Monoclonal antibodies against toxic shock syndrome toxin-1 and their use in diagnosis

Thesis

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Monoclonal Antibodies Against Toxic Shock Syndrome Toxin-1 And Their Use In Diagnosis.

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Thesis submitted to the Open University for the degree of Master of Philosophy.

Date of award: 7th September 1998
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<tr>
<td>%CV</td>
<td>Coefficient of variance</td>
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<tr>
<td>ABTS</td>
<td>2,2’-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>BT</td>
<td>Biotinylated tyramine</td>
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<tr>
<td>CAMR</td>
<td>Centre for Applied Microbiology and Research</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>DCM</td>
<td>Dichloromethane</td>
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<tr>
<td>DMF</td>
<td>Dimethyl formamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
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<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
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<tr>
<td>H₂SO₄</td>
<td>Sulphuric acid</td>
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<tr>
<td>HEPES</td>
<td>(N-2-Hydroxyethylpiperazine-N’-2-ethanesulfonic acid)</td>
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<tr>
<td>HGPRT</td>
<td>Hypoxanthine guanine phosphoribosyl transferase</td>
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<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>IFN-γ</td>
<td>Interferon gamma</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IVIG</td>
<td>Intravenous immunoglobulin</td>
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<tr>
<td>KS</td>
<td>Kawasaki syndrome</td>
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<tr>
<td>Mab</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin resistant Staphylococcus aureus</td>
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<td>NaOH</td>
<td>Sodium hydroxide</td>
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<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T/FCS</td>
<td>Phosphate buffered saline + 0.1% Tween 20 with 5% foetal calf serum</td>
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<td>RED</td>
<td>Recalcitrant erythematous desquamating disorder</td>
</tr>
<tr>
<td>RER</td>
<td>Rough endoplasmic reticulum</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
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<tr>
<td>RPLA</td>
<td>Reversed passive latex agglutination</td>
</tr>
<tr>
<td>SAg</td>
<td>Superantigen</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SE</td>
<td>Staphylococcal enterotoxin</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TK</td>
<td>Thymidine kinase</td>
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<tr>
<td>TMB</td>
<td>3,3',5,5' Tetramethylbenzidine</td>
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<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TSS</td>
<td>Toxic shock syndrome</td>
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<td>Toxic shock syndrome toxin-1</td>
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Acknowledgements.

I would like to thank my supervisors Howard Tranter, Ravi Acharya and Cliff Shone for their help and advice during the course of this project. I would also like to thank Jim Chadwick for giving me the initial impetus to start this work. The greatest vote of thanks however goes to Bassam Hallis without whose advice, encouragement and terrific sense of humour this project would never have been completed.

Declaration

I declare that the research presented in this thesis is all my own work except where otherwise indicated and has not been submitted elsewhere for a research degree.

Stephen Elvin

June 1998
Dedication.

This thesis is dedicated to my girlfriend Paula without whose patience and understanding I would never have completed this project and my parents Eric and Marian for all their support over the last few years.
Abstract

Toxic shock syndrome toxin -1 (TSST-1) is a 22kDa extracellular protein produced by some strains of *Staphylococcus aureus*. It is implicated in the pathogenesis of toxic shock syndrome, an acute life-threatening multisystem disease.

Eight hybridoma cell lines producing monoclonal antibodies with high binding affinity to TSST-1 have been produced and a monoclonal antibody based enzyme-linked immunosorbant assay developed. This assay is specific for TSST-1 and no cross reaction with a number of other bacterial toxins has been observed. The ELISA has been amplified enzymically using biotinylated tyramine and streptavidin peroxidase to detect as little as 0.1ng ml⁻¹ of toxin.

Competitive binding studies have indicated the presence of at least three antigenic sites on the toxin. The ability of these antibodies to inhibit the mitogenic activity of the toxin in T-cell proliferation assays using mouse and human lymphocytes has been assessed and results of this work indicate that one of the major antigenic sites has an important role in the mitogenicity of the toxin. Binding of the antibodies has been localised to the carboxyl terminal region which has been implicated in the biological activity of the toxin and several antigenic determinants were identified in this region by epitope mapping using polyclonal antibody.
Chapter 1

Introduction
Chapter 1.

Introduction

1.1 The staphylococci.

The staphylococci are members of the family Micrococcaceae. They are Gram positive, non motile, facultatively anaerobic, glucose-fermenting cocci, which grow as irregular clusters caused by cell division occurring in more than one plane. This gives rise to the characteristic "bunch of grapes" appearance on microscopic analysis. They have a pentaglycine cross bridge in their peptidoglycan, and the cell wall usually contains glycerol or ribitol teichoic acids (Schleifer and Kroppenstadt, 1990) which have structural functions and are also involved in phage adsorption (Schleifer and Steber, 1974) and protein A which can bind immunoglobulin molecules and thus agglutinate sera (Moks et al., 1986). Staphylococci are able to tolerate a wide variety of environmental conditions, including temperatures from 7 - 48°C (optimum 37°C), a pH range from 4 - 10 (optimum 7-7.5) and up to 12% (w/v) salt. Growth of these organisms on solid media produces mucoid, smooth, circular, opaque colonies that can be white, yellow, grey, or brown in colour. The guanine plus cytosine (G+C) content of staphylococci is approximately 30-40% and many staphylococci also carry plasmid and phage DNA capable of conferring resistances to antibiotics and heavy metals. For instance staphylococci are commonly resistant to penicillin due to a phage encoded penicillinase (Arvidson, 1983). A growing problem is resistance to the β-lactam drugs, including methicillin. Methicillin resistant *Staphylococcus aureus* (MRSA) have altered proteins in the cell wall which have a very low affinity for virtually all β-lactams. MRSA strains are resistant to virtually all other classes of antibiotics and account for 30-60% of hospital isolates worldwide (Brickner, 1997).
Most species are natural inhabitants of the mammalian skin and mucous membranes and it is estimated that as many as 40% of humans carry these organisms (Noble and Somerville, 1974). Coagulase production by these organisms has been found to correlate with pathogenicity and all coagulase-positive staphylococci of human origin are grouped together as *Staphylococcus aureus*. The ability to produce a polysaccharide capsule is also a feature of clinically important strains. These organisms cause a variety of suppurative disease in humans including superficial and deep abscesses, wound infections and infection of various internal organs. In addition they produce a variety of extracellular virulence factors including hyaluronidase, leucocidin, lipase, protease, haemolysins (A-D), exfoliative toxins, staphylococcal enterotoxins (SEA-SEH), and toxic shock syndrome toxin-1 (TSST-1; Tranter and Brehm, 1994).

1.2 Toxic Shock Syndrome - Epidemiology and Causes.

Several thousand cases of toxic shock syndrome (TSS) have been reported, mainly in the USA and to a lesser extent in Europe (Alouf et al., 1991). The majority of cases are epidemiologically associated with tampon use in menstruating young women vaginally infected with *S. aureus* (Shands et al., 1980; Davis et al., 1980; Blomster-Hautamaa and Schlievert, 1988). However up to 30% of cases may occur in other females or males with focal, non-genital *S. aureus* wound or soft tissue infections (Schlievert 1983; Blomster-Hautamaa & Schlievert, 1988). The disease results from release of a toxin (TSST-1) and illness results if the individual has no or very low antibody titre to the toxin (Crass & Bergdoll, 1986). Fatality rates for menstrual toxic shock syndrome are currently around 6% (Gaventa et al., 1989; Alouf et al., 1991). In non-menstrual cases the SE's, especially SEB can also be causative agents of toxic shock syndrome (Schlievert, 1986) and it has also been
demonstrated (Arbuthnott *et al.*, 1990; Humphreys *et al.*, 1989) that enterotoxin production is more frequent than expected in septicaemia cases compared with strains from healthy carriers. Indeed in cases of non-menstrual TSS isolation of bacteria that do not produce TSST-1 is common and there is a higher fatality rate for men and non-vaginally infected women compared to vaginal cases (Broome, 1989). Studies of the relation between tampon use and TSS suggest that both the degree of absorbency and the chemical composition of the tampon are involved although the mechanisms by which the risk of TSS is increased are not fully understood (Schlievert, 1993). There is also evidence to suggest that the use of contraceptive sponges and diaphragms may be linked to an increased risk of non-menstrual TSS (Broome, 1989). TSS resulting from the SE's may be significant in *S. aureus* infected burns (Frame *et al.*, 1985) although TSST-1 accounts for many burns-related cases of TSS (Cole and Shakespeare, 1990). Adults who do not make a full clinical recovery from TSS have been shown to suffer numerous illnesses including a permanent loss of hearing and extensive tissue necroses which have been attributed to capillary leakage. Streptococcal pyrogenic exotoxins can also be an important cause of toxic shock syndrome (Sriskandan and Cohen, 1995; Schlievert, 1993), causing symptoms similar to those caused by TSST-1 (Schlievert, 1993). This similarity in disease symptoms could be due to the small amount (10-15%) of sequence homology between the staphylococcal toxins and the streptococcal toxins (Marrack and Kappler, 1990) and their common biological properties.

1.3 Clinical symptoms of TSS and susceptibility to disease.

The onset of clinical TSS is abrupt, with symptoms of moderate to severe disease including fever, chills, malaise, headache, sore throat, myalgias, muscle tenderness, fatigue, vomiting, diarrhoea, abdominal pain and, orthostatic dizziness (Chesney *et al.*, 1984).
Between 24 and 48 hours of the onset of symptoms diffuse erythema, severe watery diarrhoea, decreased urine output, cyanosis and oedema of the extremities may be observed. Cerebral ischaemia, oedema or a toxin-mediated effect on the central nervous system (CNS) rapidly results in somnolence, confusion, irritability and agitation (Chesney et al., 1984).

Susceptibility to TSS is very difficult to predict. In menstrual cases there appear to be some pre-disposing factors such as age, tampon usage and vaginal colonisation with TSST-1 producing strains of *S. aureus*. In non-menstrual cases risk factors are unknown and colonisation with *S. aureus* strains producing toxins other than TSST-1 is common.

It has been shown that around 90% of the population will have antibody to TSST-1 by age 20 (Vergeront et al., 1983) and there is no difference between males and females. However it has been shown that 96% of TSS cases reported to the Centres for Disease Control in Atlanta have occurred in women (Vergeront et al., 1983). The lack of circulating antibody to TSST-1, even in cases of infection with TSST-1 producing *S. aureus* does not predispose TSS however. In one study only 25% of such cases actually developed symptoms of TSS, and the risk of disease was lowest in those who had intravascular infection and higher in individuals with cutaneous infections (Jacobson et al., 1989).

In the early 1980's strict criteria were laid down for the clinical diagnosis of TSS, and in the absence of a rapid reliable test for TSS these criteria (see Table 1.1) remain the only way of making a diagnosis. It is also a priority in cases of TSS to find the focus of infection and all *S. aureus* isolates should be tested for their ability to produce TSST-1. Acute and convalescent sera can be assayed for the presence of
antibody to TSST-1 however elevated levels of anti-TSST-1 in acute phase serum are unusual (Chesney et al., 1984). Diagnosis must be carefully undertaken so as not to confuse clinical conditions such as septic shock (particularly meningococcaemia), scarlet fever and urinary tract infections which can present a similar clinical picture to TSS.

Frequently however not all the criteria shown in Table 1.1 are fulfilled by a particular case. In these instances the illness may be described as probable or possible TSS (Marples and Wieneke, 1993). Comparison of symptoms in these cases with those seen in confirmed cases may help in diagnosis. In menstrual TSS recurrent episodes of the disease may occur and these are often milder than the initial episode (Chesney et al., 1984), symptoms such as fever, headache, rash, sore throat, diarrhoea, vomiting, dizziness or myalgias should raise the possibility of TSS.

Severe invasive group A streptococcal infections can also produce a toxic shock-like syndrome (TSLS; Schlievert, 1993). TSLS is defined by hypotension or shock including evidence of two or more multi-organ components (renal impairment, coagulopathy, liver involvement, adult respiratory distress syndrome, rash, soft tissue necrosis) and the presence of group A streptococci (Breiman et al., 1993). If group A streptococci are isolated from an otherwise sterile site TSLS is confirmed whereas if the organism is isolated from a nonsterile body site the case is considered probable.

TSST-1 producing staphylococci are also implicated in the pathology of a number of other diseases. For example, Kawasaki syndrome (KS) is the main cause of acquired heart disease in children (Leung et al., 1993) and is associated with the clonal expansion of Vβ 2 and Vβ 8.1 expressing T-cells (Schlievert, 1993). In most cases of KS, TSST-1 producing staphylococci are isolated (Leung et al., 1993).
suggesting that the superantigenic activity of the toxin is a probable cause of the illness. TSS and KS share common clinical symptoms; both syndromes induce high fever, rash, conjunctivitis, inflammation of the mucous membranes and erythroderma. KS is normally a self-limiting disease, but in as many as 25% of cases coronary artery abnormalities can develop (Leung et al., 1993).

Recalcitrant erythematous desquamating disorder (RED) is a variant of TSS seen in AIDS patients (Dondorp et al., 1993) and is characterised by prolonged erythema, extensive cutaneous desquamation, hypotension, tachycardia and, multiple organ involvement (Cone et al., 1991). The isolation of toxigenic staphylococci is a common feature of RED cases (Cone et al., 1991). The illness may continue for many weeks, often resulting in the patients death (Schlievert, 1993).

Influenza-associated TSS appears to be caused by viral damage to the upper respiratory tract followed by subsequent infection with S. aureus. Although the infection sites are often non-pyogenic the pH will be near neutrality due to the buffering capacity of the blood (Schlievert, 1993) which is ideal for toxin production (Schlievert and Blomster, 1983). The fatality rate for influenza associated TSS is around 90% in children but lower in adults (Schlievert, 1993).

1.3.1 Therapy.

The most serious problems encountered in the first 24-48 hours of a TSS episode include hypotension, myocardopathy, pulmonary oedema, respiratory distress, electrolyte disorders and acute renal failure. The rapid restoration of intravascular volume is most important and can be achieved by the administration of hypotonic fluids by the intra-venous route although it is equally important to maintain
respiratory stability. Specific therapy should also be administered to eradicate the bacteria. This can be achieved by intravenous administration of antibiotics such as oxacillin, piperacillin and clindamycin to kill the micro-organisms and prevent re-infection.

Studies using the rabbit model of TSST-1 (Bonventre et al., 1988; Best et al., 1988) have indicated that the use of intravenous mouse monoclonal antibody (Mab) to TSST-1 can alleviate symptoms of TSS and reduce the mortality compared to unimmunised animals. This has lead to the investigation of intravenous immunoglobulin (IVIG) as a possible therapeutic agent. Pooled human IgG preparations have been shown to have high antibody titres to the staphylococcal enterotoxins and TSST-1 (Takei et al., 1993) and to be able to neutralise the mitogenic effects of these toxins *in vitro*. The administration of IVIG has also been shown to be a highly effective therapy in cases of streptococcal toxic shock, after the failure of conventional therapy to improve the patients condition (Barry et al., 1992; Perez et al., 1997). Administration of intravenous immunoglobulin during TSS neutralises any circulating toxin which can then be cleared from the bloodstream. To alleviate symptoms this should be administered along with antibiotic therapy to clear the microbial contamination and ensure that no further toxin was produced.

There are currently no therapeutic agents to deal with the effects of the toxin.

1.3.2 Assays for TSST-1.

A number a methods have been developed to detect and quantify TSST-1. Solid phase radioimmunoassay (RIA) techniques (Cohen et al., 1983; Hayes et al., 1984) have previously been used to detect TSST-1 in culture supernatant fluids. In
this method the supernatant fluids were placed on a nitrocellulose membrane, dried and reacted with rabbit anti-TSST-1 antibody overnight. The bound antibody was then detected using $^{125}$iodine labelled staphylococcal protein A which binds to the Fc portion of the antibody molecules. Reeves et al. (1984) used a radial immunodiffusion technique utilising rabbit anti-TSST-1 to detect the toxin in clinical isolates, using purified toxin as a standard. The sensitivity of this assay was determined to be 5µg ml$^{-1}$ which is not adequate to detect toxin in clinical samples where toxin levels can be very low (around 7ng ml$^{-1}$; Wells et al., 1987).

Since then a reverse passive latex agglutination assay (RPLA) assay has been developed (Igarashi et al., 1986) which has proved useful when identifying toxinogenic cultures of *S. aureus*, but this method was found to be unsuitable when detecting toxin in serum because the serum causes non-specific agglutination (Miwa et al., 1994).

Melish et al. (1983, 1989) developed a liquid phase RIA to detect TSST-1 in serum, with a detection limit of 0.6ng ml$^{-1}$. This assay although sensitive and reproducible (inter assay variation <7%) suffers the same disadvantages as the solid phase RIA method of Cohen and Hayes in that radioisotopes are used which in addition to being hazardous present storage and disposal difficulties. Furthermore RIAs require expensive equipment such as scintillation counters to detect the radioactivity in the samples.

Various enzyme-linked immunosorbent assay (ELISA) methods for detecting toxin have been constructed, most of them use rabbit polyclonal anti-TSST-1 (Parsonnet et al., 1985a; Rosten et al., 1987). The method developed by Parsonnet was a competitive assay method for the detection of toxin in culture supernatant fluids.
which necessitated the conjugation of purified TSST-1 to the reporter enzyme alkaline phosphatase. This method was sensitive, with a detection limit of 0.5ng ml\(^{-1}\) but involved overnight incubation of the samples on the assay plate and was therefore time consuming. Also the need to incubate overnight might compromise the usefulness of this assay when clinical samples are used as they may contain proteases or enzyme inhibitors which could affect the assay performance.

