Identification of Cross-Neutralizing Epitopes on Toxic Shock Syndrome Toxin-1 and Staphylococcal Enterotoxin B

by

LIWINA TIN YAN PANG

B.Sc., The University of British Columbia, 1995

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Experimental Medicine)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April 1999

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Experimental Medicine

The University of British Columbia
Vancouver, Canada

Date 27 April, 99.

Abstract

Toxic Shock Syndrome (TSS) is primarily caused by Toxic Shock Syndrome Toxin-1 (TSST-1), Staphylococcal Enterotoxin A (SEA), Staphylococcal Enterotoxin B (SEB), and Staphylococcal Enterotoxin C (SEC). These toxins belong to a family of pyrogenic toxin superantigens (PTAgs) produced by *Staphylococcus aureus*. These PTAgs share similar immunobiological properties, including the induction of massive release of cytokines and stimulation of T cell proliferation in a Vβ-specific manner. The crystal structures of most PTAgs are now known. They share a similar basic structure even though their primary sequences are different. Despite the structural and immunobiological similarities, no cross-reactivity between TSST-1 and other PTAgs has been demonstrated in serological assays and neutralization assays. Recently, our laboratory has developed a neutralizing murine anti-TSST-1 monoclonal antibody (MAb5) which demonstrated significant cross-reactivity with SEB in ELISA. Since previous studies have shown that MAb5 can neutralize TSST-1-induced superantigenic and lethal activities both *in vitro* and *in vivo*, the present study was undertaken to evaluate possible cross-neutralizing epitopes on TSST-1 and SEB using MAb5. Our experimental approach in addressing this question included: (1) assessment of the ability of MAb5 to cross-neutralize SEB-induced superantigenic activities *in vitro* and *in vivo*; (2) determination of the location of the cross-reactive epitopes on TSST-1 and SEB by epitope peptide mapping; (3) evaluation of the importance of the cross-reactive epitope as a functional domain by the use of synthetic peptides.

MAb5 was found to partially inhibit SEB-induced T cell mitogenesis (63%) and TNFα secretion (70%) in human blood mononuclear cells (HPBMC) in a dose dependent

manner, while a control anti-TSST-1 monoclonal antibody, MAb6, had no effect. In order to locate the cross-reactive epitopes on TSST-1 and SEB, binding of MAb5 to a set of overlapping decapeptides homologous to TSST-1 (1-194) and SEB (1-239) were determined. MAb5 recognized a linear sequence of 5 amino acids (^{52} YSPAF ^{56}) on TSST-1, as well as a linear sequence of 4 amino acids (^{85}FGAN^{88}) on SEB. These epitopes are structurally similar but are topologically located in different areas of the respective toxins. Furthermore, a 10 mer peptide corresponding to SEB (83-92), ^{83} DVFGANYYYQ ^{92} , was found to inhibit SEB-induced T cell mitogenesis (90%) and TNFα secretion (90%) from HPBMC *in vitro*, while an irrelevant, scrambled peptide had no effect. These data suggest that MAb5 recognizes structurally similar motifs of SEB and TSST-1, and contains neutralizing activity against these related PTAgs.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

APC	Antigen Presenting Cell
BSA	Bovine serum albumin
BHI	Brain Heart Infusion Broth
°C	Degree celsius
EDTA	Ethylene diaminetetra acetic acid
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Fetal bovine serum
h	Hour
^{125}I	Iodine-125
Ig	Immunoglobulin
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-7	Interleukin-7
IFN-γ	Interferon-gamma
kb	kilobase
kd	kilodalton
K_d	Dissociation constant
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MAM	M. arthriditis mitogen
min	Minute
MHCII	Major Histocompatibility Complex Class II Antigen
MLS	Minor Lymphocytes Stimulating
ng/ml	nanogram per millitre
nM	nanomolar
O.D.	Optical density
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pg/ml	picogram per millitre
pI	Isoelectric point
pM	picomolar
PTSAgs	pyrogenic toxin superantigens
r.m.s.	root mean square
SAgs	superantigens
SE	Staphylococcal enterotoxin
SEA	Staphylococcal enterotoxin A
SEB	Staphylococcal enterotoxin B
SEC	Staphylococcal enterotoxin C
SED	Staphylococcal enterotoxin D
SEE	Staphylococcal enterotoxin E
SEF	Staphylococcal enterotoxin F

SEH	Staphylococcal enterotoxin H
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
S. aureus	Staphylococcus aureus
S. pyrogenes	Streptococcus pyrogenes
SPEA	Streptococcal pyrogenic A
SPEB	Streptococcal pyrogenic B
SPEC	Streptococcal pyrogenic C
TCR	T cell receptor
TNF	Tumor necrosis factor
TSS	Toxic shock syndrome
TSST-1	Toxic shock syndrome Toxin-1
V	Volts
Vβ	Beta chain of variable region of T cell antigen receptor

Acknowledgement

I would like to thank Dr. Anthony Chow for his encouragement, advice and guidance throughout my studies. I would also like to acknowledge Dr. Winnie Kum, who aided me considerably over the course of research, especially in the *in vitro* assays, toxin purification, and animal model studies. I gratefully thank Anita Kwok for her advice in our lengthy discussions and her continual support and encouragement. I would like to express my gratitude to Dr. Natalie Strynadka and Mr. Steven Ness at the Department of Biochemistry for their knowledgeable contributions in the epitope mapping studies. Their time and effort is much appreciated. Special thanks are extended to Dr. Hermann Ziltener and Dr. Lawrence Mcintosh for serving as members of my advisory committee; and Ryan Hung for helping me with the computer programs.

Last but not least, I would also like to thank my good friend, Soo Sen Lee, who shared with me the joy and tears during my studies; my fiancée, Alex Chan, who put up with my frustrations during those difficult times; my sister, Loretta Pang, who gave me her full support by being my blood donor; and my parents, who encouraged me throughout the entire studies. Without their continual support and encouragement, this thesis would never be accomplished.

Chapter 1

Literature Review

1.1. Introduction

A. Historical Overview

The term "superantigen" was first introduced by White et al. in 1989 to describe a group of bacterial toxins which induced massive proliferation of T cells in a Vβ specific manner (White et al., 1989). This was quickly followed by the insight that minor lymphocytes stimulating (MLS) antigens, a class of murine endogenous antigens which were known since the early 1970s, had similar functional properties (Kappler et al., 1988 and MacDonald et al., 1988). MLS antigens were discovered by Festenstein in 1973 because of their enormous T cell stimulatory capacity in mixed lymphocyte reactions with cells from major histocompatibility complex (MHC)-identical strains of mice (Festenstein, 1973). Since the genes encoding MLS antigens were mapped outside the MHC loci, they were named "minor" lymphocyte stimulating antigens to differentiate from MHC antigens. However, the in vivo consequences of expression were not realized until 1988, when two groups of investigators reported the absence of Vβ8.1 and Vβ6 bearing T cell subsets from the peripheral lymphoid tissues in mice expressing MLS antigens (Kappler et al., 1988; MacDonald et al., 1988). Clearly, the MLS antigens were recognized by the T cell receptor (TCR), and the major determinant of MLS reactivity appeared to be the expression of a particular Vβ gene segment. This property distinguished MLS antigens from conventional peptide antigens, in which their recognition by T cells require all the variable elements of TCR (i.e. Vα, Jα, Vβ, Dβ, and Jβ regions). Furthermore, MLS antigens were recognized as self-antigens since MLS reactive T cell subsets are deleted from the repertoire during thymic maturation as part of the self-tolerance process (Kappler et al., 1988).

Staphylococcal enterotoxins (SEs), toxic shock syndrome toxin-1 (TSST-1) and streptococcal pyrogenic exotoxins (SPEs), which were known for years as the major cause of food poisoning and toxic shock syndrome in human, were found to have immunobiological properties with striking similarities to MLS antigens. In fact, as early as 1970, Peavy et al. have reported the ability of staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin B (SEB) to induce T cell mitogenesis of mouse spleen cells and human peripheral lymphocytes (Peavy et al., 1970). The significance of these T cell responses and the superantigenic nature of these toxins were not, however, appreciated immediately. This breakthrough in understanding was dependent on the development of monoclonal antibodies (MAbs) that are specific for T cells expressing a particular Vβ domain. With such MAbs, these toxins were found to stimulate massive proliferation of T cells expressing a particular set of Vβ gene segment and injection of SEs into neonatal mice produced particular T cell Vβ subset deletion as in MLS (Janeway, 1989 and White et al., 1989). As a result, the connection between MLS antigens and these bacterial toxins was established and they were all designated as "superantigens".

