 10% | 1.6 ml |
| β-mercaptoethanol | 0.4 ml |
| Bromophenol Blue (0.1 %) | 0.2 ml |
| Deionised water | 4.5 ml |

**Stacking Gel Buffer (1.0 M Tris HCl pH 6.8)**

| | |
|---|---|
| Tris base | 12.11 g |
| Deionised water | 100 ml |

Adjust to pH 6.8 with concentrated HCl and make up to final volume.

**Resolving Gel Buffer (1.5 M Tris HCl pH 8.8)**

| | |
|---|---|
| Tris base | 36.3 g |
| Deionised water | 200 ml |

Adjust to pH 8.8 with concentrated HCl and make up to final volume.

**10% Ammonium Persulphate (APS)**

| | |
|---|---|
| APS | 0.1 g |
| Deionised water | 1.0 ml |

Make up fresh before use.

**Electran 2.6 Acrylamide/Bisacrylamide Premix**

| | |
|---|---|
| Acrylamide | 29.2 g |
| Bisacrylamide | 0.8 g |
| Deionised water to final volume | 100 ml |

**Water-Saturated Butanol**

| | |
|---|---|
| Butanol | 10 ml |
| Deionised water | 10 ml |

Mix well and allow settle. Use top layer.

**10X SDS-PAGE Running Buffer**

| | |
|---|---|
| Tris base | 30 g |
| Glycine | 114 g |
| SDS | 5 g |

Dissolve in deionised water to a final volume of 1 L and dilute 1:10 in deionised water before use.

**Coomassie Blue Gel Stain**

| | |
|---|---|
| Coomassie Brilliant Blue R-250 | 2.5 g |
| Methanol | 554 ml |
| Glacial Acetic Acid | 92 ml |
| Deionised water | 554 ml |

Dissolve the Coomassie Blue in the methanol, add the water and finally add the acetic acid. Filter through a Whatman # 1 filter and store in a lightproof container.

**De-stain Solution**

| | |
|---|---|
| Methanol | 400 ml |
| Glacial Acetic Acid | 70 ml |
| Distilled water | 530 ml |

## Western Blotting Reagents

**Transfer Buffer**

| | |
|---|---|
| Tris base | 2.9 g |
| Glycine | 1.45 g |
| SDS | 0.185 g |

Dissolve in 350 ml deionised water, add 100 ml of methanol and make up to final volume of 500 ml.

**Blocking Solution**

| | |
|---|---|
| Skimmed dried milk powder (Marvel) | 5 g |
| PBS | 100 ml |
| Polyoxyethylenesorbitan monolaureate (Tween 20®) | 100 µl |

Make up fresh before use.

**PBS/0.1% Tween Washing Solution**

| | |
|---|---|
| PBS | 500 ml |
| Tween 20® | 500 µl |

**Primary and Secondary Antibody Diluent Solution**

| | |
|---|---|
| Skimmed dried milk powder (Marvel) | 5 g |
| PBS | 100 ml |

**Amido Black Protein Detection**

| | |
|---|---|
| Amido Black | 0.5 % (w/v) |
| Isopropanol | 25 % (v/v) |
| Acetic Acid | 10 % (v/v) |

Staining was for 5 min and de-staining was done by several washes in distilled water

## Cloning and Expression Reagents

**Ethidium Bromide (EB) Stock (10 mg/ml)**

| | |
|---|---|
| Ethidium bromide | 100 mg |
| Deionised water | 10 ml |

**Tris Acetate EDTA (TAE) Buffer (10X)**

| | |
|---|---|
| Tris base | 24.2 g |
| Glacial Acetic Acid | 5.71 ml |
| Ethylenediaminetetraacetic acid (EDTA), pH 8.0 (0.5 M) | 20 ml |

Make up to final volume of 1 L with deionised water.

**TAE Running Buffer**

| | |
|---|---|
| TAE (10X) | 50 ml |
| Deionised water | 450 ml |

**Rapid Plasmid Screening Solutions**

**Solution II**

| | |
|---|---|
| NaOH (1 M) | 1 ml |
| SDS 10% | 0.5 ml |
| Sterile deionised water | 3.5 ml |

Prepare fresh.