Rosten et al. (1987) used a non-competitive assay for the detection of toxin in culture supernatant fluids and in vaginal washings from healthy controls as well as from women with suspected TSS. Their method had a detection limit of 0.5ng ml\(^{-1}\) and was successfully used to detect TSST-1 in the clinical samples.

Wells et al. (1987) reported the detection of TSST-1 in urine using an ELISA which utilised a rabbit polyclonal capture antibody and a mouse monoclonal antibody conjugated to horseradish peroxidase (HRP) as the detection reagent. This assay had a sensitivity of 0.5ng ml\(^{-1}\) and was also used to detect toxin in culture supernatant fluids. Kuffner et al. (1988) developed the ELISA one step further, testing a number of Mabs as both capture and detection reagents before optimising conditions for a two site Mab based assay. This assay was found to be more sensitive than polyclonal antibody based assays, possibly because competition between polyclonal capture and detection reagents for common epitopes on the toxin molecule resulted in a lowering of the sensitivity of the assay system. The assay was however only applied to the detection of toxin in culture supernatant fluids and was not used with clinical samples.

Miwa et al. (1994) returned to the use of rabbit polyclonal anti-TSST-1 as the capture antibody, but used a biotinylated mouse monoclonal antibody as the detection reagent. Avidin-HRP was used as an amplification reagent and a detection limit of
0.01 ng ml\(^{-1}\) toxin in phosphate buffer was claimed using this system. The assay was then applied to the detection of toxin in serum samples taken from TSS patients and 28 of 60 samples tested were positive, the authors even reported toxin in samples from patients who, having had antibiotic therapy were no longer \textit{S. aureus} culture positive. Furthermore these workers suggested that the bacteria may not produce the toxin continually and so the timing of samples taken for analysis could be crucial.

Despite the development of all these different assay methods a sensitive, rapid and reliable two site Mab based ELISA for the detection of toxin in clinical samples is not yet commercially available.

1.4 Structure and characteristics of TSST-1.

TSST-1 is thought to be encoded on a transposon (Kreiswirth \textit{et al.}, 1989) or other mobile genetic element which has integrated into the chromosome, like SEB which originated on a phage or plasmid (Johns and Khan, 1988) and has also integrated into the chromosome, or SED which is encoded by a plasmid which also codes for penicillin resistance (Bayles and Iandolo, 1989).

TSST-1 is a single chain polypeptide which does not contain any nucleic acid, carbohydrate, lipid or cofactor moieties. It has a molecular weight of 22,049 Da as deduced from the nucleotide sequence, which translates into 194 amino acids (Blomster-Hautamaa \textit{et al.}, 1986a). TSST-1 does not contain any cysteine residues and shares approximately 20\% sequence homology with the SE (Betley \textit{et al.}, 1992) however X-ray crystallographic studies have shown that there is significant tertiary structure homology between SEB and TSST-1, even though TSST-1 lacks the di-sulphide loop conserved in the SE structure. The absence of a cysteine loop is
possibly the reason for the lack of emetic activity in TSST-1 compared to the enterotoxins (Tranter et al., 1995).

Purified TSST-1 can exist at 2 different isoelectric points, 7.08 and 7.22, possibly due to deamidation during production or purification (Blomster-Hautamaa et al., 1986b). The toxin is resistant to hydrolysis with trypsin but can be cleaved using papain (Edwin et al., 1988) or pepsin (Reiser et al., 1983). A secondary structure analysis of the far-UV circular dichroic spectrum of TSST-1 revealed 6.25% α helix, 51.25% β pleated sheets, 9.0% β turns, and 33.5% random turns (Singh et al., 1988a). This low α helix and high β sheet/β turn content has also been found in staphylococcal enterotoxins A, B, C1, and E (Singh et al., 1988b; Singh and Betley, 1989).

The overall structure of TSST-1 as defined by X-ray crystallography is very compact, measuring 45x45x58Å (Acharya et al., 1994) and reveals that both the carboxyl and amino terminal domains contain regions involved in the binding of Major Histocompatibility Complex class II (MHC II) molecules and that the C terminal domain is implicated in T-cell receptor (TCR) binding (see Fig 1.1).

The area involved in MHC binding encompasses the N-terminal β barrel (Kim et al., 1994), in conjunction with residues 170-180 from the C terminal domain (Acharya et al., 1994), which fold against the N terminal. Three areas of contact between TSST-1 and the human MHC II molecule DR1 have been observed (Kim et al., 1994) by X-ray crystallographic studies, compared to only two contact areas between SEB and DR1 (Jardetzky et al., 1994). Despite the proximity of these binding sites it is not thought that SEB and TSST-1 compete for binding to DR1 (Papageorgiou and Acharya, 1997). The site of TCR binding is believed to be made
up of several structures of the C terminal domain and these areas are on the opposite side of the molecule to the MHC binding site (Acharya et al., 1994). The putative TCR binding site has also been implicated in the biological activity of the toxin by mutagenesis studies. A TSST-1 mutant with an alanine substituted for the histidine at position 135 (H135A) lost its mitogenic effects for T-cells and was unable to induce expression of interleukin-2 (IL-2), interferon-γ (IFN-γ) or tumour necrosis factor β (TNF β; Cullen et al., 1995). This inability to stimulate T-cells was not due to a lack of binding to MHC II however (Cullen et al., 1995) but rather due to the disruption of the TCR binding site as revealed by X-ray crystallographic studies using H135A (Papageorgiou et al., 1996). Other studies (Bonventre et al., 1993; Murray et al., 1996; Hurley et al., 1996) have also used site-directed mutagenesis to implicate residues in this area in the mitogenicity of the toxin. Table 1.3 lists amino acid residues known to be involved in the biological activity of the toxin and the effects of mutation of these residues.

A variant TSST-1 produced by S. aureus isolated from an ovine mastitis-associated infection has been reported (Ho et al., 1989). This variant toxin has the same molecular weight as human TSST-1, however it has an isoelectric point of 8.5. Nucleotide sequence analysis revealed that TSST-ovine (TSST-0) had 14 nucleotide differences that changed 9 amino acid residues. One nucleotide difference, at position 514, caused a change to the amino acid charge because glutamic acid at position 132 was changed to lysine in TSST-0. There is also a change from isoleucine to threonine at position 140. Both these changes are in the region implicated in the biological activity of TSST-1 and could explain the differences in biological activities between the two toxins. Like TSST-1, TSST-0 is mitogenic but it is not pyrogenic and does not
enhance endotoxin shock. It is also unable to induce TSS in a rabbit infection model (Lee et al., 1992).

1.5 The Biological Activity of TSST-1.

TSST-1 is one of a number of bacterial and viral products (see Table 1.4) which are classified as superantigens (SAgs) because of their ability to stimulate T-cells to proliferate non-specifically through interaction with the MHC II on antigen-presenting cells (APC) and then the variable region of the β chain of the TCR (see Figure 1.2). A superantigen is able to by-pass the normal antigen processing which is carried out by APCs such as macrophages, B-cells and dendritic cells (Goodglick and Braun, 1994) and bind directly to relatively conserved regions of the MHC II molecule (Woodland et al., 1997). Normally antigens are proteolytically cleaved by the APC and peptides are displayed in a cleft between the α and β chains of the MHC II on the cell surface. The T-cell then interacts with the complex on the cell surface through the antigen specific TCR which causes release of cytokines such as IL-1 (Parsonnet et al., 1986; 1985b) INFγ and TNF (Parsonnet, 1989) and mediates the immune response.

SAgs however, do not require processing by APCs but interact directly with areas of the MHC II outside the normal antigen binding cleft (Hewitt et al., 1992). Recent studies however have indicated that peptides already bound in the MHC II antigen binding cleft may have influence over the binding of TSST-1, dictating which MHC subsets are preferentially bound by the SAg (Woodland et al., 1997, Wen et al., 1997). This is supported by crystallographic studies which show that part of the TSST-1 molecule is positioned over the top of the HLA DR1 and possibly interacts.
with MHC bound peptides (Kim et al., 1994). The allele of Class II has less impact in SAg presentation than in recognition of conventionally processed peptide + MHC II by T-cells (Kappler et al., 1989). The SAg MHC II complex then interacts with the TCR such that the SAg binds to the TCR outside the groove area which recognises conventionally processed antigens. The toxin does not stimulate all T-cells to respond, it is specific for particular Vβ sequences and this specificity could explain the differential sensitivities of individuals to the toxin (Kappler et al., 1989). TSST-1 is specific for the Vβ2 region and so all Vβ2 bearing T-cells are stimulated by TSST-1, which results in stimulation of up to 20% of all T-cells leading to massive cytokine release and toxic shock (see Table 1.5 for biological properties of TSST-1).

This theory of SAg activity is supported by work utilising genetically engineered mutant TSST-1 toxins. Mutant H135A which was derived by site-directed mutagenesis (Bonventre et al., 1993) was unable to stimulate a mitogenic response in T cells, and does not stimulate the cells to express IL-2, INF-γ or TNF β, although it is still able to bind to MHC II (Cullen et al., 1995). There was also no production of tumour necrosis factor α which is important in the development of lethal shock. A similar mutation at histidine 141 also resulted in a substantial loss of mitogenic activity (Blanco et al., 1990), indicating that this area is also critical in the biological activity of the toxin. In another study (Murray et al., 1996) the mutant toxins Q136A and E132K/Q136K (double mutant) were compared with native toxin and mutant H135A for superantigenicity. It was found that all mutants lost the ability to induce lethal TSS, while Q136A was superantigenic but not lethal. These results further support the previous observations that this region is involved in the biological activity of the toxin.
The toxicity of recombinant TSST-1 and mutant toxins in vivo has been studied (Bonventre et al., 1993), and the ability of the toxin to stimulate murine T cells in vitro and its expression of toxicity leading to lethal shock in rabbits are related phenomena.

The high frequency of T-cell activation leads in turn to the overproduction of cytokines such as IL-2 and IFN-γ from T-cells and IL-1 and TNF from macrophages and monocytes. These cytokines are implicated in the pathogenesis of TSS, for although they are beneficial in small doses they are known to be toxic at high levels (Norgren and Eriksson, 1997), causing fever, weight loss and osmotic imbalances that can sometimes lead to death (Parsonnet, 1989). The release of these cytokines is self perpetuating as the IL-1 induces production of IL-2 which in turn activates cells to produce more cytokines.

Although hypotension during the disease process can be explained by capillary leakage it is not clear what causes this leakage. Several hypotheses have been put forward to explain the involvement of endotoxin in TSS including the release of cytokines such as TNF. Fast et al. (1988 and 1989) showed that in the presence of T-cells pyrogenic toxins can cause sustained release of TNF although Lee et al. (1989 and 1991) also showed that TSST-1 can alter capillary permeability directly. The ability of TSST-1 to amplify endotoxin shock (Parsonnet, 1989; Schlievert, 1993) may also be important in TSS. Humans have a predominantly Gram negative gut flora and there is an increase in Gram negative vaginal colonisation in menstrually-associated TSS (Chow et al., 1984a and 1984b) which may indicate that endotoxin is important in the cause and severity of hypotension in TSS. Another observation made during TSS is the suppression of antibody synthesis; this has been attributed to the
massive release of IFN-γ and may explain why there is a failure to generate antibody to the toxin and why so many TSS patients have recurrent episodes of the disease (Osterholme, 1982).

1.6 Antibodies, synthesis and structure.

1.6.1 Antibody synthesis.

Antibodies or immunoglobulins (Ig) are synthesized exclusively by B lymphocytes. Membrane-bound Ig expressed by B lymphocytes binds antigen in a specific reaction, which stimulates the cells to differentiate into effector cells that produce Ig.

Ig light and heavy chains in common with other secreted and membrane-bound proteins are synthesized on membrane bound ribosomes in the rough endoplasmic reticulum (RER). The covalent association of the light and heavy chains probably occurs in the RER as well. The proteins are glycosylated in the RER and the Golgi complex before transportation to the plasma membrane in vesicles. The Ig molecules then become anchored in the cell membrane or are secreted in a process of reverse pinocytosis.

1.6.2 Antibody structure.

All antibodies have a similar overall structure (for general information see Abbas et al., 1991 and Roitt, 1994) which accounts for their common physiological features such as charge and solubility. All antibodies have a common core structure of two identical light chains (24 KDa), and two identical heavy chains (50 or 70 KDa). Both the light and heavy chains contain a series of repeating homologous units of
about 110 amino acids which fold independently to form immunoglobulin domains (see Fig 1.3).

Antibody molecules can be divided into a small number of distinct classes and sub-classes, based on minor differences in physiochemical characteristics such as size, charge and, solubility. In man the classes are A, D, E, G and, M. Members of each class have the same isotype. The A and G classes also have sub-classes; $A_1$ and $A_2$ and $G_1$ to $G_4$. In mice the IgG subclasses are numbered as 1, 2a, 2b and, 3.

Shared regions of the heavy chain amino acid sequence are responsible for the common physiochemical and antigenic properties of antibodies of a particular isotype. These regions are also responsible for common abilities such as binding to certain cell surface receptors or other macromolecules such as complement, which leads to the activation of particular immune effector functions. Isotypes and sub-types determine the effector functions of humoral immunity. There are two isotypes of antibody light chains, Kappa ($\kappa$) and Lambda ($\lambda$), but they do not mediate effector functions.

1.6.3. Monoclonal and Polyclonal antibodies.

A solution of monoclonal antibody contains a single antibody specificity and affinity and a single immunoglobulin isotype, whereas a polyclonal antibody preparation contains a variety of antibody molecules directed against the antigen, as well as antibodies which do not react with the antigen of interest.

Polyclonal antibodies are therefore an "average" of the characteristics of all the antibodies in the solution (Zola, 1985). There may be many different antibodies that react with the antigen of interest, but minor differences in amino acid sequence can
result in changes in affinity for the antigen which makes standardisation from one batch of reagent to the next extremely difficult.

Mabs however will have a single isotype and a single affinity which can be utilised in the development of an ELISA. As each different Mab will have one specific epitope on the antigen of interest two non-competing Mabs can be used, one as a capture antibody, and the other conjugated to a reporter enzyme such as horseradish peroxidase as a detection reagent. The Mabs must be carefully screened to ensure suitability as ELISA reagents, as epitopes common to a range of antigens, for instance the staphylococcal enterotoxins, could allow cross reactivity to occur. Mabs also provide excellent opportunity for standardisation as once a clone is established it should be possible to produce identical antibody in unlimited amounts.

1.7 Aims of this study.

The aim of this study was to produce and characterise a panel of monoclonal antibodies to TSST-1 and to utilise these antibodies in the development of a rapid and sensitive ELISA for the detection of TSST-1 in clinical samples such as serum, urine and burns fluids.

A number of techniques including epitope mapping (Geysen et al., 1984 and Geysen, 1990) and western blot analysis of papain generated fragments of TSST-1 were used to probe the TSST-1 molecule to identify any antigenically significant regions and to provide information on the binding sites of the antibodies.

Finally the antibodies produced in this study were used to provide information on the biological activity of the toxin following construction of a T-cell proliferation
assay using both mouse spleen cells and human peripheral lymphocytes to study the superantigenic activities of TSST-1.
Table 1.1. Toxic shock syndrome case definition.

<table>
<thead>
<tr>
<th>Case Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Fever (temperature &gt;38.9°C)</td>
</tr>
<tr>
<td>2. Rash (diffuse macular erythroderma)</td>
</tr>
<tr>
<td>3. Desquamation, 1-2 weeks after the onset of illness, particularly palms and soles.</td>
</tr>
<tr>
<td>4. Hypotension (blood pressure ≤ 90mm Hg)</td>
</tr>
<tr>
<td>5. Involvement of three or more of the following organ systems:</td>
</tr>
<tr>
<td>Gastrointestinal (vomiting or diarrhoea at onset of illness).</td>
</tr>
<tr>
<td>Muscular (severe myalgia or raised creatine phosphokinase level).</td>
</tr>
<tr>
<td>Mucous membrane (vaginal, oropharyngeal, or conjunctival hyperaemia).</td>
</tr>
<tr>
<td>Renal</td>
</tr>
<tr>
<td>Hepatic</td>
</tr>
<tr>
<td>Haematologic (platelets &lt; 10^5 ml^-1)</td>
</tr>
<tr>
<td>Central nervous system (disorientation or alterations in consciousness without focal neurologic signs when fever and hypotension are absent).</td>
</tr>
</tbody>
</table>

Chesney et al., 1984.
Table 1.2 Comparison of detection limits of assays for TSST-1.