B. Hallmark of Superantigens

Superantigens differ from conventional antigens in several ways: (1) Superantigens do not require processing by antigen presenting cells (APCs). In contrast, conventional antigens are internalized and processed by APCs before being loaded into the peptide binding groove formed by the α- and β- chains of class II MHC molecule. (Dellabona et al., 1990; Fleischer and Schrezenmeir, 1988; Fleischer, 1991) (Fig. 1); (2) Superantigens bind directly to relatively invariant regions on MHC class II molecules outside the conventional peptide-binding groove, and thus presentation to T cells is not class II

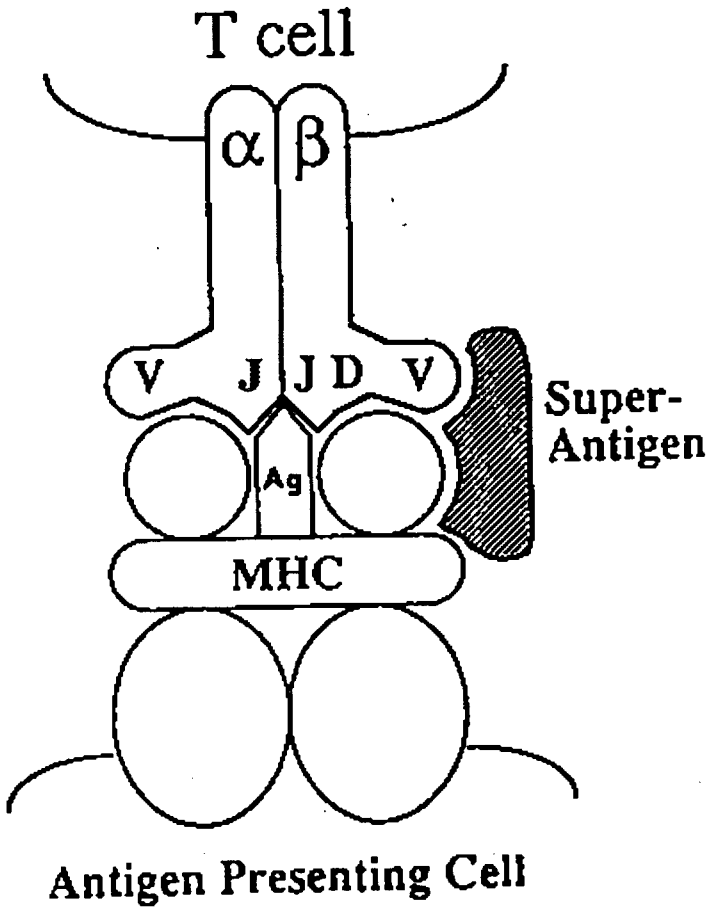


Figure 1. Hypothetical structure for the complex of MHC class II molecule, TCR, and superantigens as proposed by Marrack and Kappler, 1990. Figure taken from Herman et al., 1991.

restricted, violating the rule of MHC restriction seen in all other peptide-antigen-specific T cell responses (Dellabona et al., 1990; Kotzin et al., 1993); (3) The recognition of superantigen/MHC class II complex by TCR depends entirely on the variable domain of the TCRβ chain (Vβ), while a conventional peptide antigen requires a very specific interaction with the third hypervariable region of the TCR (Marrack and Kappler, 1990). This latter region of the TCR consists of the joining elements of both VJα and VDJβ; thus, the responding frequency to a conventional antigen is usually about 1 in 10,000. On the other hand, since the relative number of Vβ genes is limited, a given superantigen is capable of interacting with as many as 5 to 30% of the entire T cell repertoire (Drake and Kotzin, 1992).

C. Classes of Superantigens

I. Virally Encoded Superantigens

Superantigens are a diverse collection of molecules that share the ability to activate specific T cell subsets. Two groups of superantigens have been described, namely viral and bacterial superantigens. At the beginning of the 1990s, the exciting discovery was made that murine MLS antigens are encoded by the murine mammary tumor virus (MMTV) proviral DNA that had been integrated into the germline (Frankel et al., 1991 and Dyson et al., 1991). Expression of endogenous superantigens during T-cell ontogeny in mice causes physical and functional deletion of T cells bearing Vβ elements specific for those antigens (MacDonald et al., 1988; Woodland et al., 1991; Pullen et al., 1988). However, to date, an analogous system has not been found in humans. While only the MMTV-encoded viral superantigen has been well defined, there are reports of association of superantigen activity with other viruses. The nucleocapsid of rabies virus has been shown to be a superantigen

in humans and also in mice (Lafon et al., 1992 and Lafon et al., 1994). In addition, superantigenic activity has been attributed to two human tumor viruses, cytomegalovirus (CMV) and Epstein-Barr virus (EBV) (Dobrescu et al., 1995; Sutkowski et al., 1996). Furthermore, a number of different groups have proposed that human immunodeficiency virus (HIV), the cause of human acquired immunodeficiency syndrome (AIDS), encodes a superantigen that may be involved in host $CD4^+$ T cell depletion (Dadaglio et al., 1994; Akolkar et al., 1995).

II. Bacterial Superantigens

A large number of bacterial toxins made up the growing list of bacterial superantigens. Bacterial superantigens can be subdivided into pyrogenic toxin superantigens (PTSAgs) and those that are not pyrogenic toxins. The PTSAgs, including TSST-1, SEs, and group A SPEs, are probably the most well studied superantigens. PTSAgs are distinguished from other bacterial superantigens due to their shared three-dimensional structure and immunobiological properties, which will be discussed in detail later.

Superantigens that do not belong to the PTSAgs family include staphylococcal exofoliative toxins (ExFT), Mycoplasma arthritidis mitogen (MAM), Yersinia enterocolitica superantigen (YES), Yersinia pseudotuberculosis mitogen (YPM), and streptococcal M protein (Schlievert, 1997; Stuart and Woodland, 1992; Abe et al., 1993; Tomai et al., 1990).

1.2. Pyrogenic Toxin Superantigens (PTSAgs)

PTSAgs form a subfamily within the larger superantigen family that includes a large group of simple, nonglycosylated proteins, secreted by *Staphylococcus aureus*, group A streptococci, and certain non-group A streptococci. Well characterized members of this family include TSST-1 and SEs serotypes A, B, Cn, D, E, G, and H produced by *S. aureus*; SPEs serotypes A, B, C, and F (mitogenic factor), and streptococcal superantigen (SSA) produced by group A streptococci (Bohach et al., 1990; Schlievert et al., 1995). They are grouped under the same subfamily based upon their shared biochemical, immunobiological, and three-dimensional structural properties, as well as their involvement in related illnesses such as Toxic shock syndrome (TSS) and other immune-mediated diseases (Kotzin et al., 1993).

Biochemically, these toxins are small polypeptides with a neutral to basic isoelectric point (Blomster-Hautamaa et al., 1986; Bergdoll, 1993; Schlievert et al., 1990). They are generally resistant to acid, heat, and protease (Bohach et al., 1990). The biochemical properties of the PTSAgs are summarized in table I. In addition to sharing biochemical properties, the PTSAgs share many immunobiological activities, such as the ability to induce fever, to enhance host susceptibility to endotoxin shock, and to stimulate T cell proliferation via a Vβ specific manner (Bohach et al., 1990). While all PTSAgs possess these common immunobiological properties, each subclass of toxins exhibits unique characteristics as well. The shared and unique biological activities of PTSAgs are summarized in Table II.

Table I.

Biochemical properties of Toxic Shock Syndrome Toxin-1, Staphylococcal Enterotoxins, and Streptococcal Pyrogenic Toxins.

Toxin	Isoelectric Point	Number of Amino Acids (mature protein)	Molecular Weight (kDa)
TSST-1	7.2	194	22 049
SEA	7.3	233	27 100
SEB	8.5	239	28 336
SEC1	8.5	239	27 531
SEC2	7.0	239	27 589
SEC3	8.0	239	27 563
SED	7.4	228	26 360
SEE	7.0	230	26 425
SEH	5.65	217	25 210
SPEA	5.0-5.5	221	25 787
SPEB	8.0-9.0	253	27 588
SPEC	6.7-7.0	208	24 354

Derived from Bergdoll,1983; Schlievert et al., 1990; Hauser and Schlievert, 1990, and Ren et al., 1994.

Table II.

Biological Properties of Pyrogen Toxin Superantigens

Property	Subfamilies of PTSAgs		
	TSST-1	Staphylococcal Enterotoxin	Streptococcal Pyrogenic Exotoxins
Pyrogenicity	+	+	+
Enhancement of endotoxin Shock	+	+	+
Superantigenicity	+	+	+
Interference with liver clearance function	+	+	+
Emesis	-	+	-
Cardiotoxicity	+	-	-
Ability to bind endothelial cells	+	?	+

Source: Bohach et al., 1990; Leonard and Schlievert, 1992; Schwab et al., 1995; Lee et al.,1991.