**Lysis Solution**

| | |
|---|---|
| Solution II | 4 ml |
| Gel loading dye | 0.9 ml |
| Sterile deionised water | 1.1 ml |

**Solution III**

| | |
|---|---|
| Formic acid (1.8 M) | 1.22 g |
| Potassium Acetate (3 M) | 2.94 g |

Make up to 10 ml final volume with deionised water.

**Gel Loading Dye**

| | |
|---|---|
| Xylene cyanol | 50 mg |
| Bromophenol Blue | 75 mg |
| Glycerol | 25 ml |
| EDTA (0.5 M) | 5 ml |
| Deionised water | 20 ml |

Mix and store at room temperature.

**1% Agarose Gel**

| | |
|---|---|
| Agarose | 0.3 g |
| TAE running buffer | 30 ml |

Boil solution to dissolve agarose.

**100 mM $CaCl_2$**

| | |
|---|---|
| $CaCl_2$ | 2.7 g |

Make up to 100 ml final volume with deionised water and sterile filter (0.2 μ).

**100 mM $MgCl_2$**

| | |
|---|---|
| $MgCl_2.6H_2O$ | 10.33 g |

Make up to final volume of 500 ml with deionised water and sterilise by autoclaving.

**Luria-Bertani (LB) Broth**

| | |
|---|---|
| Gibco LB powder | 37 g |
| Deionised water | 1000 ml |

**Luria-Bertani (LB) Agar Plates**

| | |
|---|---|
| Gibco LB powder | 37 g |
| Agar | 15 g |
| Deionised water | 1000 ml |

**Antibiotic Selection**

| | |
|---|---|
| Ampicillin | 100 mg/ml |

Prepare in deionised water, sterile filter (0.2 μ), aliquot and store at -20° C. Use at 100 μg/ml in medium.

| | |
|---|---|
| Chloramphenicol | 30 mg/ml |

Prepare in ethanol, aliquot and store at -20°C. Use at 30 μg/ml in medium.

**Arabinose Stock Solution (20%)**

| | |
|---|---|
| Arabinose | 2 g |
| Deionised water | 10 ml |

Sterile filter (0.2 μ). Add 2 ml to 200 ml cultures to give a final concentration of 0.2%.

## *C. difficile* Genomic DNA Reagents

**Solution I**

| | |
|---|---|
| Glucose (1 M) | 1.0 ml |
| Tris HCl (1 M), pH 8.0 | 0.5 ml |
| EDTA (0.5 M), pH 8.0 | 0.4 ml |

Make up to 20 ml with deionised water.

**Soltuion II**

| | |
|---|---|
| SDS (10 %) | 1.0 ml |
| NaOH (2 N) | 1.0 ml |

Make up to 10 ml with deionised water.

## Protein Purification Reagents

**BSA (Bovine Serum Albumin) Stock Solution (20 mg/ml)**

| | |
|---|---|
| BSA | 20 mg |

Make up to 1 ml with deionised water

**Recombinant Enterokinase Cleavage Buffer (10X)**

| | |
|---|---|
| Tris HCl (1 M) pH 7.4 | 100 ml |
| NaCl (5 M) | 50 ml |
| $CaCl_2$ | 1.4702 g |

Make up to 500 ml with deionised water. Dilute 1:10 in deionised water before use.

**20 mM Tris HCl pH 8.5**

| | |
|---|---|
| Tris base | 2.422 g |

Dissolve in 950 ml deionised water, adjust pH to 8.5 with HCl, and complete to 1 L.

**20 mM Tris HCl, 0.5 M NaCl, pH 8.5**

| | |
|---|---|
| Tris base | 2.422 g |
| NaCl | 29.22 g |

Dissolve in 950 ml deionised water, adjust pH to 8.5 with HCl, and complete to 1 L.

**20 mM Tris HCl, 500 mM NaCl, pH 7.8**

| | |
|---|---|
| Tris base | 2.422 g |
| NaCl | 29.22 g |

Dissolve in 950 ml deionised water, adjust pH to 7.8 with HCl, and complete to 1 L.