<table>
<thead>
<tr>
<th>Method</th>
<th>Detection limit (ng ml(^{-1}))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radial immunodiffusion</td>
<td>5000</td>
<td>Reeves et al., 1984</td>
</tr>
<tr>
<td>Radioimmunoassay</td>
<td>0.6</td>
<td>Melish et al., 1983</td>
</tr>
<tr>
<td>Competitive ELISA</td>
<td>0.5</td>
<td>Parsonnet et al., 1985</td>
</tr>
<tr>
<td>Non-competitive ELISA</td>
<td>0.5</td>
<td>Rosten et al., 1987</td>
</tr>
<tr>
<td>Mab based ELISA</td>
<td>0.06</td>
<td>Kuffner et al., 1988</td>
</tr>
<tr>
<td>Non-competitive ELISA</td>
<td>0.01</td>
<td>Miwa et al., 1994</td>
</tr>
</tbody>
</table>
Table 1.3 Residues implicated in the biological activity of TSST-1.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Mutation</th>
<th>Location</th>
<th>Biological effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>Y→L</td>
<td>α1 helix, NH₂ Terminal</td>
<td>Loss of T-cell stimulation</td>
</tr>
<tr>
<td>15</td>
<td>S→W</td>
<td>α1 helix, NH₂ Terminal</td>
<td>Loss of T-cell stimulation</td>
</tr>
<tr>
<td>15</td>
<td>G→V</td>
<td>α1, β1 loop, NH₂ Terminal</td>
<td>Loss of T-cell stimulation</td>
</tr>
<tr>
<td>31</td>
<td>G→R</td>
<td>α1, β1 loop, NH₂ Terminal</td>
<td>Reduced MHC II binding</td>
</tr>
<tr>
<td>32</td>
<td>S→P</td>
<td>β2 loop, NH₂ Terminal</td>
<td>Reduced MHC II binding</td>
</tr>
<tr>
<td>135</td>
<td>H→A</td>
<td>α2 helix, COOH Terminal</td>
<td>Loss of T-cell stimulation</td>
</tr>
<tr>
<td>136</td>
<td>Q→A</td>
<td>α2 helix, COOH Terminal</td>
<td>Superantigenic, not lethal</td>
</tr>
<tr>
<td>132.136</td>
<td>E→K,Q→K</td>
<td>α2 helix, COOH Terminal</td>
<td>Loss of T-cell stimulation</td>
</tr>
<tr>
<td>137</td>
<td>L→V</td>
<td>α2 helix, COOH Terminal</td>
<td>Loss of T-cell stimulation</td>
</tr>
<tr>
<td>139</td>
<td>Q→K</td>
<td>α2 helix, COOH Terminal</td>
<td>Loss of T-cell stimulation</td>
</tr>
<tr>
<td>141.144</td>
<td>H→A,Y→A</td>
<td>α2 helix, COOH Terminal</td>
<td>Loss of T-cell stimulation</td>
</tr>
</tbody>
</table>

Information from Acharya et al. (1994), Bonventre et al. (1993), Hurley et al. (1995) and Kum et al. (1996).

See Appendix 1 for single letter amino acid abbreviations.
Table 1.4 Suggested bacterial and viral superantigens.

<table>
<thead>
<tr>
<th>Superantigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcal toxic shock syndrome toxin-1</td>
</tr>
<tr>
<td>Staphylococcal enterotoxins</td>
</tr>
<tr>
<td>Staphylococcal exfoliative toxins</td>
</tr>
<tr>
<td>Streptococcal pyrogenic exotoxins</td>
</tr>
<tr>
<td>Mycoplasma arthritidis mitogen</td>
</tr>
<tr>
<td>Human immunodeficiency virus proteins</td>
</tr>
<tr>
<td>Mouse mammary tumour virus proteins</td>
</tr>
<tr>
<td>Rabies virus nucleocapsid proteins</td>
</tr>
</tbody>
</table>
Table 1.5. Biological properties of TSST-1

Capacity to induce fever mediated by cytokines from macrophages.

Enhancement of host susceptibility to lethal endotoxin shock.

Reticuloendothelial system blockade.

Enhancement of delayed type hypersensitivity skin reactions.

Nonspecific T lymphocyte mitogenicity.

Suppression of immunoglobulin synthesis.

Suppression of chemotaxis of polymorphonuclear leucocytes, induced by release of tumour necrosis factor from macrophages.

(Schlievert, 1989)
Figure 1.1 The polypeptide fold for TSST-1. The MHC II binding region is shaded in black while the residues involved in mitogenicity and TCR binding (C-terminal domain) are highlighted (Tranter et al., 1995).
Figure 1.2 T-cell stimulation by superantigen. Model for antigenic stimulation of T-cells by superantigens. (A) T-cell recognition of specific antigenic peptide presented in conjunction with MHC II by antigen presenting cell, (B) TSST-1 superantigen binding to MHC II and TCR Vβ at sites distinct from the conventional peptide recognition sites.
Figure 1.3 basic antibody structure. The amino-terminal end of the molecule is characterised by sequence variability (V) in both the heavy (H) and light (L) chains. The rest of the molecule has a relatively constant (C) structure, divided into 3 domains. All domains are stabilised by internal disulphide bonds. The light and heavy chains are held together by disulphide bonds (S) and the hinge region (SS) allows the two antigen binding sites to operate independently. Ca denotes carbohydrate moieties.
Chapter 2

Primary Characterisation of Monoclonal Antibodies
Chapter 2.

**Primary characterisation of monoclonal antibodies.**

2.1 Introduction.

Production of monoclonal antibodies can be achieved because each B lymphocyte produces antibody of a single specificity. Myelomas are B cell tumours, which can occur spontaneously in man and be experimentally induced in animals; they also produce single specific antibodies. Myeloma-derived antibodies have proved useful for elucidating the structure of immunoglobulin proteins and immunoglobulin genes were initially isolated from these cells. The specificity of myelomas however is not usually known as the transformation process that affects the B lymphocytes occurs randomly. Since normal B lymphocytes of known specificity cannot survive indefinitely, Kohler and Milstein (1975) developed a procedure to immortalise the B cell by fusing a normal antibody-secreting B cell to a non-secreting myeloma (somatic hybridisation). Kohler and Milstein's technique is dependent on the availability of cultured myeloma cells that grow in normal growth medium but not in defined selection medium because they lack certain functional genes required for DNA synthesis. Fusing the myeloma cell to a B cell will provide the necessary genes and allow the hybridomas to survive, moreover the B cell is rendered immortal. Myelomas can be treated with anti-folate drugs such as aminopterin which block the formation of purines and thymidylate, thus blocking DNA synthesis by the *de novo* pathway. Normally these cells would use a salvage pathway to synthesize purine from exogenously supplied hypoxanthine using the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT). Thymidylate can be synthesized from...
thymidine using the enzyme thymidine kinase (TK) so that the hybridoma cells grow
normally in media supplemented with thymidine and hypoxanthine (HAT medium).

Myeloma cell lines can be made defective in HGPRT and TK synthesis by
mutagenesis which means that they die in HAT medium. Normal cells fused to
HGPRT or TK negative cells provide the necessary enzymes to enable the resulting
hybridomas to synthesize DNA and grow normally in HAT medium. Mouse myeloma
cells can be fused to normal B cells from immunised mice using a viral system
(Kohler and Milstein, 1975) or polyethylene glycol (Galfré et al., 1977) and any
resulting hybrids can be selected using HAT medium. Any cells that do not fuse die
while culture wells with growing, fused cells can be tested for antibody to the antigen
used and positive wells cloned by limiting dilution. The fused cells are known as
hybridomas and such cells secreting antibody of the correct specificity can be
selected. The antibodies produced by such hybridomas are called monoclonal
antibodies (Mab).

Antibodies of the correct specificity identified during the cloning procedure
must then be characterised prior to purification to ensure that they are of the desired
immunoglobulin sub-class (Dunbar and Skinner, 1990). Most fusions result in the
production of either IgG or IgM antibodies. Determination of the Ig sub-class is
important since it may influence the antibody purification strategy. For example,
Protein A chromatography which is commonly used in antibody purification binds the
mouse IgG\textsubscript{1} sub class with low affinity and for such antibodies affinity
chromatography on Protein G which strongly binds all mouse IgG sub classes is
preferred. Any cloned hybridomas producing antibody of the desired specificity can
be expanded and grown in large volumes in cell culture or as ascitic tumours in syngeneic mice to produce large amounts of antibody (Manil et al., 1986).

Further work to characterise the binding of the antibodies to their specific antigen which may involve a degree of epitope mapping should also be carried out as these studies provide valuable information for the development of immunoassays. It is important to select antibodies which bind strongly and are highly specific as false positives must be avoided if the assay is to be of any use as a diagnostic tool. On the other hand the Mab must provide the desired assay sensitivity and bind to any antigenically different forms of the antigen to avoid false-negative assay results. IgG antibodies are considered to be the most useful in ELISA development due to the high affinity or strength of binding that these molecules have for their specific antigens. IgM antibodies bind with great avidity due to their pentameric structure but with a lower affinity. They are also particularly sensitive to mild reduction by thiol agents at neutral pH, which destroys their high avidity, and makes them less suitable for use in ELISA (Tijssen, 1985).
2.2 Materials and Methods.

2.2.1 Production of monoclonal antibodies to TSST-1.

Anti-TSST-1 monoclonals were produced by immunising BALB/c (Harlan Olac) mice with purified TSST-1 (Passalacqua et al., 1992) using a modification of the method of Stahli et al. (1983). Four mice were immunised intraperitoneally (i.p.) with 25μg of TSST-1 in 0.5ml of a 1:1 mixture of Freunds complete adjuvant (Sigma) and phosphate buffered saline (PBS). At intervals of three and six weeks the mice were re-immunised by the same method, but using Freunds incomplete adjuvant (Sigma). Prior to the fusions single mice were immunised with 50μg of TSST-1 in 200μl PBS intravenously (i.v.) four days before being culled for spleen removal.

Spleen cells were fused with established P3X63/Ag8.653 myeloma cells (ECACC, CAMR, Porton Down, Salisbury), also derived from BALB/c mice as described by Newell et al. (1988). The spleen cells and myeloma cells were mixed in a ratio of 3:1 and the cell pellet treated with 50% (v/v) polyethylene glycol solution to fuse the cells. The resulting mixture was subsequently resuspended in RPMI selective medium (Gibco-BRL) for fused cells containing 20% (v/v) foetal calf serum (FCS; Sigma) and 1% (v/v) hypoxanthine-aminopterin-thymidine solution (ICN). The cells were dispensed at a concentration of 1.5x10^5 myeloma cells per well (100μl/well) in 96 well tissue culture plates (Nunc) containing 6x10^3 mouse peritoneal exudate cells as feeder cells. The hybridomas were then diluted to an average density of about one colony per well and the culture supernatant fluids were assayed by ELISA for production of antibody to TSST-1.
2.2.2 Assay of monoclonal antibodies to TSST-1.

Culture supernatant fluids were added directly to 96 well plates (Immulon 1, Dynatech) which had been coated (100μl/well) with 10μg ml⁻¹ TSST-1 in PBS, and blocked (100μl/well) with PBS containing 0.1% (v/v) Tween 20 (Sigma) and 5% (v/v) FCS (PBS-T/FCS). After shaking (Denly Wellwarm, 300rpm) at room temperature for 90 min the plates were washed 3 times in PBS + 0.1% (v/v) Tween 20 (PBS-T) and any antibody binding to the toxin was detected using rabbit anti-mouse antibody conjugated to horseradish peroxidase (Sigma) previously diluted 1/1000 in PBS-T/FCS. After incubation at room temperature for 90 minutes the plates were washed 3 times before the substrate solution was added (100μl/well). The substrate was prepared immediately prior to addition by dissolving 10 mg ml⁻¹ 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma) in dimethyl sulfoxide (DMSO; Sigma) and diluting 100μl in 10ml of 0.5M sodium citrate buffer, pH 5.0 containing 8μl 6% (v/v) H₂O₂. The enzymic reaction was stopped after the colour reaction had developed for 5 min by adding 50μl 2M H₂SO₄ to each well and the absorbance read at 450nm on a Dynatech MR7000 plate reader.

Culture wells showing significant antibody titres were cloned (Davis, 1986) and the supernatant fluids of these cultures tested as before. In total 8 hybridoma cell lines producing antibody to TSST-1 were selected for further study.

The isotype of the antibody present in the tissue culture supernatant fluids was determined by using a mouse monoclonal antibody isotyping kit (Sigma) according to the manufacturer’s instructions.
2.2.3 Bulk antibody production.

Hybridoma cell lines were expanded in culture by splitting the original culture (30ml) between 2 flasks (75cm² Dow Corning) and adding fresh medium to each to return it to the original volume. This process was repeated until confluent growth had been produced in 4 flasks. Each cell line was used to inoculate 4 BALB/c mice previously primed by i.p. injection with 0.2ml pristane (2,6,10,14-tetramethylpentadecane, Sigma) per mouse (Hoogenraad and Wraight 1986), using a concentration of 2-5x10⁶ cells per mouse in 0.5ml RPMI 1640 medium. On development of a tumour (generally in 2-4 weeks) a small sample of ascitic fluid (approx 200μl) was removed and tested for the presence of antibodies to TSST-1 by ELISA. On detection of antibody the tumour was drained whilst the mouse was maintained under terminal anaesthesia after which the mouse was culled before regaining consciousness. Ascitic fluids were cleared by centrifugation (MSE Microcentaur, 13000 rpm for 5 min) and stored at -20°C pending purification. One cell line (4 TSST 37.3) when injected into mice produced dry tumours and no ascitic fluid could be obtained. This hybridoma cell line was once again expanded in culture and used to seed a growth cassette in a Techno Mouse in vitro monoclonal antibody culture system (Integra Biosciences). The Techno Mouse culture system makes use of hollow fibres to supply a constant flow of culture media to the hybridoma cells growing in the cassette, and allows aseptic sampling of the culture for cell enumeration and assay of antibody production. In this study 5x10⁷ hybridoma cells in 5ml RPMI 1640 media containing 10% (v/v) FCS, 4mM L-glutamine (Sigma), penicillin/streptomycin solution (200 IU ml⁻¹ and 0.2mg ml⁻¹ respectively; ICN), 1mM sodium pyruvate (ICN), and 1% hypoxanthine thymidine solution (ICN) were
injected into the culture chamber. The hybridoma cells were maintained at a constant temperature of 37°C in an atmosphere of 5% CO₂ for 6 weeks.

2.2.4 Affinity testing of the monoclonal antibodies.

In order to test the binding affinity of the monoclonal antibodies for TSST-1, ELISA experiments were conducted in the presence of 3M urea which is a strong dissociating agent. ELISA plates (96 well) were coated with toxin at a concentration of 10μg ml⁻¹ in PBS (100μl/well) and blocked using PBS-T/FCS or PBS-T/FCS containing 3M urea (100μl/well). The antibody was diluted in the same buffer (1/5 dilutions) in the presence or absence of urea and applied to the plate (100μl/well) so that the effect of blocking with urea on antibody diluted in normal buffer could be compared with the effect of blocking with FCS on the binding of antibody diluted in both normal buffer and 3M urea. Bound antibody was detected using anti-mouse HRP conjugate (Sigma) diluted 1/1000 in PBS-T/FCS. The enzyme substrate colour reaction process was carried out as detailed in section 3.2.2.

2.2.5 Purification of immunoglobulins.

Immunoglobulins from the ascitic fluids and Techno Mouse culture were purified by affinity chromatography using protein G Sepharose Fast Flow in user prepared HR10/10 columns (Pharmacia) on a Fast Protein Liquid Chromatography (FPLC) system (Pharmacia). All reagents used were high purity, and buffers were filtered (0.22μm, Schleicher & Schuell, Anderman) and degassed prior to use. The ascitic fluids (4ml) were diluted 1/5 in 20mM sodium phosphate, pH 7.0 and filtered using a 0.22μm syringe tip filter (Schleicher & Schuell, Anderman). The sample was
then loaded onto an 8ml protein G column and any unbound material washed off by running the same buffer through the column until the chart recorder trace had returned to near zero. The IgG fraction was then eluted using 3 column volumes of 100mM citric acid, pH 2.7. Immediately following purification the antibodies were dialysed into 100mM Tris pH 8.0 for storage at -20°C. The purity of the antibody preparations was assessed by SDS polyacrylamide gel electrophoresis (SDS/PAGE) when the purified, dialysed IgG was applied to Pharmacia Phast gels (8-25%) according to the manufacturer's instructions. The antibody was prepared for electrophoresis by heating two aliquots (10μl) of each antibody at 95°C for 5 min in sample buffer, i.e. 10mM Tris, pH 8.0 (Sigma) containing 1mM EDTA (Sigma), 2% (w/v) SDS (Sigma), and 0.05% (w/v) bromophenol blue (Pharmacia) as a marker dye. Prior to the heating process 50mM dithiothreitol (DTT) was added to one sample.

2.2.6 Specificity of binding of the monoclonal antibodies.

The binding specificity of the monoclonal antibodies was determined by coating ELISA plates with each monoclonal antibody separately at a concentration of 10μg ml⁻¹ (100μl/ well), and blocking with PBS-T/FCS (100μl/ well). Purified TSST-1, SEA, SEB, SEC₂ and botulinum toxins A, B and E (supplied by R. Brehm and B. Hallis, CAMR, Porton Down Salisbury SP4 0JG) which had been diluted in the range of 100ng to 0.005ng ml⁻¹ in PBS-T/ FCS, were applied to the plates (100μl/ well) and shaken at room temperature for 90 min. After washing 3 times in PBS-T any bound toxin was detected using rabbit polyclonal anti-TSST-1 HRP conjugate diluted 1/200 in 5% (v/v) FCS in PBS-T. The conjugate had been made previously by inoculating a New Zealand White rabbit with purified TSST-1 according to the
method of de Azavedo et al. (1985), harvesting the resulting serum and purifying the immunoglobulin by the method detailed above (section 2.2.5). The immunoglobulin was conjugated to horseradish peroxidase by the procedure in section 3.2.1.

2.2.7 Monoclonal antibody competitive binding ELISA

All monoclonal antibodies were tested to determine whether they bound to different antigenic binding sights. For this, a 96 well ELISA plate (Immulon 1, Dynatech) was coated (100μl/well) with purified TSST-1 (5μg ml⁻¹) in PBS and blocked (100μl/well) with PBS-T/FCS, by shaking the plate for 90 min at room temperature. Two monoclonal antibodies were initially diluted to 20μg ml⁻¹ in PBS-T/FCS and a further nine 1/3 dilutions made. One of these antibodies in conjugated form (see Chapter 3) was initially diluted 1/200 in identical buffer. The samples were then applied to the plate (100μl/well) such that the conjugate was applied alone and also mixed in a ratio of 1:1 with each of the other non-conjugated antibodies at all dilutions. After incubation at room temperature for 90 min the plates were washed 3 times before the substrate solution was added (100 μl/well; prepared as described above). The enzymic reaction was stopped after the colour reaction had developed by adding 50μl 2N H₂SO₄ to each well, and the absorbances read at 450nm on a Dynatech MR7000 plate reader.
2.3 Results

2.3.1 Production and characterisation of anti-TSST-1 monoclonal antibodies.

Eight monoclonal antibodies (Table 2.1) were produced as a result of four hybridoma fusions. These hybridoma cell lines were expanded in culture and injected into mice to produce ascites or in the case of one cell line (4 TSST 37.3) cultured in the Integra Biosciences Techno Mouse in vitro culture system.