A. Shared Immunobiological Properties

I. Pyrogenicity

The term "pyrogenic toxin superantigens" is used for this class of superantigens because they are among the most potent pyrogens known. They are capable of inducing fever when injected into experimental animals (Kim and Watson, 1970; Schlievert et al., 1982). It is believed that PTSAgs cause fever through inducing the release of human endogenous pyrogens, interleukin-1 (IL-1) and tumor necrosis factor (TNF), from macrophages, and by having direct effects on the hypothalamus fever response control center (Fast et al., 1989; Bohach et al., 1990; Schlievert et al., 1995). PTSAgs' distinctive fever response can easily be distinguished from other pyrogens based upon the time of maximal response. PTSAgs-induced fever is characterized by a fairly linear rise of body temperature with a peak at 4 hour after injection, in contrast to the biphasic fever response to endotoxins, another well known pyrogen, with peaks at 1 and 3 hour post-injection (Bohach et al., 1990).

II. Enhancement of Lethal Endotoxin Shock

Another important biological property shared by all of the PTSAgs is the ability to enhance host susceptibility to the lethal effect of gram-negative bacterial endotoxin by up to 100,000-fold (Bohach et al., 1990). The precise mechanism underlying the enhancement phenomenon remains to be eludicated but appears to involve PTSAgs interference with liver clearance function (Schlievert et al., 1995). This is supported by the observation of fatty acid replacement of the liver on autopsy in staphylococcal TSS patients (Larkin et al., 1982; Stone and Schlievert, 1987). Moreover, SPEs have been

previously shown to inhibit RNA synthesis in isolated liver cells and their nuclei in SPEs-treated rabbits (Schlievert et al., 1979). However, whether PTSAgs blockage of RNA synthesis is the direct effect amplifying susceptibility to endotoxins is unclear.

III. Superantigenicity

The most intensively studied biological property of the PTSAgs at present is their capacity to induce dramatic T cell proliferation, which is referred to as superantigenicity. PTSAgs are able to stimulate both $CD4^+$ and $CD8^+$ T cells regardless of their antigenic specificity (Kotzin et al., 1993). Interestingly, different PTSAgs have different specificities for the variable regions of the Vβ region of the TCR (Table III). For example, TSST-1 is capable of preferentially stimulating the proliferation of human T-cell subset bearing Vβ2, which accounts for approximately 10% of all T cells (Choi et al., 1990). In an acute TSS patient, as many as 60-70% of all circulating T cells may display T cell receptors containing Vβ2 segment (Choi et al., 1990). The non-antigen-specific T cell stimulation elicit massive release of cytokines, including interleukin (IL)-1α, -2, -4, -6, -10, interferon (IFN)-γ, tumor necrosis factor (TNF)-α and β, and subsequently lead to capillary leak and hypotension in the host (Miethke et al., 1993). It is postulated that the uncontrolled release of cytokines underly the pathogenesis of TSS (Kain et al., 1993).

B. Unique Properties

I. Toxic shock syndrome toxin-1 (TSST-1)

TSST-1 was first identified by Schlievert et al. as staphylococcal pyrogenic exotoxin C (Schlievert et al., 1981) and by Bergdoll and his coworkers as enterotoxin F

Table III.

Vβ Specificities of Staphylococcal Pyrogenic Toxin Superantigens.

Toxin	Vβ Specificity	
	Human	Mouse
SEA	1.1, 5.3, 6.3, 6.4, 6.9, 7.3 7.4, 9.1, 18	1, 3, 10, 11, 12, 17
SEB	3, 12, 14, 15, 17, 20	3, 7, 8.1, 8.2, 8.3,17
SEC1	3, 12	3, 8.2, 8.3, 11, 17
SEC2	12, 13.1, 13.2, 14, 15, 17,20	3, 8.2, 10, 17
SEC3	5, 12	3, 7, 8.1, 8.2
SED	5, 12	3, 7, 8.2, 8.3, 11, 17
SEE	5.1, 6.1, 6.2, 6.3, 8, 18	11, 15, 17
TSST-1	2	3, 4, 15, 17

Source: Marrack and Kappler, 1990; Herman et al., 1991, and Uchiyama et al., 1994.

(Bergdoll et al., 1981) independently in 1981 as the causative agent of staphylococcal TSS. When staphylococcal pyrogenic exotoxin C and enterotoxin F were later shown to be identical, the term "toxic shock syndrome toxin-1" (TSST-1) was adopted to describe the toxin (Bergdoll and Schlievert, 1984). TSST-1 is produced by nearly 100% of the *S. aureus* isolated from vaginal or cervical cultures in cases of menstruation-associated TSS, and approximately 50% of *S. aureus* isolates obtained from other body sites in nonmenstrual TSS cases (Bergdoll and Schlievert, 1984; Schlievert, 1986).

The toxin is encoded by the gene *tst* present in the chromosome as a large heterologous insert (Blomster-Hautamaa et al., 1986). *tst* occurs in two major forms, *tst*H, where H refers to human isolate of *S. aureus* and the gene encodes TSST-1, and *tst*O where O refers to ovine isolate of *S. aureus* and the gene encodes TSST-O (Blomster-Hautamaa et al., 1986; Lee et al., 1992). TSST-1 and TSST-O are translated as proteins consisting of 234 amino acids of which the first 40 residues comprise signal peptides (Blomster-Hautamaa et al., 1986; Lee et al., 1992). The mature secreted toxins are single polypeptide chains with molecular weights of approximately 22,000 and isoelectric points of 7.2 and 8.5 for TSST-1 and TSST-O, respectively (Blomster-Hautamaa et al., 1986; Schlievert et al., 1995). These two proteins differ from each other by seven amino acids at position 19, 55, 57, 69, 80, 132, and 140 (Lee et al., 1992). The consequence of these differences is that TSST-1 is biologically active whereas TSST-O lacks significant biological activity (Lee et al., 1992). These toxins have no cysteines and thus no internal disulfide loop, contain a high percentage of hydrophobic amino acids, and is generally resistant to heat and proteolysis (Schlievert et al., 1995). TSST-1 shares all the biological properties as SEs except the ability to induce emesis as characterized by the SEs.

Furthermore, TSST-1 shares little sequence similarities with other members of the PTSAgs (Schlievert et al., 1995).

II. Staphylococcal Enterotoxins

The SEs form a subgroup of related toxins in the PTSAgs family. They are distinguishable from other members of the PTSAgs by their ability to induce emetic response when ingested (Bohach et al., 1990). This unique feature of the SEs has long been recognized since the 60s. Hence, they are the causative agents in staphylococcal food poisoning (SFP), a very common form of food-associated gastroenteritis in the United States and worldwide (Holmberg and Blake, 1984). In recent years, SEs have received more attention for causing TSS (Schlievert, 1986). It is now known that these toxins, particularly SEB and SEC, are responsible for 50% of nonmenstrual toxic shock syndrome (Murray et al., 1995).

Barber is generally credited for providing the first evidence for enterotoxin production by *Staphylococcus aureus* in 1914 (Holmberg and Blake, 1984). To date, seven major antigenic forms of SEs, including SEA, SEB, SEC, SED, SEE, SEG, and SEH, have been identified based primarily on their antigenic heterogeneity (Schlievert, 1997). Based on minor differences in antigenicity, SEC can be further differentiated into three subtypes (SEC1, SEC2, and SEC3). Comparison of primary amino acid sequences indicates that SEs can be divided into 2 groups: group 1 contains SEA, SED, SEE, and SEG; group 2 consists of SEB and SEC subtypes (Betley et al., 1992).

III. Streptococcal Pyrogenic Exotoxins (SPEs)

SPEs have been known to cause scarlet fever since the beginning of the century (Dick and Dick, 1924). For years, it was thought that the only property of these toxins was rash production. In the early 1960s, the discovery of their distinctive fever-producing ability led Watson to name these proteins streptococcal pyrogenic exotoxins (SPEs) A, B, C (Watson, 1959). Although the T lymphocyte proliferative effects of the toxins have been known since 1970s (Hanna and Watson, 1973), their superantigenicity was recognized only approximately 20 years later when investigators noted their specificity for the Vβ chain of the TCR (Marrack and Kappler, 1990). It is now known that these toxins are also responsible for streptococcal toxic shock syndrome (Cone et al., 1987 and Stevens et al., 1989). Unlike the SEs, the SPEs share relatively little primary sequence homology with each other or with other PTSAgs. SPEA is an exception, which shares 45-50% sequence similarity with SEB and the three SEC subtypes (Schlievet et al., 1995).