**20 mM Tris HCl, 500 mM NaCl, pH 7.8, 500 mM imidazole**

| | |
|---|---|
| 20 mM Tris, 500 mM NaCl, pH 7.8 | 100 ml |
| Imidazole | 3.404 g |

**50 mM Tris HCl, pH 7.4**

| | |
|---|---|
| Tris base | 6.055 g |

Dissolve in 950 ml deionised water, adjust pH to 7.4 with HCl, and complete to 1 L.

**50 mM Tris HCl, 8 M Urea, pH 8.3**

| | |
|---|---|
| Tris base | 0.6055 g |
| Urea | 48.048 g |

Dissolve in 90 ml deionised water, adjust pH to 8.3 with HCl, and complete to 100 ml.

**20 mM Tris HCl, 0.15 M NaCl, pH 8.0**

| | |
|---|---|
| Tris base | 2.422 g |
| NaCl | 8.766 g |

Dissolve in 950 ml deionised water, adjust pH to 8.0 with HCl, and complete to 1 L.

## Immunoassay Buffers and Reagents

**0.1 M Carbonate Buffer**

| | |
|---|---|
| Soln. A: $Na_2CO_3$ (0.1 M) | 1.06 g in 100 ml water |
| Soln. B: NaH $CO_3$ (0.1 M) | 1.68 g in 200 ml water |

Take 70 ml of soln. A and add soln. B until pH is at 9.6 (approximately 190 ml of soln. B).

**0.05 M Carbonate Buffer**

| | |
|---|---|
| Soln. A: $Na_2CO_3$ (0.05 M) | 0.52 g in 100 ml water |
| Soln. B: NaH $CO_3$ (0.05 M) | 0.84 g in 200 ml water |

Take 70 ml of soln. A and add soln. B until pH is at 9.6 (approximately 190 ml of soln. B).

**Immunoassay Blocking Buffer**

| | |
|---|---|
| Skimmed dried milk powder (Marvel) | 2 g |
| PBS | 100 ml |

**PBS, pH 7.0**

| | |
|---|---|
| NaCl | 80 g |
| KCl | 2 g |
| $Na_2HPO_4.2H_2O$ | 10.857 g |
| $KH_2PO_4$ (anhydrous) | 5.3075 g |

Dissolve in 9.5 L deionised water, adjust pH to 7.0 if necessary, and complete to 10 L. Sterilise by autoclaving.

**Assay Diluent**

| | |
|---|---|
| FCS | 10 ml |
| PBS, pH 7.0 | 90 ml |

**Assay Diluent (R&D Systems)**

| | |
|---|---|
| PBS, pH 7.2-7.4/0.05% Tween | 98.6 ml |
| Delipidised Bovine Serum | 1.4 ml |

**PBS/0.05% Tween Washing Solution**

| | |
|---|---|
| PBS | 500 ml |
| Tween 20 | 250 µl |

**Dulbecco's PBS**

| | |
|---|---|
| NaCl | 8 g |
| KCl | 0.2 g |
| $Na_2HPO_4.2H_2O$ | 1.2815 g |
| $KH_2PO_4$ (anhydrous) | 0.3811 g |

Dissolve in 950 ml deionised water, adjust pH to 7.2
if necessary, and complete to 1 L. Sterilise by autoclaving.

**PBS, pH 7.2-7.4**

| | |
|---|---|
| NaCl | 40 g |
| KCl | 1.0 g |
| $Na_2HPO_4.2H_2O$ | 7.2 g |
| $KH_2PO_4$ (anhydrous) | 1.02 g |

Dissolve in 4.5 L deionised water, adjust pH to 7.2-7.4
if necessary, and complete to 5 L. Sterilise by autoclaving.

**Tris Buffered Saline (TBS) pH 7.6**

| | |
|---|---|
| Tris base | 2.422 g |
| NaCl | 8.766 g |

Dissolve in 950 ml deionised water, adjust pH to 7.6
if necessary, and complete to 1 L. Sterilise by autoclaving.