2.3.1.1 Isotype determination.

Isotype determination carried out on tissue culture supernatant fluids showed that all of the hybridoma cell lines produced antibody of the IgG isotype. The antibody titres of the tissue culture supernatant fluids were assayed and found to be in the range of 1/3125 to 1/15625. However when assayed in the presence of 3 M urea, a strong dissociating agent, most of the Mabs showed a reduction in affinity with some titres falling as low as 1/625 (Table 2.1). Although urea can disrupt the conformation of the antibody molecule itself, it also disrupts the hydrophobic interactions which are important in antibody-antigen interactions. Antibodies which have a much lower affinity in the presence of urea may be less suitable for use in immuno-assays as components of the sample matrix may affect binding and thus accuracy of the results.

2.3.2 Purification of the monoclonal antibodies.

The Mabs were purified using protein G Sepharose affinity chromatography because of the high affinity of protein G for murine IgG. A typical elution profile obtained during antibody purification is shown in Fig 2.1. Purification of the antibodies using this method consistently produced two peaks. The first of these
contained proteins other than IgG whilst the second peak produced in response to 100mM citric acid, pH 2.7 contained the IgG fraction. The yields obtained by purification of antibody from mouse ascitic fluid are shown in Table 2.2. Although two antibodies (4 TSST 5.3 and 4 TSST 116.3) produced higher levels of antibody (3.4 and 2.7 mg ml⁻¹ respectively) all of the other hybridoma cell lines gave under 2 mg ml⁻¹ (range 1.5 - 1.9 mg ml⁻¹). Purification of culture supernatant fluid from the Techno Mouse culture of hybridoma cell line 4 TSST 37.3 produced material with a concentration of 1.8 mg ml⁻¹.

2.3.3 SDS polyacrylamide gel analysis of purified antibody.

Purification of the Mabs using protein G yielded homologous antibody preparations (as revealed by SDS PAGE gels) suitable for use in assay development and structure/function studies.

The antibody samples treated with DTT (which is used to break the disulphide bonds that hold the various heavy and light chains of the antibody molecule together) produced 2 bands on SDS PAGE gels, one corresponding to the light chains, with an apparent molecular weight of 25 KDa and one corresponding to the heavy chains with an apparent molecular weight of 50 KDa. The samples not treated with DTT produced only one band with an apparent molecular weight of 150kDa. An example of an antibody purity gel is shown in Fig. 2.2.
2.3.4 Specificity testing.

To test whether any of the Mabs could bind to toxins other than TSST-1, ELISA plates were coated (100µl/well) with the Mabs at a concentration of 10µg ml\(^{-1}\) in PBS. Development of the enzyme substrate colour reaction revealed detection of only TSST-1, with no reactivity above background level for any of the other toxin types. These tests were carried out to ensure the specificity of binding as the staphylococcal enterotoxins share around 20% primary sequence homology with TSST-1 (Betley et al., 1992).

2.3.5 Competitive binding studies.

An ELISA assay was used to determine which of the Mabs competed with each other for binding to the toxin. For simplicity the results of these tests are summarised in Table 2.3.

Competitive binding ELISA assays carried out using the Mabs conjugated to horseradish peroxidase revealed that they could be divided into 3 groups (Table 2.4). The members of each group competed with each other for binding to the toxin although not with members of other groups. An example of a typical competitive binding assay result is shown in Fig 2.3. The conjugated antibody (4 TSST 5.3 HRP) alone gives a high signal but when non-conjugated antibody of the same type is added the signal is lowered as a function of the concentration of the blocking antibody. Of the two other Mabs tested in this experiment one, 4 TSST 10.3, competed for binding to the toxin while the other, 4 TSST 23.3, did not and the signal from the conjugate was as strong as that from the conjugate alone. These results indicate the presence of at least three distinct antigenic sites on the toxin molecule, which appear to be
supported by the results of the epitope mapping experiments conducted with the rabbit polyclonal anti TSST-1 anti toxin (see Chapter 4).
Table 2.1. Isotype, titres and affinity titres of anti TSST-1 monoclonal antibodies.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Isotype</th>
<th>Titre</th>
<th>Affinity titre*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 TSST 109.3</td>
<td>IgG₁</td>
<td>1/3125</td>
<td>1/625</td>
</tr>
<tr>
<td>2 TSST 112.3</td>
<td>IgG₁</td>
<td>1/15625</td>
<td>1/625</td>
</tr>
<tr>
<td>4 TSST 5.3</td>
<td>IgG₁</td>
<td>1/3125</td>
<td>1/3125</td>
</tr>
<tr>
<td>4 TSST 10.3</td>
<td>IgG₁</td>
<td>1/3125</td>
<td>1/625</td>
</tr>
<tr>
<td>4 TSST 23.3</td>
<td>IgG₁</td>
<td>1/15625</td>
<td>1/3125</td>
</tr>
<tr>
<td>4 TSST 37.3</td>
<td>IgG₁</td>
<td>1/3125</td>
<td>1/3125</td>
</tr>
<tr>
<td>4 TSST 110.3</td>
<td>IgG₁</td>
<td>1/3125</td>
<td>1/625</td>
</tr>
<tr>
<td>4 TSST 116.3</td>
<td>IgG₁</td>
<td>1/3125</td>
<td>1/3125</td>
</tr>
</tbody>
</table>

* Determined in the presence of 3M urea.

Values shown are the mean of three replicate assays.
Table 2.2. Yield of purified monoclonal antibody produced by growth of hybridoma cells in ascites fluid.

<table>
<thead>
<tr>
<th>Hybridoma cell line</th>
<th>Antibody conc. (mg ml⁻¹)</th>
<th>Total volume of ascitic fluid (ml)</th>
<th>Total antibody yield (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 TSST 109.3</td>
<td>1.9</td>
<td>6</td>
<td>11.4</td>
</tr>
<tr>
<td>2 TSST 112.3</td>
<td>1.5</td>
<td>5</td>
<td>7.5</td>
</tr>
<tr>
<td>4 TSST 5.3</td>
<td>3.4</td>
<td>7</td>
<td>23.8</td>
</tr>
<tr>
<td>4 TSST 10.3</td>
<td>1.9</td>
<td>5</td>
<td>9.5</td>
</tr>
<tr>
<td>4 TSST 23.3</td>
<td>1.5</td>
<td>5</td>
<td>7.5</td>
</tr>
<tr>
<td>4 TSST 110.3</td>
<td>1.8</td>
<td>5</td>
<td>9.0</td>
</tr>
<tr>
<td>4 TSST 116.3</td>
<td>2.7</td>
<td>5</td>
<td>13.5</td>
</tr>
</tbody>
</table>
Table 2.3 Antibodies that compete for binding to TSST-1

<table>
<thead>
<tr>
<th>Non conjugated antibody ↓</th>
<th>2 TSST 109.3 - HRP</th>
<th>2 TSST 112.3 - HRP</th>
<th>4 TSST 5.3 - HRP</th>
<th>4 TSST 10.3 - HRP</th>
<th>4 TSST 23.3 - HRP</th>
<th>4 TSST 37.3 - HRP</th>
<th>4 TSST 110.3 - HRP</th>
<th>4 TSST 116.3 - HRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 TSST 109.3</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>2 TSST 112.3</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>C</td>
<td>C</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>4 TSST 5.3</td>
<td>N</td>
<td>N</td>
<td>C</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>C</td>
</tr>
<tr>
<td>4 TSST 10.3</td>
<td>N</td>
<td>N</td>
<td>C</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>C</td>
</tr>
<tr>
<td>4 TSST 23.3</td>
<td>C</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>4 TSST 37.3</td>
<td>C</td>
<td>C</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>C</td>
<td>N</td>
</tr>
<tr>
<td>4 TSST 110.3</td>
<td>C</td>
<td>C</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>C</td>
<td>N</td>
</tr>
<tr>
<td>4 TSST 116.3</td>
<td>N</td>
<td>N</td>
<td>C</td>
<td>C</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

C - indicates antibodies which compete  
N - indicates antibodies which do not compete
Table 2.4 Monoclonal antibody competitive binding groups.

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 TSST 109.3</td>
<td>2 TSST 112.3</td>
<td>4 TSST 5.3</td>
</tr>
<tr>
<td>4 TSST 23.3</td>
<td>4 TSST 37.3</td>
<td>4 TSST 10.3</td>
</tr>
<tr>
<td>4 TSST 110.3</td>
<td>4 TSST 116.3</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.1. Typical elution profile of FPLC purification of Monoclonal antibody. Mabs were bound to Protein G sepharose, and eluted in 100mM citric acid pH 2.7, from fraction 19. The first peak on the graph contains non-IgG proteins and the second peak is the purified IgG. Fractions (21-27) containing IgG were pooled and dialysed overnight against 100mM Tris pH 8.0, and stored at -20°C as 0.05ml aliquots.
Figure 2.2. SDS PAGE gel of protein G affinity purified Mab to TSST-1.

Purified Mab was applied to an 8-25% gradient gel both with (lane 2) and without (lane 1) DTT. The two bands in lane 2 represent the heavy chains with an apparent molecular weight of 50kDa and the light chains with an apparent molecular weight of 25kDa. The single band in lane 1 is whole antibody with an apparent molecular weight of 150kDa. The multiple bands in lane 3 are molecular weight standards (Novex) in the range of 200 - 2.5 kDa.
Figure 2.3. Competition ELISA assay using 4 TSST 5.3 HRP conjugate. The conjugate is applied alone, and with other non-conjugated antibodies to assess the effects of the non-conjugated antibody on the binding of the conjugate to the toxin. This figure shows inhibition of binding of 4 TSST 5.3 by 4 TSST 10.3 but not 4 TSST 23.3.
2.4 Discussion.

Monoclonal antibodies were generated by immunisation of Balb/c mice with highly purified TSST-1 and fusion of antibody secreting B-cells with myeloma cells. Such monoclonal antibodies can be used in the investigation of structure/function relationships of an antigen and are also useful as reagents in a variety of diagnostic methods and assays.

Before their use as reagents in assay systems antibodies must be thoroughly characterised to ensure that they are specific for the antigen being investigated. All of the Mabs used in this study were of the IgG\textsubscript{1} isotype; these molecules tend to have a higher affinity for their specific antigen than IgM antibodies (Liddell and Weeks, 1995) are more stable and easier to use. The Mabs were purified using protein G affinity chromatography which gave high purity immunoglobulins from a simple one-step procedure and has a comparable efficiency to other methods such as DEAE Affi-Gel Blue chromatography (Bruck et al., 1986) and HPLC (Burchiel, 1986).

Purification of the antibodies facilitates efficient conjugation to reporter enzymes such as horseradish peroxidase (Manil et al., 1986) for use as detection reagents in assay systems. Another advantage of using purified antibody is that concentrations of capture and detection reagents can be optimised so that a highly sensitive, reproducible assay can be produced using the minimum quantities of antibody and enzyme which may also help to reduce background noise to a minimum. The higher the quality of reagents used in an immunoassay the more specific, reproducible and accurate the results of the assay should be.
Characterisation of the Mabs is also essential as the specificity of any assay system must be absolute. If the assay is to be used as a diagnostic tool cross reactivity must be eliminated in order to achieve accuracy of results. The lack of cross reactivity of the Mabs with the staphylococcal enterotoxins suggests that the 20% homology in amino acid sequence (Betley et al., 1992) does not account for any of the antigenic sites identified by the Mabs generated in this study. This should allow detection of TSST-1 even in the presence of staphylococcal enterotoxins and keep false positive reactions to a minimum. High affinity reagents with no potential for cross reaction may also help to keep "background noise" to a minimum which is essential if the assay is to be used to detect very small amounts of antigen.

The affinity of the antibodies used for ELISA development must be high as elements of the sample matrix may interfere with antibody-antigen interactions to some extent. Furthermore it is essential during the development of a Mab-based ELISA for detection of toxin to use a pair of Mabs that do not compete for binding to the target antigen as any competition or steric hindrance will result in a loss of sensitivity in the assay system. The method of affinity testing using urea, as used in this study is by no means an absolute measure of affinity, but studies have shown that the binding of low affinity antibodies is reduced in the presence of urea (Hallis et al., 1993), and it gives an indication of the behaviour of the Mabs in the presence of interfering factors. Urea affects hydrophobic interactions and may disrupt the conformation of the antibodies thus influencing binding affinities. If however the antibody-antigen interactions rely on charge the presence of the urea will not greatly affect the binding. Of the
antibodies used in this study, 5 showed reduced binding in the presence of urea while 3 did not whilst competitive binding studies revealed that the Mabs could be divided into 3 groups, which indicated the existence of at least 3 different antigenic determinants on the toxin molecule. In order to increase the specificity and reproducibility of the assay Mabs were chosen from non-competing groups so that one antibody would not influence the binding of another to the toxin.

The information generated from the affinity studies together with that from competitive binding was used to help identify and select a pair of Mabs to use as capture and detection reagents in the development of an ELISA for TSST-1.
Chapter 3

ELISA Development
Chapter 3.

ELISA Development.

3.1 Introduction.

Since the implication of TSST-1 in the pathogenesis of toxic shock syndrome (Todd et al., 1978) various methods have been devised to detect this toxin. Most methods involve the culturing of bacterial isolates and assay of supernatant fluids for the presence of toxin, although assays for toxin in serum and urine have been developed (Wells et al., 1987; Miwa et al., 1994; Melish et al., 1983). Methods such as isoelectric focusing, double immunodiffusion, solid phase radioimmunoassay (Cohen et al., 1983; Hayes et al., 1984), reverse passive latex agglutination (RPLA; Igarashi et al., 1986) and immunoblotting have also been used, but all are subject to a number of limitations, such as labour intensive production of samples, the need for special growth media to enhance toxin production, lack of sensitivity and / or specificity and prolonged processing time. There are also limitations to use for example, as RPLA cannot be used for serum samples because of non-specific agglutination caused by serum components (Miwa et al., 1994). The aim of the work presented in this chapter was to develop a rapid monoclonal antibody-based assay to detect toxin in serum, urine and cultures of clinical isolates.

ELISA has advantages over several of the other methods in that it is a sensitive method and detection levels may be as low as pg ml⁻¹. It can also be a very rapid procedure especially when non-competitive Mabs are used as capture and detection reagents.

The use of Mabs in an ELISA for TSST-1 can increase both the specificity and sensitivity of the assay. It was found previously (Kuffner et al., 1988) that a
combination of two non-competing Mabs used as capture and detection reagents may provide a similar level of detection to polyclonal antibody reagents. Mabs also have a distinct advantage in that successively produced batches of antibody should have identical characteristics such as affinity and specificity of binding because they are produced from immortal hybridoma cell lines kept in storage at -70°C. Frozen stocks of hybridomas can be replenished by growing the cells in culture and then freezing down a fresh batch in a special preserving medium containing DMSO. Polyclonal antibodies, in contrast, can differ greatly in avidity and affinity from batch to batch due to the nature of their production (Dunbar and Schwoebel, 1990). This in turn can lead to variation in assay sensitivity and specificity, reducing the effectiveness of the assay.

It is important that Mabs be carefully characterised during the development of an assay system using screening and selection to evaluate the suitability of the reagents. Each different Mab may react differently when used in different situations, for instance a Mab that recognises its specific antigen when the antigen is bound to a polystyrene assay plate may not be able to bind the antigen when it is immobilised by another antibody (Kuffner et al., 1988). Similarly some of the Mabs may be rendered incapable of binding the antigen when used as a capture reagent but are in turn very useful as detection reagents when coupled to a reporter enzyme such as horse radish peroxidase.
3.2 Methods.

3.2.1 Conjugation of monoclonal antibody.

All of the monoclonal antibodies in this study were conjugated to horseradish peroxidase (HRP) using the method of Nakane and Kawaoi (1974). Five milligrams of horseradish peroxidase (Sigma type XII) were dissolved in 1ml of 0.3M sodium bicarbonate, pH 8.0 (BDH), containing 0.1ml of 1% (v/v) 1-fluoro-2,4-dinitrobenzene (Sigma) in absolute ethanol. The solution was mixed gently at room temperature for 1h and 1ml of 50mM sodium periodate (Sigma) added. The mixing was continued for a further 30 min before the addition of 1ml 0.16M ethylene glycol (ethanediol; BDH). After mixing for a further 1h at room temperature the solution was dialysed overnight against 2L of 0.01M carbonate/bicarbonate buffer, pH 9.5 at 4°C. During this time 5mg of purified immunoglobulin in (2-3 ml) was dialysed against 2L of the same buffer.

The dialysed antibody was added dropwise to the dialysed dinitrobenzene periodate - horseradish peroxidase mixture over a period of 30 min, mixing constantly. Then the solution was mixed for a further 2-3h at room temperature. Following this 5mg of sodium borohydride was added and the solution incubated on ice for 3h with occasional mixing. The antibody-HRP solution was dialysed overnight against 2L of 50mM Tris (Sigma) pH 8.0 containing 0.3M NaCl (Sigma). The resulting enzyme conjugate was filtered through an 0.22μm syringe tip filter (Sartorius) and purified to separate bound conjugate from unbound material by gel filtration chromatography on a Sephadex G-200 column (bed volume 145ml, diameter 16mm, height 580mm), previously equilibrated with 50mM Tris, pH 8.0 containing 0.3M NaCl. The column was eluted at a flow rate of 8ml h⁻¹ and the eluate collected
in 2ml fractions. The optical density of the fractions was monitored at 280nm for protein content and 405nm for peroxidase activity. Fractions within the $A_{405}$ peak were pooled and divided into 0.5ml and 50μl amounts for storage at -20°C. The activity of the conjugate was tested by ELISA to establish a working dilution which would give reliable, reproducible results using the minimum quantity of conjugate.