1.3. Structural Characteristics of PTSAgs

In addition to their shared immunobiological activity, the PTSAgs also have many common structural features even though there is little primary sequence similarities among the PTSAgs (Fig. 2). Recently, the three-dimensional structures of SEA (Schad et al., 1995), SEB (Swaminathan et al., 1995), SEC2 (Papageorgiou et al., 1995), SED (Sundstrom et al., 1996), TSST-1 (Prasad et al., 1993; Acharya et al., 1994; Papageorgiou et al., 1996) and SPEC (Roussell et al., 1997) were determined. These PTSAgs share strikingly similar architecture as typified by the ribbon structure of TSST-1 shown in Fig. 3. The TSST-1 structure is the simplest of the family. Basically, it is a two-domain kidney

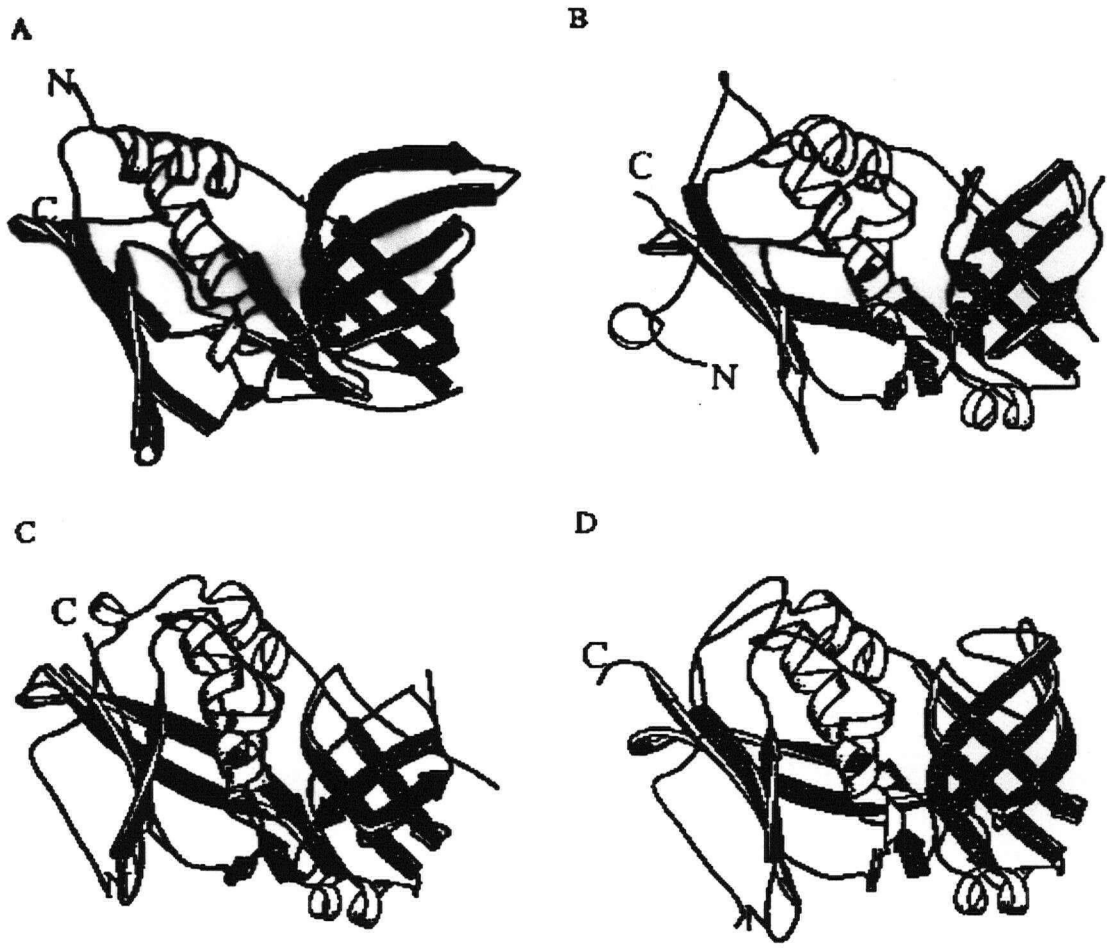


Figure 2. Three dimensional structure ribbon diagram of (A) toxic shock syndrome Toxin-1 (TSST-1); (B) Staphylococcal Enterotoxin A (SEA); (C) Staphylococcal Enterotoxin B (SEB); (D) Staphylococcal Enterotoxin C3 (SEC3), as determined by X-ray crystallographic analyses. Domain A and B of each toxins are shown on the left and right of each figure respectively.

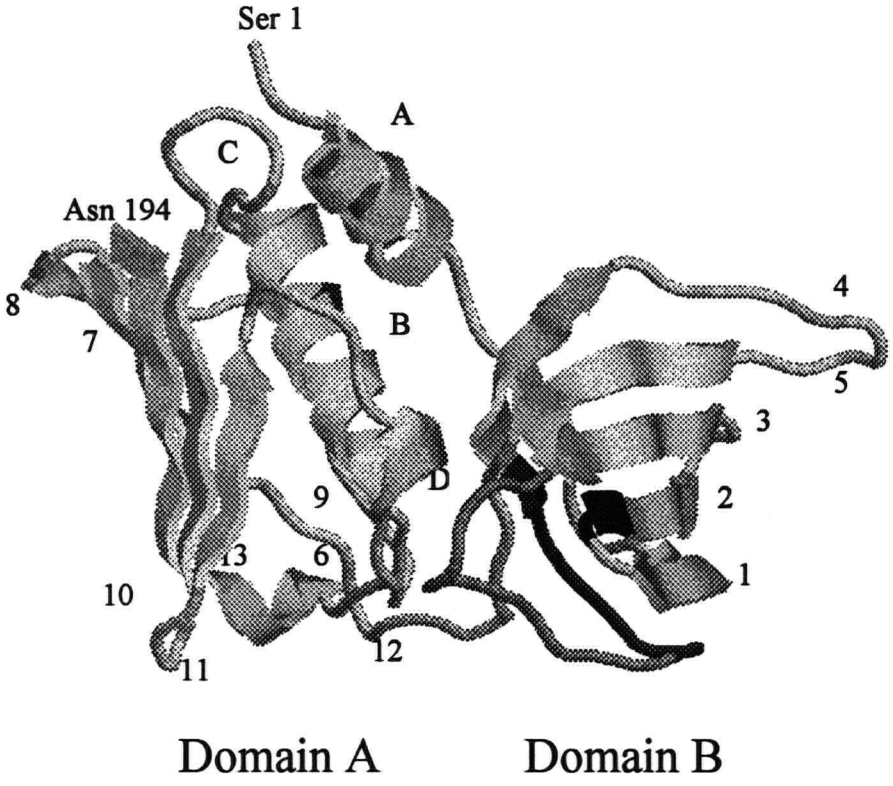


Figure 3. Ribbon diagram of TSST-1. Helices are labeled A-D and are represented as coils. β strands are labeled 1-13 and are shown as arrows. From: Prasad et al., 1993.

shaped molecule containing a long, diagonal, solvent accessible α helix spanning the center of the molecule. The C-terminal domain (domain A), the larger of the two domains, is a so-called β-grasp motif composed of a long central α helix resting against a five-stranded β sheet (Schlievert et al., 1995; Overington, 1992). Amino acid residues in domain A of TSST-1 (Murray et al., 1996; Hurley et al., 1995), SEA (Hudson et al. 1993), SEB (Jardetsky et al., 1994), SEC (Li et al., 1998), and SEE (Hudson et al., 1993) have been implicated in TCR binding. The N-terminal domain (domain B) is characterized by a "claw-like" barrel structure composed of 5 β strands. Crystallographic studies of the three-dimensional structure of PTSAgs complexed with MHC II molecules have revealed that specific regions of domain B are responsible for contacting the MHC class II molecules (Jardetsky et al., 1994; Kim et al., 1994). Key features of the TSST-1 molecule are the two grooves, referred to as the front- and backside grooves formed by the central α helix. The N-terminal α helix and its subsequent loop located on the opposite sides of the central α helix define the wall of these grooves. The larger backside groove is more exposed to the environment than the front side groove. Mutational analysis have indicated that residues within the back-side-groove, including amino acids in the central and N-terminal α helix, are important for T cell interaction (Murray et al., 1994; Deresiewicz et al., 1994).

Despite the many overall structural similarities, each toxin seems to have its unique structural features. Close analysis of their structures reveals 4 features not found in the TSST-1 structure: (Schlievert et al., 1995 and Earhart et al., 1998): (1) an elongated N-terminus of approximately 20 amino acids preceding the amino-terminal α-helix; (2) a second α helix sits between domain A and domain B; (3) a highly flexible "disulfide loop" at the top of domain B; (4) an α-helix at the bottom of domain B. It is speculated that

TSST-1 contains the minimal structural requirements for superantigenicity. Additional structural features found in SEs may be associated with their unique sub-group specific biological activities. For example, the disulfide loop present in all SEs but missing in TSST-1 has been implicated in the emetic properties of the SEs, which is absent in TSST-1 (Schlievert et al., 1995). Site-directed mutagenesis studies of SEC1 suggested that local conformations near or within the disulfide loop appear to be important for emetic activity (Hovde et al., 1994).