**TBS, pH 7.6/0.05% Tween**

| | |
|---|---|
| TBS, pH 7.6 | 1 L |
| Tween 20® | 500 µl |

**2 N $H_2SO_4$**

| | |
|---|---|
| $H_2SO_4$ (36 N) | 11.1 ml |
| Deionised water | 88.9 ml |

**2.5 N $H_2SO_4$**

| | |
|---|---|
| $H_2SO_4$ (36 N) | 13.9 ml |
| Deionised water | 86.1 ml |

**1 M HCl**

| | |
|---|---|
| Concentrated HCl (12.1 N) | 8.3 ml |
| Deionised water | 91.7 ml |

**3 M NaOH**

| | |
|---|---|
| NaOH | 12 g |

Dissolve in 90 ml deionised water and complete to 100 ml.

**1.2 M NaOH/0.5 M HEPES**

| | |
|---|---|
| NaOH | 4.8 g |
| HEPES | 11.9 g |

Dissolve in 90 ml deionised water and complete to 100 ml.

**Horseradish Peroxidase Substrate**

| | |
|---|---|
| 1,2-phenylenediamine dihydrochloride (OPD, 2 HCl) | 1 X 2 mg tablet |

Dissolve in 5 ml of deionised water and add 5 µl of 30% $H_2O_2$.

**Alkaline Phosphatase Substrate**

| | |
|---|---|
| p-nitrophenyl phosphate | 1 X 5 mg tablet |

Dissolve in 5 ml of 1 M diethanolamine buffer,
pH 9.8, containing 0.5 mM $MgCl_2$.

## Phagocytosis Assay Reagents

**Detaching Buffer**

| | |
|---|---|
| EDTA (250 mM) | 1.0 ml |
| FCS | 250 µl |
| HBSS | 48.75 ml |

**RPMI/50% FCS**

| | |
|---|---|
| RPMI 1640 | 50 ml |
| Heat inactivated FCS | 50 ml |

# Appendix B

## Publications and presentations from this thesis

Cloning, Expression and Purification of a Surface Layer Protein of *Clostridium difficile*.

**O'Brien, J.,** Ní Eidhin, D., Kelleher D.

Presented as a poster at the United European Gastroenterology Week (UEGW), Madrid, Spain, November 2003. Awarded poster of distinction prize. Published as an abstract in Gut, suppl. VI, vol. 52, 2003.

Passive Immunisation of Hamsters Against *Clostridium difficile* Infection Using Anti-Surface Layer Proteins of *Clostridium difficile*.

**O'Brien, J.,** McCabe, M., Ní Eidhin, D., Kelleher D.

Presented as a poster at the Digestive Disease Week (DDW), New Orleans, U.S.A., May 2004. Published as an abstract in Gastroenterology, suppl. 2, vol. 126, April 2004.

Passive Immunisation of Hamsters Against *Clostridium difficile* Infection Using Antibodies Against Surface Layer Proteins.

**O'Brien, J.B.,** McCabe, M.S., Ní Eidhin, D.B., Kelleher D.P.

Oral presentation at the Irish Gastroenterology Society (ISG), Kilkenny, June 2004. Received second prize for oral presentation.

Passive Immunisation of Hamsters Against *Clostridium difficile* Infection Using Antibodies to Surface Layer Proteins.

**O'Brien, J.B.,** McCabe, M.S., Athié-Morales, V., McDonald, G.S.A., Ní Eidhin, D.B., Kelleher D.P. FEMS Microbiology Letters, May 2005, vol. 246, issue 2, pages 199-205.

Antibodies Against *Clostridium difficile* Surface Layer Proteins are Not Protective in an *in vivo* Hamster Challenge Model of Infection.

**O'Brien, J.B.,** Ní Eidhin, D.B., McCabe, M.S., Athié-Morales, V., Kelleher D.P. Manuscript in preparation.

*Clostridium difficile* Surface Layer Proteins Modulate Dendritic Cell Function.

**O'Brien, J.B.,** Ní Eidhin, D.B., Loscher, C., Kelleher D.P.

Manuscript in preparation.