3.2.2 Enzyme Linked Immunosorbent Assay (ELISA) of TSST-1.

In the standard ELISA assay the 96 well plate (Immulon 1, Dynatech) was coated (100μl/well) with antibody at a concentration of 10μg ml$^{-1}$ in PBS overnight at 4°C. The antibody solution was removed by aspiration and the plates blocked with FCS/PBS-T as detailed previously (section 2.2.2). The plates were then washed once with PBS-T and either used immediately or stored (up to three months) at -20°C for future use. A standard curve ranging from 100ng ml$^{-1}$ to 0.005 ng ml$^{-1}$ of purified TSST-1 diluted in blocking buffer was prepared and applied (100μl/well) to the plate. The plates were then shaken at room temperature for 90 min, before being washed three times with PBS-T. The HRP conjugated detection antibody (diluted 1/300 in blocking buffer) was applied and the plates shaken at room temperature as before. Finally the plates were washed 3 times with PBS-T before the substrate solution was added (100 μl/well). The substrate solution was prepared previously by dissolving 10 mg ml$^{-1}$ TMB (Sigma) in DMSO (Sigma) and diluting 100μl in 10ml of 0.5M sodium citrate buffer, pH 5.0 containing 8μl of 6% $H_2O_2$ (BDH). The enzymic reaction was stopped after the colour reaction had developed by adding 50μl 2M $H_2SO_4$ to each well, and the absorbance read at 450nm on a Dynatech MR7000 plate reader.
3.2.3 Amplification of HRP in ELISA.

Amplification (Fig 3.1) of the ELISA assay was performed by coating the 96 well plate (100μl/well) with antibody at a concentration of 5μg ml⁻¹ in PBS overnight at 4°C. The antibody solution was removed by aspiration and the plates blocked with FCS/PBS-T as detailed above. The plates were then washed once with PBS-T and either used immediately or stored (up to three months) at -20°C for future use. A standard curve ranging from 10ng ml⁻¹ to 0.0005 ng ml⁻¹ of purified TSST-1 diluted in blocking buffer was prepared and applied (100μl/well) to the plate. The plates were then shaken at room temperature for 90 min, before being washed three times with PBS-T. The bound toxin was detected using mouse monoclonal anti-TSST-1-HRP conjugates diluted 1/200 in blocking buffer. After washing off any unbound conjugate 100μl of biotinylated tyramine reagent was added to the wells and shaken at room temperature for 15 min. This reagent was initially prepared by dissolving 40mg tyramine (Pierce) in 1ml DMSO (Sigma) and 100mg of N-Hydroxysuccinimidobiotin (Sigma) in 1ml DMSO before mixing them together in equal volumes. The resulting solution was then added to 10ml 50mM Tris pH8.0, containing 0.01% H₂O₂ (the amount used was determined by experiment, but was usually between 0.03 and 3.0μl per ml of buffer). The plate was washed 4 times in PBS-T and 100μl of 1mg ml⁻¹ streptavidin peroxidase (Pierce) diluted 1/1000 in blocking buffer added to the wells. The plate was then shaken for 15 min at room temperature before being washed again 4 times in PBS-T. Finally the TMB substrate was added as detailed above with the exception that the concentration of TMB used was 6mg ml⁻¹.
3.2.4 Reproducibility of the assay.

Experiments were conducted to measure the reproducibility of the assay system using two methods: intra assay variations and inter assay variations (Beer, 1993). The intra assay variation method tests the variability of results from a set of samples which are applied at a number of different points on an ELISA plate, compared to the values of a standard curve of toxin, while the inter assay variation method measures the variability of results when assays are performed on different days.

3.2.4.1 Intra assay variations.

A series of six standard concentrations (8.0, 4.0, 2.0, 1.0, 0.5 and 0.25 ng ml$^{-1}$) of toxin in PBS-T/FCS were made and stored at -20°C prior to use. The samples were thawed at room temperature and applied to an assay plate such that each dilution occupied a different position on each row of sample wells. This determined the effects on the results of positioning of the sample on the assay plate. A standard curve of toxin in the range of 10ng to 0.0005ng ml$^{-1}$ was also applied to the plate and the assay completed as described in section 3.2.3. From the results the mean, standard deviation and co-efficient of variance (%CV) were calculated.

3.2.4.2 Inter assay variations.

For this the assay was repeated 15 times using the same set of toxin dilutions at fixed concentrations on different days and the mean, standard deviation and %CV calculated. The standard toxin concentrations consisted of: 10, 3.3, 1.1, 0.37, 0.123, 0.041, 0.0137 and 0.0045 ng ml$^{-1}$. 
3.2.5 Detection of antibody to TSST-1 in human serum.

To detect the presence of antibody to TSST-1 in human serum, ELISA plates were coated with toxin at 5μg ml⁻¹ and blocked as described in section 3.2.2. The test sera (from volunteers at CAMR) were then diluted 1/50 in FCS/PBS-T and then a further nine 1/3 dilutions were made. The dilutions were applied to the assay plates (100μl/well) and incubated by shaking at room temperature for 90 min; any unbound material was removed by washing as detailed in section 3.2.2. Bound antibody was then detected using anti-human IgG HRP conjugate (Sigma) diluted 1/1000 in FCS/PBS-T. The enzyme substrate and colour reaction process were exactly as described in section 3.2.2 (ELISA).

3.2.6 Spiking experiments.

In these experiments the detection of toxin in human serum and urine was compared to the detection of toxin in buffer. The assay was carried out as described in section 3.2.3 (Amplified ELISA) but a standard curve of toxin was applied to the ELISA plate diluted in either 30% human serum in FCS/PBS-T or 30% urine in the same buffer. The serum used in these assays was from a volunteer who had previously been found to have no antibody titre to TSST-1 using the method described in section 3.2.5.

3.2.7 Detection of TSST-1 and antibody to TSST-1 in clinical samples.

A number of serum, plasma and urine samples from suspected TSS cases were obtained and tested for the presence of TSST-1 or antibody to TSST-1 using the methods described above. These samples were obtained from: Queens Medical
Centre, Nottingham, Stoke Mandeville NHS Trust, Southampton General Hospital, Russels Hall Hospital, Dudley, and from Booth Hall Children’s Hospital, Manchester.

3.2.8 Detection of TSST-1 in clinical isolates.

Ten culture supernatant fluids from clinically isolated *S. aureus* strains cultured in brain-heart infusion broth (Oxoid) were supplied by Dr. W. Al-Wali at the Sheffield PHL, Northern General Hospital, Herries Road, Sheffield S5 7BQ. These supernatant fluids were assayed for the presence of TSST-1 using the amplified ELISA system described in section 3.2.3. The supernatant fluids were diluted 1/5 in blocking buffer containing 10% normal rabbit serum and incubated at 37°C for 1h to adsorb any staphylococcal protein A from the sample. Then a further four 1/5 dilutions made using the same buffer. The diluted samples were applied (100μl/well) to 96 well assay plates in duplicate along with positive controls of purified TSST-1 at concentrations of 0.1, 1.0 and 10ng ml⁻¹.
Figure 3.1 Horseradish peroxidase amplification system.

Biotinyl-tyramine (BT) is added to the assay system. Immobilised HRP catalyses the activation of the phenolic group of the tyramine, resulting in the binding of the biotin to the solid phase. The bound biotin is then reacted with the HRP labelled streptavidin.
3.3 Results.

3.3.1 Conjugation of the monoclonal antibodies to horseradish peroxidase.

Each of the purified monoclonal antibodies were conjugated to horseradish peroxidase and purified by chromatography on a Sephadex G-200 column. A typical elution profile showing the purification of the conjugates on Sephadex G-200 is shown in Figure 3.2. The resulting purified conjugates were tested by ELISA to establish a working dilution. In all cases results showed that diluting the conjugate 1/200 - 1/300 in blocking buffer would yield adequate sensitivity and reproducibility of results in the unamplified ELISA, while in the amplified assay system the conjugate could be used at a dilution of 1/450 and still give reproducible results.

3.3.2 Monoclonal antibody based ELISA for detection of TSST-1.

The Mabs produced in this work were used in a series of initial experiments in which different pairs of non-competing antibodies were used as both capture and detection reagents. In all cases, the Mab-HRP conjugate was used at a dilution of 1/300. The limit of detection of TSST-1 in the standard ELISA assay was in the region of 1-50 ng ml\(^{-1}\) depending on which pairs of Mabs were used. To improve the detection limits of the assay to the picogram level, the enzyme signal was amplified using biotinylated tyramine and streptavidin peroxidase. Using the amplification system each pair of Mabs were further tested as both capture and detecting antibody (Table 3.1) values shown in the table represent the lowest toxin concentration to give a value of twice the mean of the blank values in the same assay. The combination of 4 TSST 5.3 as capture antibody and 4 TSST 37.3 HRP as the detection reagent proved to offer the most sensitive level of detection (60pg ml\(^{-1}\)). Figure 3.3 compares a non-
amplified and amplified ELISA showing differences in detection limits when using 4 TSST 5.3 as the capture antibody and 4 TSST 37.3-HRP conjugate as the detection reagent. After these initial observations, the amplified ELISA based on 4 TSST 5.3 and 4 TSST 37.3-HRP was studied further in an attempt to increase assay sensitivity by optimising the antibody and amplification reagent concentrations.

The capture antibody was tested at concentrations of 10, 5, 2.5 and 1.25 μg ml\(^{-1}\) (Fig 3.4) and the conjugate diluted 1/200, 1/300, 1/400, 1/500 (Fig 3.5). Furthermore biotinylated tyramine was tested (Fig 3.6) at dilutions of 0.03, 0.1, 0.2 and 0.3 μl ml\(^{-1}\) of buffer and the streptavidin-HRP reagent at dilutions of 1/1000, 1/1500, 1/2000 and 1/2500 (Fig 3.7).

As a result of this assay optimisation the best combination of reagents appeared to be: capture antibody 5 μg ml\(^{-1}\); conjugate 1/450 dilution; BT 0.1 μl ml\(^{-1}\), and shRP 1/1000. Using the reagents at these concentrations it was possible to detect as little as 10pg ml\(^{-1}\) in blocking buffer (Figs. 3.6 and 3.7).

3.3.3 Reproducibility of the assay.

3.3.3.1 Intra assay variations.

Test samples consisting of 6 different concentrations of TSST-1 were assayed using the amplified ELISA based on 4 TSST 5.3 and 4 TSST 37.3-HRP. The mean, standard deviation and %CV were calculated and are shown in Table 3.2. The results demonstrate that the assay is accurate and sensitive with good reproducibility as shown by the %CV values which are generally under 10%.
3.3.3.2 Inter assay variations.

The standard curve of toxin at concentrations of 10 - 0.0045ng ml$^{-1}$ was assayed on 15 different occasions using the method in section 3.2.4, and the results were analysed to determine the mean, standard deviation and %CV for each sample concentration. The results are shown in Table 3.3 and show high accuracy and low %CV values indicating good reproducibility.

3.3.4 Detection of antibody to TSST-1 in human serum.

These experiments were performed using Normal Human Serum (Sigma) and sera from colleagues at CAMR. An antibody titre of approximately 1/1350 was found to be present in the Sigma serum, and in most of the samples from CAMR staff. There were however some samples which showed titres as low as 1/50 and one sample from a staff member at CAMR had no detectable antibody to TSST-1 (data not shown). This latter serum was subsequently used to develop an assay for detection of toxin in the serum of hospital patients with acute phase TSS.

3.3.5 Detection of TSST-1 in human serum and urine.

The detection of toxin in normal dilution buffer was compared to detection in buffer containing either 30% serum (Miwa et al., 1994) or 30% urine. Using the optimised amplified assay system outlined in section 3.3.2 the recovery from serum which had no antibody to TSST-1 (Fig 3.8) and urine (Fig 3.9) was comparable to recovery of toxin from FCS/PBS-T buffer. It was noticeable that recovery of toxin from "normal human serum" was not possible, probably due to the presence of anti-TSST-1 antibodies (results not shown).
The amplified ELISA assay was applied to the testing of several clinical samples of serum and plasma for presence of antibody to TSST-1 and in some cases for the presence of the toxin itself. Three urine samples were also assayed for the presence of TSST-1. Results from these assays are shown in Tables 3.4 and 3.5.

3.3.6 Detection of toxin in culture supernatant fluids.

The detection of toxin in culture supernatant fluids from TSS clinical isolates using the amplified ELISA was compared to that obtained when the supernatant fluids were tested using the Oxoid RPLA kit (tests carried out according to the kit manufacturers instructions). The results shown in Table 3.6 indicate that the amplified ELISA was able to positively identify those samples in which low concentrations (<1 ng ml⁻¹) of toxin were present. Such samples were negative or would require retesting by RPLA.
Table 3.1 Sensitivity (ng ml⁻¹) of the Amplified ELISA detection of TSST-1 using a combination of monoclonal antibodies for capture and detection.

<table>
<thead>
<tr>
<th>Detecting antibody</th>
<th>2 TSST 109.3</th>
<th>2 TSST 112.3</th>
<th>4 TSST 5.3</th>
<th>4 TSST 10.3</th>
<th>4 TSST 23.3</th>
<th>4 TSST 37.3</th>
<th>4 TSST 110.3</th>
<th>4 TSST 116.3</th>
</tr>
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<tbody>
<tr>
<td>2 TSST 109.3</td>
<td>NT</td>
<td>10.0</td>
<td>0.4</td>
<td>0.6</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0.5</td>
</tr>
<tr>
<td>2 TSST 112.3</td>
<td>5.0</td>
<td>NT</td>
<td>0.4</td>
<td>0.3</td>
<td>3.0</td>
<td>NT</td>
<td>NT</td>
<td>0.3</td>
</tr>
<tr>
<td>4 TSST 5.3</td>
<td>3.0</td>
<td>1.0</td>
<td>NT</td>
<td>NT</td>
<td>1.0</td>
<td>0.13</td>
<td>0.3</td>
<td>NT</td>
</tr>
<tr>
<td>4 TSST 10.3</td>
<td>50.0</td>
<td>50.0</td>
<td>NT</td>
<td>NT</td>
<td>11.0</td>
<td>1.3</td>
<td>10.0</td>
<td>NT</td>
</tr>
<tr>
<td>4 TSST 23.3</td>
<td>NT</td>
<td>20.0</td>
<td>0.2</td>
<td>0.2</td>
<td>NT</td>
<td>1.3</td>
<td>10.0</td>
<td>0.4</td>
</tr>
<tr>
<td>4 TSST 37.3</td>
<td>NT</td>
<td>NT</td>
<td>0.06*</td>
<td>0.1</td>
<td>4.0</td>
<td>NT</td>
<td>NT</td>
<td>0.2</td>
</tr>
<tr>
<td>4 TSST 110.3</td>
<td>NT</td>
<td>NT</td>
<td>0.2</td>
<td>0.2</td>
<td>4.0</td>
<td>NT</td>
<td>NT</td>
<td>0.3</td>
</tr>
<tr>
<td>4 TSST 116.3</td>
<td>5.0</td>
<td>5.0</td>
<td>NT</td>
<td>NT</td>
<td>3.0</td>
<td>0.14</td>
<td>1.1</td>
<td>NT</td>
</tr>
</tbody>
</table>

NT - Antibody combinations determined as competitive and subsequently not tested in this assay.

*The combination of Mabs giving the greatest reproducible sensitivity was selected for further study.
Table 3.2 ELISA detection of TSST-1: Intra assay variations.

<table>
<thead>
<tr>
<th>Pool</th>
<th>Toxin standard (ng ml⁻¹)</th>
<th>Mean detectable toxin (ng ml⁻¹)</th>
<th>Standard deviation</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.00</td>
<td>7.80</td>
<td>0.55</td>
<td>7.0</td>
</tr>
<tr>
<td>2</td>
<td>4.00</td>
<td>4.10</td>
<td>0.29</td>
<td>7.0</td>
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<tr>
<td>3</td>
<td>2.00</td>
<td>2.15</td>
<td>0.14</td>
<td>6.5</td>
</tr>
<tr>
<td>4</td>
<td>1.00</td>
<td>1.10</td>
<td>0.10</td>
<td>8.9</td>
</tr>
<tr>
<td>5</td>
<td>0.50</td>
<td>0.56</td>
<td>0.06</td>
<td>10.7</td>
</tr>
<tr>
<td>6</td>
<td>0.25</td>
<td>0.25</td>
<td>0.02</td>
<td>7.2</td>
</tr>
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</table>

Values represent mean figures from 15 assays.

Table 3.3 ELISA detection of TSST-1: Inter assay variations.

<table>
<thead>
<tr>
<th>Pool</th>
<th>Toxin standard (ng ml⁻¹)</th>
<th>Mean detectable value (ng ml⁻¹)</th>
<th>Standard deviation</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.00</td>
<td>9.938</td>
<td>0.54</td>
<td>5.4</td>
</tr>
<tr>
<td>2</td>
<td>3.3333</td>
<td>3.333</td>
<td>0.16</td>
<td>4.7</td>
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<tr>
<td>3</td>
<td>1.1111</td>
<td>1.106</td>
<td>0.09</td>
<td>7.8</td>
</tr>
<tr>
<td>4</td>
<td>0.3700</td>
<td>0.367</td>
<td>0.02</td>
<td>5.1</td>
</tr>
<tr>
<td>5</td>
<td>0.1230</td>
<td>0.120</td>
<td>0.01</td>
<td>4.6</td>
</tr>
<tr>
<td>6</td>
<td>0.0410</td>
<td>0.041</td>
<td>0.00</td>
<td>8.1</td>
</tr>
<tr>
<td>7</td>
<td>0.0137</td>
<td>0.015</td>
<td>0.00</td>
<td>16.0</td>
</tr>
<tr>
<td>8</td>
<td>0.0045</td>
<td>0.004</td>
<td>0.00</td>
<td>9.0</td>
</tr>
</tbody>
</table>

Values represent mean figures from 15 assays.
Table 3.4. Test of urine samples from hospital patients with suspected TSS for presence of TSST-1.