1.4. MHC class II molecules – The Receptor for Superantigens

Convincing evidence has been provided that staphylococcal superantigens (SSAgs) bind specifically to class II MHC molecules, and that this interaction is important for T cell stimulation (Fleischer and Schrezenmeier, 1988; Fischer et al., 1989; Mollick et al., 1989; Scholl et al., 1989). Although binding to the MHC class II molecules is a shared property among SSAgs, a significant degree of heterogenity is observed in regard to: (1) affinity of PTSAg binding to MHC class II; (2) requirement of metal atoms; (3) repertoire of compatible MHC class II molecules; (4) molecular regions of MHC class II molecule and PTSAgs involved in binding.

The dissociation constants for the binding of staphylococcal superantigens to human MHC class II molecules were estimated to range from 10^{-5} – 10^{-8} M (Chintagumpala et al., 1991). The binding affinities of TSST-1, SEC1 and SEB for HLA-DR are lower than that of SEA but substantially higher than those of SED or SEE (Mollick et al., 1991 and Chintagumpala et al., 1991). A correlation is noted between the binding affinities of these toxins to MHC class II molecules and the toxins' potencies of T cell

proliferation. The affinity of the staphylococcal superantigens for MHC class II molecules is usually inversely correlated with the amount of toxins required for efficient T cell stimulation; i.e. toxins which required the lowest concentrations to elicit T cell responses displayed the highest affinity for HLA-DR1 (Mollick et al., 1991). This is true for SEA, SEB, SEC1 and SED but not for SEE and TSST-1 (Mollick et al., 1991; Chintagumpala et al., 1991).

SEA, SEC2, SED, and SEE, but not SEB and TSST-1, have been found to possess a zinc binding site (Fraser et al., 1992; Schad et al., 1995; Sundstrom et al., 1996; Papageorgiou et al., 1995). Earlier biochemical studies demonstrated that binding of radiolabeled SEA to MHC class II molecules was completely inhibited by EDTA, indicating that divalent cations were important for this interaction (Fraser et al., 1992). Zinc ions, but not magnesium ions, calcium ions, manganese ions, ferrite ions, and copper ions, were able to restore completely the binding of SEA to HLA-DR (Fraser et al., 1992). Moreover, zinc ions bound directly to SEA but not to HLA-DR, suggesting that they are required to stabilize the class II binding domain on SEA (Fraser et al., 1992).

The ability of various human and murine MHC class II molecules to present PTSAgs has been well documented (Scholl et al., 1989; Mollick et al., 1991; Scholl et al., 1990; Mollick et al., 1989). Although the presentation of PTSAgs is generally described as not being restricted to particular MHC class II proteins, the efficacy of presentation of PTSAgs to T cell varies when different alleles and isotypes of MHC class II molecules are used (Herrmann et al., 1989; Scholl et al., 1990). In general, HLA-DR is the most effective human HLA molecule for PTSAg presentation (Herman et al., 1990). However, alleles of HLA-DR were shown to have heterogeneity in binding to particular PTSAgs. SEA was

shown to bind to most HLA-DR molecules except for the DRw53 molecules (Herman et al., 1990). Yet, DRw53 displays an intact affinity for SEB or SEC (Herman et al., 1991; Karp and Long; 1992; Herrmann et al., 1989). Herman et al determined that there was a hierarchy of SEs presentation to human MHC class II molecules with HLA-DR > HLA-DQ > HLA-DP (Herman et al., 1990). However, SEC seems to be an exception to this rule. SEC_2 binds predominantly to HLA-DQ and SEC_3 to HLA-DR and HLA-DQ (Herrmann et al., 1989). Similarly, studies of the murine analogue of HLA-DR, I-E, demonstrated consistently better presentation of toxins than the murine analogue of HLA-DQ, I-A (Yagi et al., 1990). However, I-A alleles were shown to bind TSST-1 more efficiently than I-E molecules (Scholl et al., 1990). Alleles of murine class II molecule I-A were also shown to have different capacity to bind TSST-1. Specific binding of TSST-1 to $I\text{-}A^d$ and $I\text{-}A^k$ was observed but transfected cells expressing $I\text{-}A^b$ did not bind this toxin (Scholl et al., 1990).

Competition studies suggest that multiple overlapping or independent MHC class II binding sites exist for superantigens (Scholl et al., 1989; Chintagumpala et al., 1991; See et al., 1990). SEA competes with SEB and TSST-1 for binding to HLA-DR but a mixture of TSST-1 and SEB does not display SEA binding to HLA-DR, suggesting that SEA has multiple binding sites, one of which overlaps with SEB and TSST-1, and one that is unique (Fraser, 1989; See et al., 1990). Although the class II binding sites of TSST-1 and SEB (as well as SEA) overlap, the toxins do not compete with each other because they bind different subsets of DR1 molecules (Scholl et al., 1989). This data suggests that there are at least two binding sites for staphylococcal superantigens on class II molecules, one

common binding region shared by these toxins and an additional unique binding region for SEA and its related toxins (Chintagumpala et al., 1991).

1.5. MHC class II Binding Sites of PTSAgs

A. PTSAgs Bind MHC Class II Molecule Using Common Structural Strategies

The most direct information on the interaction of superantigens with MHC class II molecules has been provided by the elucidation of the three-dimensional crystal structures of the SEB:HLA-DR1 and TSST-1:HLA-DR1 complex (Jardetzky et al., 1994; Kim et al., 1994). Together with superimposition of the crystal structures of PTSAgs, a similar binding mode involving the N-terminal domain of the toxins and the α-chain of the DR1 molecules was noted (Ulrich et al., 1995; Ulrich et al.,1998). The MHC class II binding sites on PTSAgs consists of principally two structurally conserved region which are located in domain B and are essential for the molecular recognition of the α chain of HLA-DR (Fig. 4) (Ulrich et al., 1995; Ulrich et al., 1998). The first region is the hydrophobic binding loop, centered around a leucine residue (SEA L48; SEB L45; TSST-1 L30), in most of the PTSAgs (Ulrich et al., 1998). The alignment of primary sequences of PTSAgs reveals a conservation of this leucine residue among PTSAgs except SPEC, suggesting that it may serve as an important contact for PTSAg: MHC class II binding (Acharya et al., 1994; Swaminathan et al., 1992; Schad et al., 1995; Ulrich et al., 1998). The crystal structure of SEB:DR1 reveals that Phe44, Leu45, and Phe47 point into a hydrophobic pocket of the DR1 molecule (Jardetzky et al., 1994). The importance of these residues in MHC class II binding had been demonstrated by mutagenesis studies (Kappler et al., 1992).

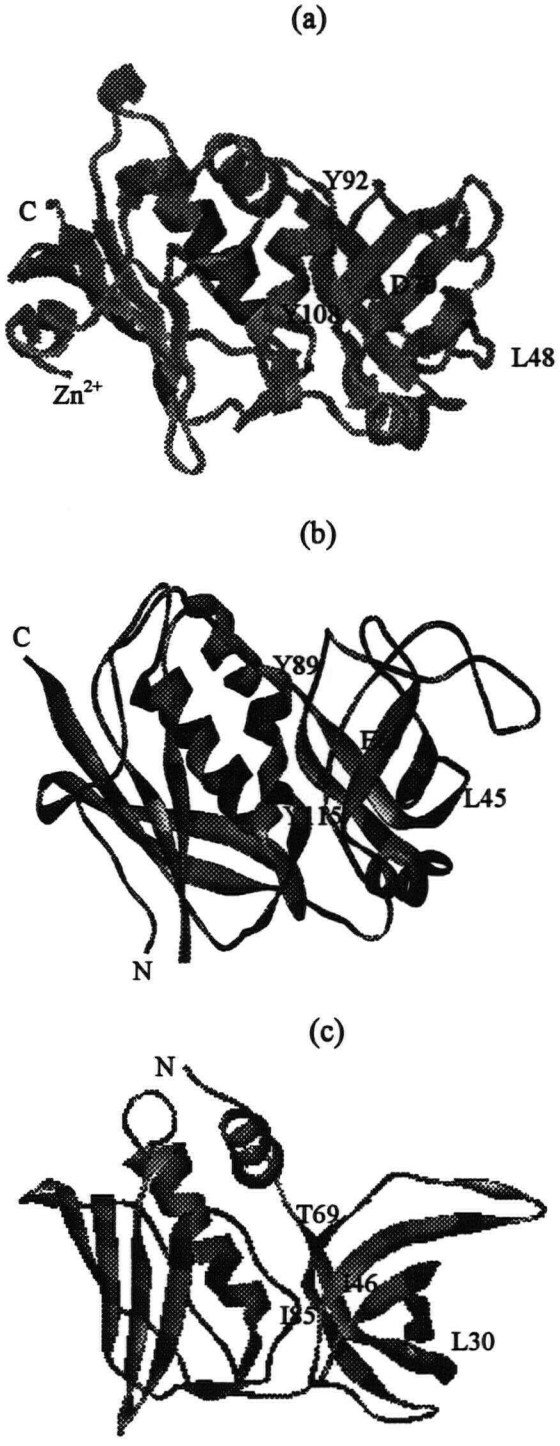


Figure 4. Common structural strategies used by SEA (a), SEB (b), and TSST-1 (c) for binding to MHC class II α subunits. C represents carboxyl-terminus; N represents amino terminus; Zn^{2+} in (a) represents bound to zinc ion.
Adapted from Ulrich et al., 1998.

In TSST-1, the corresponding region makes similar contacts to DR1 as observed in SEB:DR1 complex (Kim et al., 1994). Additional evidence for the importance of this hydophobic loop between β strands 1 and 2 of TSST-1 (residues 27-32) in complex formation comes from mutational studies performed by our laboratory and others. Site-directed and random mutagenesis studies demonstrated that TSST-1 G31R mutant and TSST-1 G31S/S32P double mutants were unable to bind an HLA-DR1 molecule or stimulate human peripheral blood lymphocytes (Hurley et al., 1995; Kum et al., 1996).

Although the SEA:DR1 crystal complex is not yet available, the modeled DR1-SEA complex also presents similar interaction in the analogous region (Ulrich et al., 1995). Site-directed mutagenesis indicated that changes of residue Phe 47 in SEA to either alanine or serine resulted in a six to seven fold decrease in MHC class II binding (Abrahmsen et al., 1995; Hudson et al., 1995). Moreover, alanine substitution of His 50 in SEA reduced MHC class II binding by threefold (Abrahmsen et al., 1995). In addition, Phe-47-Gly, Leu-48-Gly, Phe-47-Ala, and Phe-47-Ser mutation drastically reduced T cell proliferation (Harris et al., 1993; Harris and Betley, 1995, Abrahmsen et al., 1995; Hudson et al., 1995). The SEA residues Phe 47, Leu 48, and His 50 are equivalent to residues Phe 44, Leu 45, and Phe 47 on SEB, which are all important in the interface between SEB and HLA-DR1 (Jardetzky et al., 1994). Furthermore, Thibodeau et al have recently shown that mutations in the α 1 domain of HLA-DR affected the binding of SEA (Thibodeau et al., 1997).

The second structural binding motif is a polar pocket (SEA Y92, Y108, D70; SEB Y89, Y115, E67), derived from three β-strand elements located at the upper part of domain B, that accommodates Lys39 of DR1α chain (Ulrich et al., 1995; Ulrich et al., 1998). Site-directed mutagenesis studies showed that the substitution of alanine for SEB Y89 or SEA

Y92 eliminated the hydrogen bond with Lys39 of DR1α chain and resulted in 100 fold reduction in DR1 binding (Ulrich et al., 1995). In addition, the importance of αLys39 of DR1α chain in MHC class II binding had also been demonstrated by mutagenesis studies. By mutation of αLys39 to alanine, the binding of both TSST-1 and SEB are abolished (Jardetzky et al., 1994). Although Lys39 of DR1 α chain hydrogen bonds to TSST-1 residues Pro50 and Ser53 at the TSST-1:DR1 interface(Kim et al., 1994), TSST-1 is the only PTSAg which lacks this particular motif, as represented by the positioning of residues T69, I85, and I46 in Fig. 4c (Ulrich et al., 1998). The polar pocket combined with other surface elements, such as the disulfide loop, stabilize the PTSAg:HLA-DR complexes (Ulrich et al., 1998). Although the disulfide loops differ in structure between SEA and SEB, both SEA A97 and SEB Y94 located within the loop apparently bind in a similar manner to DRα chain (Ulrich et al., 1995). Replacement of these residues with alanine disrupted MHC class II binding (Ulrich et al., 1995).

Additional contact region involving the α-helix of DR1 β-chain and TSST-1 residues 73-80 was identified in the TSST-1:DR1 interface (Kim et al., 1994). These TSST-1 residues form part of a mobile loop to optimize TSST-1 contacts with both the DR1β chain (residue 55-63) and the peptide antigen (Papageorgiou et al., 1996) (Fig. 5b, 5d). Thus, TSST-1 binding is likely to be peptide-dependent (Hsu and Huber, 1995). In contrast, SEB interacts entirely with the external surface of the α 1 domain of HLA-DR1 and no contacts with the DR1 β chain and peptide was observed in the complex (Fig. 5a, 5c).

Comparison of the TSST-1:DR1 complex with the SEB:DR1 complex reveals that 11 of the 17 DR1 residues in the TSST-1:DR1 interface are common to the SEB:DR1

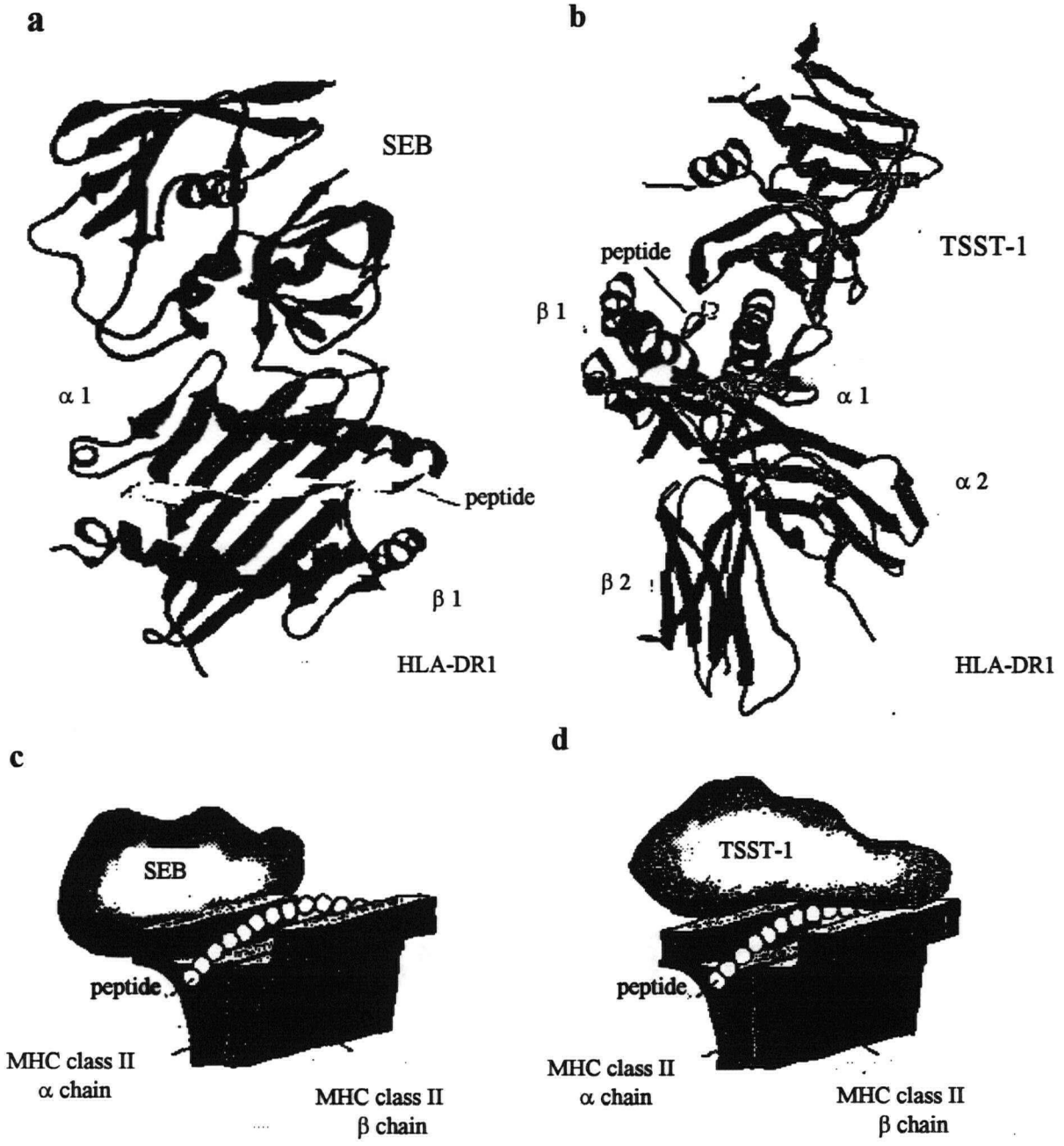


Figure 5. Structures of complexes between HLA-DR1 and the superantigens SEB and TSST-1. (a) Crystal structure of SEB-DR1 complex; (b) Crystal structure of TSST-1-DR-1 complex; (c) Schematic structure of the SEB-DR1 complex, showing the exclusive binding of SEB to the α chain of DR1, primarily off one edge of the peptide binding groove; (d) Schematic structure of the TSST-1-DR1 structure, showing how TSST-1, in contrast to SEB, binds to both α and β chains of DR1 and contacts part of the bound peptide. Taken from Hsu et al., 1995.