<table>
<thead>
<tr>
<th>CAMR reference No.</th>
<th>Toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP 356</td>
<td>ND</td>
</tr>
<tr>
<td>DP 357</td>
<td>ND</td>
</tr>
<tr>
<td>DP 358</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND - below limit of detection of this assay (0.06ng ml⁻¹)
Table 3.5. Test of serum and plasma samples from hospital patients with suspected TSS for presence of antibody to TSST-1 and/or presence of toxin.

<table>
<thead>
<tr>
<th>CAMR reference No.</th>
<th>Antibody titre</th>
<th>Toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABL 1609</td>
<td>1/50</td>
<td>ND</td>
</tr>
<tr>
<td>DP 349</td>
<td>1/450</td>
<td>NT</td>
</tr>
<tr>
<td>DP 350</td>
<td>1/50</td>
<td>ND</td>
</tr>
<tr>
<td>DP 351</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>DP 352</td>
<td>1/4050</td>
<td>NT</td>
</tr>
<tr>
<td>DP 353</td>
<td>1/450</td>
<td>NT</td>
</tr>
<tr>
<td>DP 354</td>
<td>0</td>
<td>ND</td>
</tr>
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<td>DP 355</td>
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<tr>
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<td>1/4050</td>
<td>NT</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>DP 377</td>
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<td>ND</td>
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<tr>
<td>DP 379</td>
<td>1/450</td>
<td>NT</td>
</tr>
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</table>

ND - below limit of detection of this assay (0.06ng ml⁻¹)  
NT - not tested
Table 3.6. Detection of TSST-1 produced by clinical TSS isolates of *S. aureus*.

<table>
<thead>
<tr>
<th>*Sample number</th>
<th>RPLA result</th>
<th>ELISA result (ng ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>++</td>
<td>≥10</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>8.0</td>
</tr>
<tr>
<td>3</td>
<td>++</td>
<td>≥10</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>+/-</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>++</td>
<td>≥10</td>
</tr>
<tr>
<td>7</td>
<td>++</td>
<td>≥10</td>
</tr>
<tr>
<td>8</td>
<td>+/-</td>
<td>0.7</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>4.0</td>
</tr>
</tbody>
</table>

RPLA results are shown as ++; strong positive, +; positive, +/-; weak positive, or -; negative as defined in the kit instruction leaflet.

*Samples are culture supernatant fluids produced by growing the *S. aureus* isolates in brain-heart infusion broth.
Figure 3.2. Elution profile of conjugation of IgG to HRP. Purified monoclonal antibodies were conjugated to horseradish peroxidase and the conjugate purified on a Sephadex G-200 column. The protein and enzyme content of each fraction were measured at 280 and 405nm respectively. Fractions containing conjugate were pooled, aliquoted and stored at -20°C.
Figure 3.3. Detection of TSST-1 using the amplified and non-amplified ELISA systems. In both cases 4 TSST 5.3 was used as the capture antibody and 4 TSST 37.3 as the detection reagent. Values represent the mean of three replicates.
Figure 3.4. Optimisation of 4 TSST 5.3 capture antibody concentration in ELISA for TSST-1. Values represent the mean of four replicate assays.
Figure 3.5. Optimisation of 4 TSST 37.3 HRP conjugate dilution in ELISA for TSST-1. Values represent the mean of four replicate assays.
Figure 3.6. Optimisation of biotinylated tyramine dilution in the amplified ELISA for TSST-1. Values represent the mean of four replicate assays.
Figure 3.7. Optimisation of the streptavidin HRP conjugate dilution in the amplified ELISA for TSST-1. Values represent the mean of four replicate assays.
Figure 3.8. Detection of toxin in human serum. Values represent the mean of three replicates.
Figure 3.9. Detection of toxin in urine. Values represent the mean of three replicates.
3.4 Discussion.

From a panel of eight Mabs, two non-competing antibodies were chosen after an extensive selection process to develop an amplified ELISA which can reliably detect picogram levels of toxin. A two Mab ELISA for TSST-1 has been developed previously (Kuffner et al., 1988) but these workers appeared to apply this assay to the detection of toxin in culture supernatant fluids only and did not verify the assay using clinical samples. The detection limit of that assay was 60pg ml\(^{-1}\), which is almost certainly sensitive enough for the detection of toxin in clinical samples. The assay described here would have potential diagnostic value if it could be used to detect TSST-1 in the body fluids of hospital patients with suspected TSS. In order to investigate this possibility 3 urine and 29 serum and plasma samples from hospital patients were assayed for the presence of TSST-1. Despite a sensitive assay for the toxin no TSST-1 was detected in any of the clinical samples.

Urine is perhaps the most likely body fluid in which to find the toxin due to the cumulative effects of the kidneys (J. Parsonnet, personal communication). This is supported by previous observations during research into sudden infant death syndrome (SIDS) when i.v. injection of TSST-1 and staphylococcal enterotoxins into rats resulted in toxin concentration in the kidneys (Malam et al., 1992). A number of SIDS cases have also been shown to carry increased levels of toxigenic \textit{S. aureus} (Telford et al., 1989) and SEC and TSST-1 have also been detected in kidney tissues from SIDS cases (Tranter and Brehm, 1994), indicating a possible role for these toxins in the pathogenesis of this disease. Although in all cases the clinical symptoms suggested possible TSS and bacteriology samples revealed infection with staphylococci which were TSST-1 producers, toxin was not detected in any of the
plasma or urine samples. The reason for this may have been that the toxin was unavailable as it was bound to antibody which was present in most of the serum and plasma samples assayed. Such results are at variance from those of previous workers (Wells et al., 1987) who reported the detection of TSST-1 in the urine of patients with suspected TSS. The amount detected (7ng ml⁻¹) in their study was easily within the capabilities of the assay developed in this study.

That toxin was not detected in the serum samples was surprising, considering detection of toxin in human serum has previously been reported (Miwa et al., 1994) using a rabbit polyclonal capture antibody and a biotinylated mouse monoclonal antibody detection reagent. It is entirely possible however that a toxic molecule or bacterial product other than TSST-1 was responsible for the symptoms shown by the patient, for instance staphylococcal enterotoxin B or a streptococcal pyrogenic toxin which have both been implicated in TSS-like illnesses (Schlievert, 1986, Perez et al., 1997). In support of this some of the patients from whom samples originated were also later found to be infected with S.pyogenes. Another possible explanation may be that toxin is degraded too rapidly for it to be detected in an antibody-based assay if the samples are not processed immediately; prolonged storage or transport at ambient temperatures could allow toxin degradation to occur. The majority of samples tested in this study had been sent unrefrigerated through the normal post and so could have been at ambient temperatures for a considerable time. This would have had an adverse effect on chances of detecting toxin in these samples and if an assay was to be used in a clinical setting strict control of sample transport and storage would have to be maintained. Furthermore it is possible that the toxin was present in such low
quantities in the samples tested in this study that although it was able to cause clinical symptoms detection was not possible.

The amplified ELISA for TSST-1 developed during this work can however be used to detect TSST-1 in culture supernatant fluids from clinical isolates and results are comparable to, or better than those obtained with a commercial RPLA kit which has a sensitivity of 1 ng ml\(^{-1}\) of TSST-1. The ELISA is also a more accurate method for the quantification of toxin should this be necessary. The amplified ELISA is not only highly specific for TSST-1, but has low %CV values which indicates accuracy and reproducibility of results. This is an important consideration if the assay were to have a clinical application.
Chapter 4

*In vitro* Assay Development and Structure/Function Studies
Chapter 4.

**In vitro assay development and structure/function studies.**

4.1 Introduction.

A principle effect of bacterial superantigens (SAg) such as TSST-1 is the acute stimulation of T cells leading to the release of a broad range of cytokines, including interleukin-2 (IL-2; Kum *et al.*, 1993) tumour necrosis factor (TNF; Fast *et al.*, 1989), gamma interferon (IFN-γ; Jupin *et al.*, 1988), and IL-1 (Parsonnet *et al.*, 1985b) from the activated cells. These cytokines are possibly responsible for many of the toxic effects of the SAg (Schlievert, 1993). The SAg is able to achieve this situation by bypassing the normal antigen-specific T-cell activation mechanisms and stimulating a relatively large proportion (up to 20%) of the total T-cell population.

The SAg is able to bind to the MHC II on an antigen presenting cell (APC), outside the normal antigen binding groove without undergoing any of the proteolytic degradation which normally occurs during this process. The SAg then cross links the MHC II to the TCR on a T-cell via a conserved region outside the normal antigen binding cleft on the TCR, and activates the T-cell, initiating the cascade of cytokine release.

A variety of methods have been employed to study the structure/function relationships of active proteins. For example chemical fragmentation of TSST-1 using cyanogen bromide (Kokan-Moore and Bergdoll, 1989) and enzymic hydrolysis using papain (Edwin *et al.*, 1988; Edwin and Kass, 1989) were used to generate toxin fragments which could be purified and assayed for biological activity. Mutagenesis studies have also been carried out using either random chemical mutagenesis (Kum *et al.*, 1996) or point mutations (Murray *et al.*, 1995; Hurley *et al.*, 1995) to induce
changes in the DNA sequence which change the amino acid sequence of the protein. The effects of these changes to the biological activity of the toxin molecule can then be assessed and compared to the native protein.

Synthetic peptides representing particular regions of the toxin molecule can be made and used in biological assays to assess the importance of the regions they represent in the activity of the holotoxin (Soos et al., 1993). Another approach has been to generate monoclonal antibodies to the toxin, determine their binding sites on the toxin molecule and then assess their effect on the biological activity of the toxin (Shimonkevitz et al., 1996). Information from X-ray crystallographic studies (Acharya et al., 1994; Prasad et al., 1993) can also be invaluable in defining the structure/function relationships of a protein molecule.

The usual approach to combating toxic shock syndrome is to administer antibiotics to clear the bacterial infection and hypotonic fluids to counter the fluid loss characteristic of this illness. An alternative strategy could however include the administration of toxin specific antibodies during the acute stage of the illness. For an antibody to have any potential as a therapeutic agent in this disease scenario it must be able to prevent binding of toxin to either the TCR or the MHC II and therefore prevent cross-linking and stimulation of the T-cell. The work presented in this chapter was undertaken to establish whether the Mabs previously described were able to neutralise the mitogenic activity of the toxin and therefore limit production of cytokines. Another objective was to map the epitopes on the toxin molecule that the Mabs bound to. The high specificity of the Mabs suggested that this was feasible and a variety of techniques were employed during the mapping process.
Inhibition of the mitogenic response to TSST-1 by antibodies has been reported previously (Bonventre et al., 1988). The Mabs which successfully blocked the *in vitro* proliferation of cells and production of cytokines were also effective in attenuating the disease process in rabbits experimentally exposed to TSST-1 in bacterial culture supernatant fluids or bacterial cultures producing TSST-1 (Best et al., 1988). These workers however did not identify to which epitopes the Mabs were directed; such information could be very useful if antibody therapy for the disease condition became a practical option in the clinical situation.

Epitope mapping experiments involve the synthesis, on polyethylene pins, of protein molecules in a series of overlapping amino acid peptides. The peptides displayed on these pins are then reacted with antibodies which bind to epitopes that they recognise. Such information can be compared with data from X-ray crystallographic studies which may allow localisation of binding sites and give some indication of the possible effect antibodies may have on the biological activity of the protein.

Cleavage of the toxin by means of enzymic hydrolysis with papain can also be used to analyse the binding of the Mabs to the toxin. Papain has been shown to cleave TSST-1 into three fragments of 10, 12, and 16 KDa (Edwin et al., 1988) and these fragments have been sequenced. Separation of the fragments by SDS-PAGE and then performing Western blots using horseradish peroxidase conjugated antibodies may allow localisation of binding to a particular region of the toxin molecule and by reference to the crystal structure the significance of the binding site could be deduced.
4.2 Materials and Methods.

4.2.1 Papain cleavage of TSST-1.

This work was performed primarily according to the method of Edwin et al. (1988).

Solid phase papain, covalently bound to agarose beads (Sigma) was re-suspended in sterile ultra-pure distilled water at a concentration of 10 mg ml$^{-1}$, mixed gently for 10 min and then centrifuged (12000 rpm, 3 min) in a microfuge (MSE) to sediment the enzyme. The wash step was repeated and the enzyme re-suspended at a concentration of 2 mg ml$^{-1}$ in activation buffer consisting of freshly prepared 50mM Tris HCl, pH 6.8 containing 50mM L-cysteine HCl and 20mM EDTA. The solution was adjusted to pH 7.4 with 10M NaOH immediately before use.

TSST-1 (2mg ml$^{-1}$) was then mixed with the enzyme slurry such that the ratio of toxin to enzyme was 2:1. The enzyme / toxin mixture was then incubated on a shaking platform at 37°C for 4h. After this time the reaction mixture was centrifuged as detailed above to sediment the solid phase enzyme and the supernatant fluid was removed for analysis or storage at -20°C.

4.2.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE).

Samples of toxin and the toxin fragments generated by papain hydrolysis were prepared for electrophoresis by heating at 95°C for 5 min in 10mM Tris buffer, pH 8.0 (Sigma) containing 1mM EDTA (Sigma), 2% (w/v) SDS (Sigma), 10% (v/v) glycerol (Prolabo) and 0.05% (w/v) bromophenol blue (Pharmacia) as a marker dye.

Samples were electrophoresed in 15% polyacrylamide gels (BioRad) using a BioRad Mini Protean II discontinuous system as described by Laemmeli (1970).
Approximately 10µg of protein was loaded into each well. The electrophoresis buffer consisted of 192mM glycine (Fisons) and 25mM Tris (Sigma), pH8.3, containing 0.2% (w/v) SDS (Sigma).

Samples were run at 100V for 90 minutes before the gels were removed and stained in 40% methanol (Hayman Ltd.), 10% acetic acid (BDH) containing 0.1% (w/v) Coomassie blue R-250 (Pharmacia) for 2h. This was followed by destaining overnight in 40% methanol (Hayman Ltd.) containing 10% acetic acid (BDH). The relative molecular mass of the toxin fragments was estimated by comparison of their mobility with that of the protein standards (6.5-200 KDa, Boehringer Mannheim) and the toxin control.

4.2.3 Western blotting.

The method of Towbin et al. (1979) was followed, with some modifications. Following SDS-PAGE of the toxin fragments and whole toxin the staining step was omitted and the gel was sandwiched between filter papers and nitrocellulose membrane (Amersham Hybond), pre-soaked in transfer buffer consisting of 25mM Tris (Sigma) and 192mM glycine (Fisons); pH8.3 containing 20% (v/v) methanol (Hayman Ltd.). The gel sandwich was placed in the blotting apparatus with the nitrocellulose oriented towards the anode. Proteins were then transferred electrophoretically at 50V for 2h using the BioRad Mini Protean II transblot system. After transfer of the proteins the nitrocellulose membrane was incubated at room temperature overnight in blocking buffer (2% BSA w/v (Sigma) in PBS-T) to block any protein binding sites. This was followed by continuous shaking at room temperature with either rabbit polyclonal anti-TSST-1 conjugated to horseradish...
peroxidase (diluted 1/500 in PBS-T), or mouse monoclonal anti-TSST-1 HRP conjugate (diluted 1/1000 in PBS-T) for 1h.

Unreacted antibody was removed by three 10 minute washes in PBS-T, before detection of bound enzyme by the addition of the substrate solution consisting of 12.5mg TMB (Sigma) dissolved in 5ml methanol (Hayman Ltd), 10ml dioctyl sulphosuccinate (10mg ml⁻¹; Sigma), 0.5ml 1M HEPES pH 8.0, 34.5ml distilled H₂O and 12.5 μl 30% H₂O₂). After colour development (5-10 min) the membranes were washed in distilled water and photographed.

4.2.4 Epitope mapping.

4.2.4.1 Octapeptide synthesis on pins.

The TSST-1 molecule was synthesised on polyethylene pins as a series of octapeptides encompassing the entire amino acid sequence (Blomster-Hautamaa et al., 1986a) using a commercially available epitope mapping kit (Cambridge Research Biochemicals, Northwich, Cheshire) according to the methods developed by Geysen (Geysen, 1990; Geysen et al., 1984). Synthesis involved sequential ester coupling of F-moc (fluorenyl methoxy-carbonyl) protected L-amino acids with t-butyl side chain protection. The supplied pins incorporate a flexible protected β-alanine residue attached to the pin via hexamethylene diamine acrylic acid.

4.2.4.2 Deprotection of pins.

F-moc N-terminal protection was removed by mild base cleavage. For this the pins were gently shaken in a solution of 20% (v/v) piperidine (Sigma) in neat dimethyl formamide (DMF; Sigma) for 10 min, washed once in DMF (5 min) and
four times (2 min per wash) in HPLC grade methanol (Hayman Ltd). The pins were allowed to air dry for at least 10 min and washed again in DMF for 5 min before insertion into the activated amino acid solutions.

4.2.4.3 Coupling of amino acid residues.

Prior to coupling, the protected amino acids were activated by dissolving the freeze dried amino acid in neat DMF containing 30 mmols of catalytic 1-hydroxybenzotriazole to give a final amino acid concentration of 30mM. Aliquots (100µl) of amino acid residues were dispensed into the appropriate wells of polypropylene microtitre trays supplied with the kit. The deprotected pins were then inserted into the wells and incubated at 4°C overnight for the coupling reaction to occur. Post reaction the pins were washed once in neat DMF (2 min), four times in HPLC grade methanol (2 min per wash) and once in neat DMF (2 min) before the next round of deprotection.