interface, and 13 of the 19 residues on TSST-1 that contact DR1 molecule are homologous to residues on SEB that contact DR1, demonstrating that there is overlap between the MHC class II binding sites for TSST-1 and SEB (Table IV) (Kim et al., 1994). Despite the similarity and the apparent overlap of their respective MHC-binding sites, TSST-1 and SEB do not compete with each other for binding to HLA-DR (Scholl et al., 1989). It was proposed that the affinity of TSST-1 and SEB for MHC class II molecules might be influenced by the nature of the peptide contained in the conventional peptide-binding groove (Kim et al., 1994; Thibodeau et al., 1994). Hence, only a portion of MHC class II molecules carrying the appropriate peptides could bind the toxins and present them to T cells (Wen et al., 1996). Another possible explanation would be the existence of a second binding site on DR1 for SEB, or TSST-1. Although the SEB:DR1 complex reveal only one MHC class II binding site on SEB, several groups of investigators have independently implicated that residues outside the binding interface defined in the crystal complex are involved in class II binding. Using random mutagenesis, Kappler and his associates had identified mutants at positions 14 and 17 of SEB that caused a 100-fold reduction in HLA-DR binding (Kappler et al., 1992). Moreover, peptides containing residues 1-33 and 9-20 were shown to compete with SEB for binding to HLA-DR, implicating that these regions are involved in MHC class II binding (Soos and Johnson, 1994; KoMLSar et al., 1994). These implicated residues are located on the outside edge and back of the large β-grasp domain, which somewhat overlaps with the zinc coordinated MHC class II binding site. However, zinc atom is absent in the SEB crystal structure (Swaminathan et al., 1992).

Table IV.

The TSST-1:DR1 interface. Underlined residue numbers are also found in the DR1:SEB interface.

TSST-1 residue	SEB residue	Location	DR1 contact residues
D27	D42	β1	α–Q18; α–Y13; α–K67
S29	F44	β1β2 loop	
L30	L45	β1β2 loop	α–M36; α-I63
G31	Y46	β1β2 loop	
S32	F47	β2	α–A64
R34	L49	β2	α–E71; α–A68
I42	N63	β3	
L44	R65	β3	
I46	E67	β3	α–A61; α-Q57
F47	F68	β3	α–K39
P48	K69	β3β4 loop	α–L60
P50	K71	β3β4 loop	α–K39; α–K38
S53	A74	β3β4 loop	α–K39
K58		β3β4 loop	α–Q18
T69	Y89	β4	
K70	Y90	β4	
	Q92	β4	
Q73	C93	β4	β–Y60; β–B64
H74	T94	β4	
T75	F95	β4	p-A13
S76	S96	β4β5 loop	p-A13
E77	K97	β4β5 loop	
I81	K111	β5	
F83	C113	β5	α–V65
I85	Y115	β5	

Modified from Kim et al., 1994.

B. SEA, SEC2 and SED: the role of zinc

In addition to the PTSAg-DRα binding mode, several PTSAgs have evolved a distinct, non-overlapping binding site involving a zinc complex with the DRβ chain (Fraser et al., 1992; Papageorgiou et al., 1998). The recently reported structures of SEA (Schad et al., 1995), SEC2 (Papageorgiou et al., 1995), SED (Sundstrom et al., 1996) revealed that the zinc bindings sites in these molecules were situated far from the normal MHC class II binding site, as observed in SEB-DR1 complex (Jardetzky et al., 1994). SEA binds MHC class II receptor with high affinity and the binding of SEA to DR1 is reduced to a level comparable to SEB by chelation of ionic zinc with EDTA (Ulrich et al., 1995). As a result, it is now clear that SEA has two binding sites for class II: a high affinity site involving zinc (K_d = 100 nM) and a low affinity SEB-like site (K_d = 10μM) (Abrahmsen et al., 1995; Hudson et al., 1995; Thibodeau et al., 1997; Papageorgiou and Acharya, 1997). Analysis of SEA crystal structure illustrates the presence of a metal ion bound in a coordination motif involving residues Ser 1, His 187, His 225, and Asp227 situated at lower part of domain A, far from the equivalent binding site of TSST-1 and SEA (Schad et al., 1995). Site-directed mutagenesis further showed that residues His 187, His 225, Asp227 and Asn 128 play an important role in binding to MHC class II molecules (Abrahmsen et al., 1995; Hudson et al., 1995). On the class II molecules, the histidine at position 81 of the HLA-DR β chain (β81His) was demonstrated to be involved in high affinity zinc co-ordinated binding of SEA (Herman et al., 1991; Karp and Long, 1992). This β81His is conserved among all DR alleles except for DRw53 allele, which was extremely poor at binding and presentation of SEA, as well as SEE. Presumably, binding of SEA to class II MHC molecule is facilitated by the "zinc bridge" cross-linking the two molecules.

A zinc-binding site has also been found in SEC2 (Papageorgiou et al., 1995), formed by His 118, His 122, Asp83 from one molecule and Asp9 from a neighbouring SEC2 molecule in the crystal lattice. Although the zinc-binding site is distinct from that of SEA, the dissociation constant of the zinc ion is comparable to that observed for SEA (Papageorgiou et al., 1997). The zinc atom provides a potential mechanism for MHC class II to bind in this region of the toxin. At this point, it is still unclear whether SEC binding to MHC class II requires zinc. This possibility is currently being actively investigated.

Another variation in superantigen binding to MHC class II molecules was recently revealed in the crystal structure of SED (Sundstrom et al., 1996). In SED, the zinc binding site comprises SED residues Asp182, His220, and Asp222 (Sundstrom et al., 1996). The zinc ion induces homodimerization of SED with each monomer able to interact with MHC class II molecules in a SEB-like manner (Papageorgiou et al., 1997). However, under certain conditions, SED may appear as a monomer and bind MHC class II molecules in a SEA-like mode (Papageorgiou et al., 1997).

1.6. TCR-Binding Sites of PTSAgs

The TCR molecules are important binding targets for the PTSAgs. Structural insights into the interactions of superantigens with the TCR were recently provided by the crystal structures of SEC2:TCRVβ, SEC3:TCRVβ, and SEB:Vβ complex (Fields et al., 1996; Li et al., 1998). The TCR binding site lies in the shallow cleft between the two domains of the toxins, making contact with the complementarily-determining region 2 (CDR2) of the TCR Vβ chain and, to lesser extents, CDR1 and hypervariable region 4 (HV4) of the TCR (Fields et al., 1996; Li et al., 1998). The TCR binding sites for

Table V.

Contacts between the TCR Vβ 8.2 Chain and Staphylococcal Superantigens.

Vβ residue	SEB residue	SEC3 residue
H47	L20 F177	
Y50	Y91	Y91
G51		Y91
A52	Y90	Y90
G53	N23 Q210	N23 Y26 Q210
S54	N23	N23 V91
T55	L20 N23 E22 F177	T20 N23
E56	L20 N23	T20
K57	T18 G19 L20	G19 T20
Y65	F177	
K66	F177	F176
A67	F177	F176
P70	N60	N60 L58
S71	N60	N60

Modified from Li et al., 1998.

SEC2/SEC3 and SEB are adjacent to, but distinct from, the MHC class II binding sites (Jardetzky et al., 1994; Fields et al., 1996; Li et al., 1998). The major contacts between the TCRβ chain and these PTSAgs are shown in table V. The importance of some of these residues has also been demonstrated by mutational studies. For example, mutations in SEB at position 23, 60, and 61 affect T-cell stimulation but not MHC class II binding, indicating that these residues are involved in TCR interaction (Kotzin et al., 1993; Fields et al., 1996, Kappler et al., 1992; Hayball et al., 1994). Interestingly, residue Asn23 is conserved among all the SEs (equivalent to residue Asn25 in SEA) but not TSST-1. It is possible that this Asn residue is a conserved TCR contact site for all SEs. Moreover, this TCR binding cavity seems to be a common feature of all staphylococcal superantigens. Mutational analysis, synthetic peptide studies, and structural information have all suggested topologically similar TCR binding sites for SEA (Hudson et al., 1993, Schad et al., 1995) and SEE (Hudson et al., 1993; Mollick et al., 1993).