Deprotection and coupling steps were repeated until peptides of the desired length had been generated. The terminal amino acid was deprotected before the N-terminal acetylation was performed.

4.2.4.4 N-terminal acetylation.

N-terminal acetylation of the completed peptides was carried out by gently shaking the pins in a mixture of DMF, acetic anhydride and diisopropylethylamine (50:5:1 v/v/v of the neat solutions) for 90 min. The pins were then washed once in neat DMF (2 min) and four times in HPLC grade methanol (2 min per wash) and then allowed to air dry.
4.2.4.5 Side chain deprotection.

Side chain deprotection was performed by washing the pins in a mixture of trifluoroacetic acid, phenol and ethane diol (95:2.5:2.5 v/w/v of neat solutions) for 4h followed by 2 washes (2 min each) in neat dichloromethane (DCM), two washes (5 min each) in DCM:diisopropylethylamine (95:5 v/v of neat solutions) and one wash in neat DCM (5 min). The pins were then allowed to air dry.

The pin bound peptides were washed in HPLC grade methanol for 18h and dried under vacuum. A duplicate series of octapeptides with seven residue overlap was synthesised on a separate set of pins. Both sets of pins contained sufficient overlapping octapeptides to encompass the entire amino acid sequence of TSST-1.

4.2.4.6 Octapeptide screening.

Peptides were screened using a variation of the manufacturers recommended ELISA procedure. Non-specific binding was minimised using 175μl of blocking buffer (1% ovalbumin, 1% bovine serum albumin, 0.1% Tween 20, 0.01% sodium azide in PBS) per well for 1h at room temperature. The pin bound peptides were then placed in 96 well reaction trays containing 150μl/well of the appropriate dilution of test antibody in blocking buffer (1/500 dilution of ascites) or neat hybridoma tissue culture supernatant fluid, sealed in plastic bags and incubated at 4°C overnight. The pins were washed four times (10 min per wash) in PBS-T, before transfer to reaction trays containing 150μl/well of Fab fragments of goat anti-mouse antibody conjugated to horseradish peroxidase (Amersham International) diluted in PBS-T. The pins were incubated at ambient temperature for 1h on a rotary shaker and then washed in PBS-T as before. The pins were then transferred to reaction trays containing 150μl/well of
substrate solution consisting of 50mg 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma) dissolved in 0.1M disodium hydrogen orthophosphate (BDH) and 0.1M citric acid (Prolabo), adjusted to pH 4.0. The trays were shaken at ambient temperature for up to 30 min to allow colour development. Finally the reaction was halted by removal of the pins and the absorbance was read at 405nm using a Dynatech MR7000 plate reader.

The resultant peptide-antibody complexes were disrupted for 30 min at an initial temperature of 65°C using a sonication bath (Pulsatron 125, Kerry Ultrasonics) containing approximately 500ml of disruption buffer (1% SDS and 0.1M sodium dihydrogen orthophosphate adjusted to pH 7.0 (BDH) to which 0.1% (v/v final concentration) 2-mercaptoethanol (BDH) was added). The pins were then washed twice for 1 min in hot distilled water (60-70°C) and then submerged in gently boiling methanol for 2 min. Finally the pins were allowed to air dry before re-use or storage at 4°C over silica gel.

The reproducibility of the peptide screening assay was assessed each time by the inclusion of two control peptides conjugated to pins provided by the kit manufacturer. One of these (a positive control) consisted of a pin of a pre-synthesized tetramer (PLAQ) which reacts with a mouse monoclonal antibody (provided by the manufacturer), the other (negative control) tetramer (GLAQ) does not react with this mouse monoclonal antibody. Both of these control tetramer peptides were synthesized de novo at the same time as the TSST-1 octamers to monitor the effectiveness of the peptide synthesis.
4.2.5 T-cell proliferation assay.

A murine spleen cell assay was developed so that the ability of the monoclonal antibodies to inhibit the mitogenic activity of the toxin could be assessed. For each assay a BALB/c mouse was killed by cervical dislocation and the spleen was removed and placed in a universal containing 10ml of warmed (37°C) medium (RPMI 1640 containing 10% FCS, 2mM L-glutamine, penicillin / streptomycin solution (200IU ml⁻¹/200µg ml⁻¹ final concentration), 5mM HEPES and 1mM sodium bicarbonate). The spleen cells were harvested by forcing the spleen through a cell strainer (Falcon) and then centrifuging (5 min at 1000rpm) in 15ml of warmed medium. The supernatant fluid was poured off and the cells resuspended in 1ml of red blood cell lysing solution (Sigma) and gently mixed for 1 min prior to the addition of 14ml of medium. The cells were harvested by centrifugation as before, the supernatant fluid was removed and the cells resuspended for counting in 10ml of medium. The cells were diluted to 8x10⁶ cells ml⁻¹ and 50µl of cell suspension added to each well of a 96 well tissue culture plate. Dilutions (1/3) of TSST-1 in culture medium were added to the wells (in triplicate), covering a range of final concentrations from 100ng to 0.005ng ml⁻¹. Normal culture medium was used as a negative control, and Concanavalin A (Sigma) added to other wells to act as a positive control. The plate was incubated at 37°C in 5% CO₂ for 72h, after which 10µl of Alamar blue™ (Serotec) was added to each well and incubation continued as before for 4h. The absorbance of the plate was then read at 570nm and 600nm on a Multiskan plate reader (Labsystems). The mean value for each of the triplicate samples was calculated and those at 600nm subtracted from the mean 570nm values. The resulting values were used to prepare a graph of mitogen concentration vs absorbance. This assay was
also used to assess the ability of the monoclonal antibodies to inhibit cell proliferation by adding 10μg ml⁻¹ (final concentration) monoclonal antibody to wells when the toxin was added and measuring the response of the cells to the toxin both in the presence and absence of antibody.

4.2.6. Interleukin 2 assays

The T-cell mitogenicity assay described above was also employed to study the effect of TSST-1 on interleukin 2 (IL-2) production by spleen cells by removing the culture supernatant fluids and assaying for the presence of IL-2.

For this 96 well ELISA plates were coated (100μl/well) overnight with a rat anti-mouse IL-2 monoclonal capture antibody (Pharmingen) at a concentration of 2μg ml⁻¹ diluted in 10mM sodium carbonate pH 8.0 and blocked with 10% FCS in PBS (200 μl/well) for 2h at room temperature. Culture supernatant fluids from mouse spleen cells stimulated by TSST-1 in the presence or absence of monoclonal antibody were then transferred directly to the ELISA plates, which were incubated at room temperature for 4h. After 4 washes with PBS-T a biotinylated rat anti-mouse IL-2 detection antibody (2μg ml⁻¹ ; Pharmingen) was added, diluted in PBS-T containing 10% FCS (100μl/well) and incubated at room temperature for 45 min. The plates were then washed 6 times and 1mg ml⁻¹ avidin-peroxidase (Sigma) previously diluted 1/400 in PBS containing 10% FCS was added, and the plates incubated for 30 min at room temperature. After 8 washes with PBS-T, the ABTS substrate solution (see Section 4.2.4.6) in 0.1M citric acid, pH 4.35 containing 10μl H₂O₂ per 10ml of substrate solution was added (100μl/well). The absorbance of the resulting reaction was determined at 405nm on a Dynatech MR7000 plate reader.
4.2.7 Human peripheral blood lymphocyte assay.

A sample of blood (up to 30ml) was taken from a volunteer and diluted 1/2 with RPMI 1640 medium (Gibco-BRL) containing penicillin/streptomycin (ICN; 200IU ml⁻¹ /200μg ml⁻¹ final concentration respectively), 10ml 5.5% sodium bicarbonate (ICN), 5ml 1M HEPES buffer, pH 7.5 (ICN) and 5ml 200mM L-glutamine (Sigma) per 500ml. The diluted blood (20ml aliquots) was layered onto 15ml Lymphoprep (Nycomed Ltd) and centrifuged at 400xg (RT 600B centrifuge; Dupont) for 30 min at 4°C.

The layer containing the lymphocytes was removed using a pipette into a fresh tube and the volume made up to 40 ml with RPMI 1640 as above, but also containing 10% FCS (Sigma). The tubes were then centrifuged again as before and the supernatant fluid discarded. Finally the pellet containing the lymphocytes was resuspended in 15ml medium (as above) for counting. The cells were diluted to \(1 \times 10^7\) cells per ml and 50μl of cell suspension added to each well of a 96 well culture plate. Dilutions (1/3) of TSST-1 in culture media were added to the wells (in quadruplicate), covering a range of final concentrations from 100ng to 0.005ng ml⁻¹. Normal culture medium was used as a negative control and Concanavalin A added to other wells to act as a positive control. The plate was incubated at 37°C in 5% CO₂ for 96h.

The proliferation induced by the toxin was measured using a Biotrak™ cell proliferation assay kit (Amersham), according to the manufacturers instructions.
4.3 Results.

4.3.1 Papain cleavage of TSST-1.

Proteolysis of purified TSST-1 using papain resulted in the production of three peptides of approximately 10, 12, and 16 KDa due to cleavage of the tyrosine 52-serine 53 and glycine 87-valine 88 peptide bonds (Edwin et al., 1988). These fragments were separated electrophoretically on SDS PAGE gels, as shown in Figure 4.1.

4.3.2 Western blot analysis of papain generated TSST-1 fragments.

The papain generated TSST-1 fragments were electrophoresed and then transferred to nitro-cellulose membranes which were probed subsequently with the anti-TSST-1 HRP conjugates in an attempt to localise the binding of the Mabs on the toxin molecule. All of the Mabs appeared to bind to the 12 and 16 KDa fragments. Figure 4.2 shows an example of a Western blot experiment comparing binding of monoclonal antibody 4 TSST 5.3-HRP and rabbit polyclonal anti TSST-HRP to the TSST-1 fragments. It can be seen that there is still a band of uncleaved toxin above the fragments and the rabbit polyclonal binds to all three toxin fragments, whereas the Mab identifies only the 12 and 16 KDa fragments. The 12 KDa fragment makes up 75% of the 16 KDa fragment, and these fragments are situated at the carboxyl end of the holotoxin. These results indicate that the Mabs all bind within the COOH terminal domain of the toxin where several antigenic determinants were also identified using rabbit polyclonal anti-serum in the epitope mapping experiments (see section 4.3.3). This region has also been implicated in the biological activity of the toxin (Edwin et al., 1988, Cullen et al., 1995).
4.3.3 Epitope mapping.

Initial epitope mapping experiments were carried out by reacting the epitopes displayed on the pins with rabbit polyclonal anti-TSST-1 serum in order to establish the antigenic profile of the toxin and to allow a direct comparison with any epitopes which may have been highlighted by the Mabs. The antigenic profile of the toxin as defined by the rabbit polyclonal anti-serum is shown in Fig 4.3. The polyclonal serum identifies three major antigenic sites, in addition to four minor epitopes. A positive reaction was deemed to be one where the absorbance value was at least twice the value of the background binding which was defined as an average of the lowest 25% of absorbance figures. Residues of the primary sequence corresponding to the antigenic sites are detailed in Table 4.1. Reaction of the TSST-1 pins with the Mabs failed to highlight any of the linear epitopes displayed on the pins, with no reactivity above background levels seen in any assay.

As a control the rabbit polyclonal antibody was re-tested after testing all of the Mabs to make sure that the pins were still working satisfactorily and that the procedure had been carried out correctly. The second assay (results not shown) with the rabbit antibody yielded results identical to those of the first test, proving the reproducibility of the assay technique and the integrity of the pins. The results again highlighted the 7 antigenically important epitopes. One epitope, which includes residues 137-148 overlaps a region of the molecule containing residues 132-144 which are known to affect mitogenic activity (Acharya et al., 1994, Blanco et al., 1990, Bonventre et al., 1993). Also identified by this antibody is an epitope between residues 172 and 191 which includes residues 170-180 predicted to be involved in MHC II binding (Acharya et al., 1994).
4.3.4 T cell proliferation assays.

The T cell proliferation assay was developed to show the mitogenic activity of the toxin. Once the protocol had been established Mabs were introduced to assess their ability to neutralise the mitogenicity of the toxin *in vitro*.

A comparison of the Mabs in an attempt to neutralise the activity of TSST-1 showed that not all of them were able to limit the mitogenic activity of the toxin (see Table 4.2). The ability to neutralise the toxin *in vitro* corresponded with the grouping of the Mabs in the competitive binding experiments (see section 2.3.5), in that one group neutralised the toxin while the other two groups did not. Although the Mabs were able to neutralise the toxin *in vitro* these results did not indicate whether the Mabs were binding to the epitope of the toxin which binds to the Vβ segment of the TCR or if they were binding to the MHC II binding region of the toxin. Polyclonal rabbit anti TSST-1 was also used in the proliferation assay to provide a direct comparison with the Mabs.

The effects of the Mabs on the mitogenicity of the toxin can be seen in Fig 4.4. Monoclonal antibody 4 TSST 5.3 has neutralising ability when used at a concentration of 10μg ml⁻¹, and cell proliferation is prevented while 4 TSST 37.3 does not affect the mitogenicity of the toxin when used at the same concentration.

4.3.5 Interleukin 2 assays.

The effects of the Mabs on production of IL-2 by T-cells proliferating in response to stimulation with TSST-1 was investigated as a follow up to the study on inhibition of mitogenicity. Results showed that all of the Mabs which inhibited mitogenicity also inhibited expression of IL-2, while those that did not inhibit cell
proliferation had little or no effect on cytokine production. This result concurs with the findings of other workers (Bonventre et al., 1988) who found that cytokine production was inhibited by neutralising antibodies. Figure 4.5 illustrates the production of IL-2 in the presence of TSST-1 and also when neutralising (4 TSST 5.3) and non-neutralising (4 TSST 37.3) Mabs were present.

4.3.6 Human Lymphocyte proliferation assays.

This assay was developed to demonstrate the mitogenic effect of the toxin on human peripheral blood lymphocytes and assess the ability of the Mabs to neutralise the effects of the toxin in vitro.

The results of this work were similar to the results of the mouse T-cell proliferation assays (Section 4.3.4) in that the Mabs ability to neutralise the toxin corresponded to their competition groups. Mabs 4 TSST 5.3, 10.3, and 116.3 were able to neutralise the toxin, while the other Mabs had no effect on the mitogenicity of the toxin. Figure 4.6 shows the proliferative effects of the toxin on the lymphocytes and the effects of neutralising (4 TSST 5.3) and non-neutralising (4 TSST 37.3) Mabs on mitogenicity.
Figure 4.1. Electrophoresis of papain generated TSST-1 fragments.

Papain generated TSST-1 fragments (see section 4.2.1) were applied to a 15% SDS-PAGE gel (Bio-Rad), lane 2, and untreated TSST-1 to lane 3. The samples were electrophoresed at 100V for 90min. Lane 1 shows molecular weight markers in the range 6.5 - 202kDa (Boehringer Mannheim).
Figure 4.2. Western blot analysis of papain generated TSST-1 fragments.

TSST-1 and papain generated toxin fragments were electrophoresed and transferred to nitrocellulose membranes. These were then probed with either rabbit polyclonal anti-TSST-1 HRP conjugate (lanes 2 and 3) or mouse monoclonal anti-TSST-1 HRP conjugate (lanes 5 and 6). The polyclonal antibody reacts with all three fragments as well as the holotoxin while the Mab (4 TSST 5.3) reacts with only the 16 and 12 kDa fragments and holotoxin. Pre-stained molecular weight markers (Bio-Rad) were used (range 202-6.9 kDa; lanes 1 and 4).
Table 4.1 Identification of antigenic sites during epitope mapping of TSST-1 octapeptides using rabbit polyclonal anti-toxin.

<table>
<thead>
<tr>
<th>Antigenic site*</th>
<th>Octapeptide numbers</th>
<th>Corresponding amino acid residues of primary sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4-9</td>
<td>4-16</td>
</tr>
<tr>
<td>2</td>
<td>40-52</td>
<td>40-59</td>
</tr>
<tr>
<td>3*</td>
<td>57-62</td>
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<td>7*</td>
<td>172-182</td>
<td>172-189</td>
</tr>
</tbody>
</table>

* An antigenic site was one which gave a significant absorbance reading above the background level which was defined as an average of the lowest 25% of absorbance figures.

* indicates major antigenic site as identified in Figure 4.3
Figure 4.3. Antigenic profile of TSST-1 identified by epitope mapping of primary sequence octapeptides using rabbit polyclonal antibody.
Table 4.2 Identification of neutralising monoclonal antibodies by *in vitro* inhibition of TSST-1 induced proliferation of mouse spleen cells *in vitro*.

<table>
<thead>
<tr>
<th>Neutralising antibodies</th>
<th>Non-neutralising antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 TSST 5.3</td>
<td>2 TSST 109.3</td>
</tr>
<tr>
<td>4 TSST 10.3</td>
<td>2 TSST 112.3</td>
</tr>
<tr>
<td>4 TSST 116.3</td>
<td>4 TSST 23.3</td>
</tr>
<tr>
<td></td>
<td>4 TSST 37.3</td>
</tr>
<tr>
<td>Rabbit polyclonal</td>
<td>4 TSST 110.3</td>
</tr>
</tbody>
</table>
Figure 4.4. The mitogenicity of TSST-1 in a mouse spleen cell assay, and neutralising effect of Mab 4 TSST 5.3, compared to the effect of Mab 4 TSST 37.3 (final antibody concentration 10µg ml$^{-1}$).
Figure 4.5. Production of IL-2 by TSST-1 stimulated mouse spleen cells, in the presence and absence of neutralising (4 TSST 5.3) and non-neutralising (4 TSST 37.3) monoclonal antibody (final antibody concentration 10μg ml⁻¹).
Figure 4.6. Neutralisation of TSST-1 in the human lymphocyte proliferation assay. Mab 4 TSST 5.3 neutralises the mitogenic effect of the toxin while 4 TSST 37.3 does not (final antibody concentration 10μg ml⁻¹).
4.4 Discussion.

Epitope mapping experiments failed to indicate the binding sites within TSST-1 of any of the Mabs. The reasons for this may be several fold but may include that the Mabs bind to conformational epitopes, or that the octapeptides did not produce long enough linear epitopes for recognition by the Mabs. The antigenic profile generated by the rabbit polyclonal antibodies was of great interest, as it highlighted several epitopes throughout the linear peptide sequence. These results complemented the findings of structure-function studies of other workers by highlighting epitopes in regions shown to be involved in the biological activity of the toxin. For instance residues 50 and 53 in the TSST-1 amino acid sequence have been shown to interact with the human major histocompatibility molecule DR1 (Kim et al., 1994) and these residues are highlighted in a region encompassing residues 40-59 in the antigenic profile. Similar interactions are seen between SEB and DR1, which are also known to interact (Kappler et al., 1992).

Early studies using cyanogen bromide generated fragments (Blomster-Hautamaa et al., 1986b) revealed that a fragment composed of amino acids 34-158 bound to antibodies that blocked the mitogenic and immunosuppressive functions of TSST-1. There are several epitopes within this peptide that were highlighted by the polyclonal antibody epitope mapping (peptide numbers 40-52, 57-62, 75-98, 133-145 and 151-168) and some of these epitopes are also included in the 12kDa COOH terminal region of the toxin molecule spanning residues 88-194 identified by Edwin et al. (1988 & 1989) to be important in the biological function of the toxin. The 12kDa papain generated fragment studied by Edwin et al. (1988) was found to be
serologically active by means of a competitive ELISA and capable of inducing a proliferative response in human peripheral blood lymphocytes.

Much work has also been done using site-directed mutagenesis to identify biologically important regions of the toxin. The work of Blanco et al. (1990) and Bonventre et al. (1993) has implicated residues between 115 and 144 in the toxin's mitogenic activity. Mutation of the histidine residue at position 135 abolishes mitogenic activity although it does not affect recognition of the mutant toxin molecule by a Mab that neutralises mitogenicity of the native toxin in vitro and in vivo (Bonventre et al., 1993). Substituting the histidine residue at position 141 with alanine also affects the mitogenicity of the toxin, although unlike residue 135, histidine 141 is buried in the structure of the toxin and mutation here probably results in conformational changes to the structure of the toxin (Acharya et al., 1994). Other studies have also shown the importance of the region including residue 135. Hurley et al. (1995) have shown that the TCR binding site consists of a region contained within residues 127-150, which is located on the central long α helix and residues 10-16 which are in the NH₂ terminal short α helix and loop structure. There is also possible involvement in TCR binding of residues in the loop formed by residues 97-116 and mutation of the tyrosine at position 115 has been shown to diminish binding of a neutralising antibody (Blanco et al., 1990). All of these areas are highlighted as important epitopes in the antigenic profile revealed by the rabbit polyclonal antibodies.

Involvement of residues 127-150 in TCR binding is also supported by the work of Cullen et al. (1995) who found that substitution of alanine for histidine at position 135 does not inhibit binding to MHC II bearing A20 cells. This mutation
does however affect interaction with the TCR and there is failure of the T-cell to produce IL-2, INF-γ, or TNF-β.

The MHC II binding region of the toxin is thought to lie in the NH₂ terminal domain, within residues 31-45 (Hurley et al., 1995), and mutation of the glycine residue at position 31 has been shown to abrogate binding to human monocytes (Kum et al., 1996). This re-enforces the findings of Soos et al. (1993) who used synthetic peptides of TSST-1 to block binding of ¹²⁵-labelled TSST-1 to MHC II displaying Raji and A20 cells. This work identified peptides 39-68 and 155-194 as being of important for MHC II binding. There is also X-ray crystallographic data which supports these results and demonstrates TSST-1 binding to the human MHC II molecule DR1 by interaction between residues 50 and 53 on TSST-1 and residue 39 of DR1 (Kim et al., 1994).

The Mabs studied in this project appear to bind to the 12 and 16 kDa papain generated fragments (residues 88-194 and 53-194 respectively) in the western blot experiments, which do not include the NH₂ terminal section of the toxin and as such would seem to indicate that they are not binding to epitopes involved in MHC II interactions. The results of the proliferation assays with both the mouse spleen cells and human peripheral blood lymphocytes show that not all the Mabs are effective in preventing proliferation. In fact only three of the eight Mabs (4 TSST 5.3, 10.3 and, 116.3) have any neutralising ability, and therefore they must bind to or very close to a biologically important epitope. This is most likely in the TCR binding region of the toxin molecule, and this is supported by the lack of IL-2 expression when these Mabs are used. IL-2 production is an early event in T-cell activation (Takei et al., 1993) and precedes proliferation. Taken as a whole the results of the Western blot analyses, T-
cell assays and the published information on the structure and function of the toxin indicate that these Mabs are most probably binding to the TCR binding region of the toxin which is contained within the COOH terminal region of the molecule (Hurley et al., 1995, Cullen et al., 1995). Whilst it cannot be ruled out entirely it appears that interaction of the toxin with the MHC II is probably not affected by these Mabs.

The use of Mabs as potential therapeutic agents would require that the Mab prevent toxin mediated cell proliferation and production of cytokines. This would minimise vascular leakage and fever induced by the over production of cytokines such as IL-1 and TNF. To this end optimum inhibition of toxicity may be achieved by an antibody which blocks binding to the TCR thereby preventing T-cell activation. An antibody which bound to the MHC II interaction site on the toxin may well "present" the toxin to the TCR itself, resulting in T-cell activation (Shimonkevitz et al., 1996). The results demonstrated here indicate that the Mabs which neutralise in vitro may be suitable for further study as potential therapeutic agents.
Chapter 5

General Discussion
Chapter 5.

General Discussion.

5.1 Toxic shock syndrome.

Toxic shock syndrome has been noted in many cases where staphylococcal or streptococcal infections are evident. These include menstrual TSS (Shands et al., 1980; Davis et al., 1980), infected burns (Cole and Shakespeare, 1990; Childs et al., 1994; Edwards-Jones et al., 1996) and some autoimmune diseases such as atopic dermatitis (Hofer et al., 1995). The toxin-mediated effects can include cytokine release, proliferation of immune cells, vascular leakage, fever, nausea and dizziness (Chesney et al., 1984). These symptoms have a rapid onset and are potentially life-threatening (Gaventa et al., 1989; Alouf et al., 1991). Survivors can often suffer recurrence of illness (Chesney et al., 1984) and in menstrually-associated TSS they do not appear to retain immunological memory of the toxin (Chesney, 1989; Arvand and Hahn, 1996).

Current therapy involves the use of antibiotics to clear the microbial infection (Resnick, 1990, Chesney, 1989) and administration of hypotonic fluids to counter the loss of fluid caused by the illness (Resnick, 1990). In severe cases IVIG may be administered (Barry et al., 1992; Lamothe et al., 1995; Perez et al., 1997) to clear pre-formed toxin from the bloodstream, but this treatment relies on the blood products used containing antibody to TSST-1 which is not always the case (Childs and Edwards-Jones, 1994). There is also the possibility that early administration of IVIG could blunt the immune response to this and other antigens (Chesney, 1989).

A specific, sensitive, rapid and reliable assay for the toxin in the blood or other body fluids would aid diagnosis and therapy in these clinical situations. This would,
in turn aid the choice of treatment and more effective measures could be taken as early as possible in the disease process which may enhance patient survival rates.

5.2 Production, Purification and Primary Characterisation of Monoclonal Antibodies.

Mabs produced in this study were all of the IgG\textsubscript{1} isotype, and primary characterisation revealed that they did not cross react with other bacterial toxins including the staphylococcal enterotoxins. Purification was carried out by Protein G affinity chromatography to provide homogenous antibody solutions as assessed by SDS PAGE gel analysis. Two Mabs were chosen for assay development on the basis of their high binding affinities for TSST-1 in the presence of the chaotrophic agent 3M urea, and they did not compete for binding to the toxin.

5.3 ELISA Development.

Conjugation of the Mabs to HRP by the method of Nakane and Kawaoi (1974) produced detection reagents for use in ELISA assay development. This work allowed selection of the two Mabs which gave the highest sensitivity in the assay system which was improved further by using biotinylated tyramine and streptavidin HRP (Bobrow \textit{et al.}, 1989, 1991; Helle \textit{et al.}, 1991) to amplify the reaction so that as little as 10pg ml\textsuperscript{-1} of toxin could be detected in buffer. The reproducibility of the assay was then tested using inter and intra assay variation methods (Beer, 1993). These tests showed the assay to have good reproducibility with very low %CV values which indicates that the accuracy of the results is high.
The amplified assay system was applied to the detection of toxin in serum and urine samples from healthy donors which had been spiked with purified toxin. These tests revealed that detection of toxin to the levels (0.5-0.01 ng ml\(^{-1}\)) reported by other workers (Wells et al., 1987; Miwa et al., 1994) was achievable and so the ELISA was applied to the detection of TSST-1 in clinical samples. Urine, serum and plasma were obtained from various cases of suspected TSS where toxigenic staphylococci had been isolated.

The assay developed as part of this study failed to detect toxin in any of the clinical samples available, despite successful detection in tests using spiked control serum and urine. When the samples were analysed for antibodies to TSST-1 they were detected in most of the serum and plasma samples tested which may indicate that TSST-1 was not a causal agent in the disease process in these cases. The lack of toxin detection could however be due to other factors, for instance the samples used in this study were all transported at ambient temperature which may have permitted proteolytic degradation of the toxin, making it undetectable, or it is possible that free toxin was not circulating at the time the samples were taken.

The procurement of suitable clinical samples has proved extremely difficult, but if more could be obtained the suitability of the assay system could be further investigated. The lack of sufficient data is currently preventing a full evaluation of the efficacy of this method of detection for this particular toxin. If successful the assay may have applications in other disease scenarios where TSST-1 may be involved such as atopic dermatitis (Hofer et al., 1995; Lester et al., 1995) autoimmune disease involving B-cell activation and apoptosis (Hofer et al., 1996), recalcitrant erythematous desquamating disorder (Cone et al., 1992; Dondorp et al., 1994) and
Kawasaki syndrome (Leung et al., 1993). Such an assay may also offer a possible veterinary application as TSST-1 and/or the staphylococcal enterotoxins have been implicated in pathogenesis of bovine mastitis (Yokomizo et al., 1995; Takeuchi et al., 1996).

5.4 Biological activity

The results of the western blot experiments revealed that the Mabs were binding within the carboxyl terminal region of the toxin which has been identified as important in the biological function of the toxin (Edwin et al., 1988; Cullen et al., 1995), and the epitope mapping studies carried out using rabbit polyclonal anti-TSST-1 revealed several antigenic determinants in this area.

The carboxyl terminal region of the toxin is thought to be involved in TCR binding (Cullen et al., 1995; Hurley et al., 1995) which is a requisite for the biological activity of the toxin (Marrack and Kappler, 1990; Saha et al., 1996). Cross-linking of the TCR with MHC II molecules displayed on APC's by TSST-1 leads to T-cell activation which plays an essential role in pathogenesis (von Bonin et al., 1995). That the neutralising (in vitro) Mabs described in this study are binding to the TCR binding region of the toxin could be a very important feature. It has been shown that TSST-1 binds to the Vβ2 region of the TCR (Shimonkevitz et al., 1996) and it can induce expression of Vβ2 by activated T-cells (Takahashi et al., 1995; Hu and Zhu, 1996). The use of anti- Vβ2 Mab does not necessarily interfere with TSST-1 binding to the TCR (Makida et al., 1996). This suggests that in a potential therapeutic application the use of a Mab, or synthetic peptide which bound to the TCR to prevent toxin binding and MHC II cross linking may not necessarily prevent cell activation and the
consequent release of cytokines characteristic of this disease (Goodglick and Braun, 1994; Saha et al., 1996). A Mab such as those described here which binds to the toxin and prevents it binding to the TCR could however be a useful therapeutic tool.

Neutralisation of the toxin *in vivo* will reduce the cytokine activation normally associated with TSS (Goodglick and Braun, 1994). It has been demonstrated that T-cells bearing selected Vβ2 elements can bind and respond to TSST-1 directly without the involvement of MHC II structures (Dennig et al., 1996) although T-cells responding to TSST-1 in the presence of APC's do produce much higher amounts of IL-2 (Takahashi et al., 1995; Dennig et al., 1996). In view of these facts a Mab which could bind to the toxin and prevent its interaction with the TCR could be very effective in limiting the severity of the illness.

Although binding of the Mabs to the toxin has been localised to a 12KDa segment at the COOH terminal end of the toxin the precise epitope to which the Mabs bind has not been identified. Indeed, there are several antigenic determinants within this region which were identified by epitope mapping experiments using polyclonal antibody. The lack of results from this procedure when carried out using the Mabs probably indicates that these antibodies bind to conformational rather than linear epitopes.

None of the Mabs produced during this study appear to bind to the toxin within the MHC II binding region. This is disappointing as binding to the MHC II or human HLA is important in the disease process and it has been shown that HLA-DR expression is increased in human TSS caused by TSST-1 (Arvand and Hahn, 1996). An antibody which was able to prevent binding to MHC II molecules may have proved very useful in studying the biological activity of the toxin. Eliminating binding
to accessory cells would allow the effect of the toxin alone to be assessed. Since it has been determined that even without APC activity T-cells can bind and respond to TSST-1 (Dennig et al., 1996) or that a Mab bound to the MHC II binding region could 'present' the toxin to the TCR (Shimonkevitz et al., 1996) the use of Mabs as a therapeutic tool may be best achieved by using a combination of 2 antibodies, one binding to the TCR binding epitope and one to the MHC II epitope. This would ensure effective neutralisation of the toxins biological activity. Using Mabs as therapeutic agents in a toxin-mediated disease scenario would give a direct approach to therapy whereas the use of IVIG is more empirical - it is by no means definite that antibody to the agent in question is present in the Ig (Childs and Edwards-Jones, 1994).

5.5 Conclusion.

Overall this project has successfully achieved some of its aims. The monoclonal antibodies have been characterised and a rapid very sensitive ELISA for TSST-1 has been developed. This assay can be used to detect small amounts of toxin in *S. aureus* culture supernatant fluids and early studies suggest the assay may be suitable for use with clinical samples from TSS patients after further evaluation.

The antibodies have also been used in *in vitro* assays using mouse spleen cells and human peripheral blood lymphocytes to assess their ability to neutralise the mitogenic activity of the toxin. Three of the Mabs have been found to neutralise the toxin in these assays.

The binding of the Mabs to the toxin has been localised to a 12KDa fragment encompassing the COOH domain of the molecule, which is believed to be involved in
TCR binding. The ability of the Mabs to neutralise the toxin \textit{in vivo} has yet to be established.
5.5 Future work.

A primary objective of any future work should be to test more clinical samples in order that the suitability of the assay as a clinical tool can be fully assessed. Serum, plasma, urine or burns fluids could all be tested in this assay and results compared with clinical findings and other assay methods.

The second objective should be to test the effect of the Mabs on the toxin and its function in an in vivo study to establish whether the mitogenic effect of the toxin could be abrogated. There have been several attempts to establish an animal model of TSS, including the use of mice and primates, but rabbits are the only species to show TSS-like symptoms (de Azevedo, 1989). Most studies have used either sub-cutaneous whiffle balls (Scott et al., 1983) or mini osmotic pumps (Bonventre et al., 1988), although an intra-vaginal tampon model in rabbits has been developed (Melish et al., 1989). Whichever infection model is used TSST-1 producing strains and purified toxin induce death in rabbits (Scott et al., 1983; Bonventre et al., 1988) and symptoms observed in the rabbits are similar to those seen in humans. These include; fever, conjunctival hyperaemia, respiratory distress and shock, and blood chemistry changes such as increased creatinine and urea (de Azevedo, 1989). Post mortem changes parallel those seen in humans, e.g. congestion and oedema of various tissues, erythrophagocytosis in lymphoid tissue and liver triaitis (de Azevedo, 1989).

The use of purified TSST-1 in rabbits using the mini osmotic pump method (Bonventre et al., 1988) allows the toxic effects of TSST-1 to be evaluated without interference from other bacterial products, and delivery of small amounts of toxin over a period of time, such as might occur in infection. The pump method also allows a set amount of toxin to be delivered reproducibly.
As an alternative to the methods above, micro-encapsulated toxin could be used to induce symptoms of TSS. The toxin can be encapsulated in micro-particles of poly(lactide-co-glycolide) polymer (PLG; Jones et al., 1995 and 1996) which when injected sub-cutaneously into the animal undergoes non-enzymic hydrolysis to give lactic acid and glycolic acid and release the toxin. This method has all the advantages of the pump method, delivering a known quantity of toxin without interference from other bacterial products and the added advantage that surgical implantation is not required, which reduces stress on the animals.
References.


Appendix.

Single letter amino acid abbreviations.

- Alanine: A
- Valine: V
- Leucine: L
- Isoleucine: I
- Proline: P
- Phenylalanine: F
- Tryptophan: W
- Methionine: M
- Cysteine: C
- Glycine: G
- Asparagine: N
- Glutamine: Q
- Serine: S
- Threonine: T
- Tyrosine: Y
- Lysine: K
- Arginine: R
- Histidine: H
- Aspartate: D
- Glutamate: E