The crystal structures of these complexes provide explanation for the unique Vβ specificity profile for these PTSAgs. Superimposition of PTSAgs of known structures shows that the putative binding sites of SEA, TSST-1, and SPEC, which do not activate murine Vβ8.2 T cells, are markedly different from those of SEB and SEC (Li et al., 1998). Conversely, a comparison of Vβ domains of known three-dimensional structure reveals that the relative positions of CDR2 and FR3 in Vβs that recognize SEB or SEC are markedly different from their position in Vβs that do not (Li et al., 1998). SEB and all three SEC subtypes share similar Vβ specificities (Deringer et al., 1996). However, SEB and SEC1 react with human Vβ3 but not Vβ13.1, whereas SEC2 and SEC3 react well with Vβ13.1 but only weakly with Vβ3 (Deringer et al., 1996). Although SEC1 and SEC2 only

differed by seven residues, they have widely different reactivities to Vβ13.1 and Vβ3. Site-directed mutagenesis studies have localized this reactivity differences to residue 26 (valine in SEC1 and SEB and tyrosine in SEC2 and SEC3) (Deringer et al., 1996). An SEC1 mutant, in which the valine at position 26 is mutated to the corresponding SEC2 residue (tyrosine), induces a stimulatory profile of T cell subsets identical to that of SEC2 (Deringer et al., 1996).

The putative TCR binding site of TSST-1 is distinct from that of SEs and is believed to be located in the C-terminal domain. The presumed TCR sites extends from the back of the short amino terminal helix αA into a cleft formed with the β7-β9 loops and central helix αB (Fig. 6) (Papageorgiou and Acharya, 1997, Earhart et al., 1998). In contrast, for SEB and SEC, the TCR sites are mainly formed by the loop covering the front of domain A and extend only to amino terminal helix αA. Mutations outside the MHC class II binding site which confer a loss in superantigenic activity provide a clue of which residues may be important in TCR interaction. Several residues, including Glu132, His135, Ile140, His141, and Tyr144, along the backside of the central α helix have been shown to be responsible for mitogenicity (Acharya et al., 1994; Murray et al., 1996; Deresiewicz et al., 1994). For example, a point mutation at residue His135 results in a significant decrease in both superantigenicity and lethal activity (Murray et al., 1996; Blanco et al., 1990; Bonventre et al., 1995).

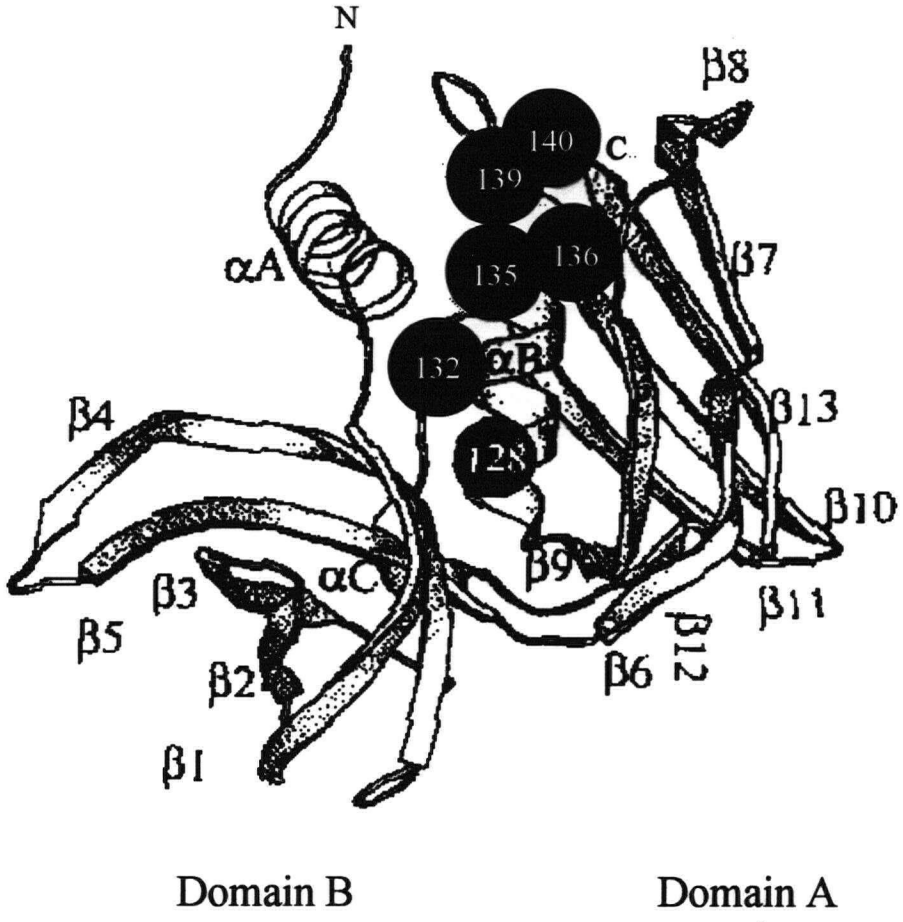


Figure 6. Ribbon diagram of TSST-1 shown in back-side view. Residues that have been mutated and lead to alteration of biological activity are indicated by numbered balls.
Taken from: Earhart et al., 1998.

1.7. Superantigens and Human Diseases

A. Biological Responses to Superantigens

Recently, much attention has focused on the contribution of PTSAgs in human diseases. The discovery of superantigens and characterization has provided new insights into potential mechanisms by which infectious agents may cause disease. Several immune mechanisms have been proposed to explain how PTSAgs can take part in the pathogenesis of human diseases (reviewed by Kotzin et al., 1993). First, PTSAgs bind directly without processing to the MHC class II molecules on APCs such as macrophages or dendritic cells and lead to proliferation of Vβ specific T cells. This can have serious physiological consequences due to the massive systemic release of proimmflammatory cytokines by activated APCs and Vβ specific T cells.

Second, PTSAgs may cause inflammatory or autoimmune responses through the activation of B cells. Several groups of investigators have suggested that PTSAgs stimulate autoantibody or IgE production by bridging the MHC class II molecules on B cells with the TCRs on T cells (Hofer et al., 1995; He et al., 1992). This form of B cell activation is likely to be non-antigen specific. PTSAgs can either activate or suppress immunoglobulin synthesis depending on the concentration of PTSAgs present in the local environment. Staphylococcal superantigens, in nanogram range or higher, inhibit B-cell activation by inducing apoptosis, whereas lower concentrations induce B-cell activation (Hofer et al., 1996).

Third, PTSAgs may activate normally quiescent auto-reactive T cells that escape thymic clonal elimination (Murray et al., 1995). Once activated, these T cells may continue to proliferate due to the presence of autoantigens along with APCs. They may

mediate damage via cytokine mechanisms or the secretion of proinflammatory cytokines. Since PTSAgs have different Vβ specificity profile, they may differ in their capability to expand autoreactive T cells. Under certain conditions, T cell expansion by PTSAgs may be followed by anergy and/or deletion of the activated T cells (MacDonald et al., 1991).

The particular response of T cells to PTSAgs depends on a variety of factors, including their stage of development and activation, TCR avidity of the specific PTSAg, and costimulatory signals provided by the PTSAgs-presenting cells. Thus, the potential role of PTSAgs in the pathogenesis of human diseases is associated with inflammation, autoimmunity, and/or immunodeficiency. However, due the complexities to establish cause-and-effect in the clinical setting, information on the role of PTSAgs in mediating human diseases remains unclear. By comparison, the pathogenesis of TSS is relatively well understood.

B. PTSAgs and Disease Association

I. Toxic Shock Syndrome

Toxic shock syndrome (TSS) is a life threatening disease characterized by rapid onset of fever, rash, shock, and multiorgan involvement (Chesney,1989; Bohach et al., 1990; Chow et al., 1995). Although the term "toxic shock syndrome" was first introduced by Dr. James Todd in 1978, sporadic case reports of illness resembling the syndrome had appeared in the medical literature dating as far back as 1927 (Todd et al., 1978). A rise in incidence of cases of TSS caught the attention of the public and medical community in early 1980, since they occurred particularly among young and previously healthy high-absorbency tampon users during or immediately following menstruation (Reingold et al.,

1982). TSST-1 was the first toxin to be implicated in TSS and was characterized in 1981 shortly after the TSS epidemic (Schlievert et al., 1981; Bergdoll et al., 1981). Over 95% of *S. aureus* strains isolated from menstruation-related cases produce TSST-1 (Chow et al., 1995).

Prior to 1981, the association of TSS with menstruating women and tampon usage was the main focus of clinical professionals and the general public. However, in recent years, nonmenstrual TSS is becoming more prevalent than menstrual TSS (Chow, 1993). Unlike menstrual TSS, nonmenstrual cases had occurred in males and females of all ages and races (Murray et al., 1995). TSST-1 accounts for approximately half of nonmenstrual cases of TSS; SEB and to a lesser extent SEC account for the remainder (Bohach et al., 1990; Kotzin et al., 1993). Nonmenstrual TSS occurs in a wide variety of clinical settings and is often associated with *Staphylococcus aureus* infections at a variety of sites. It includes nonsurgical focal infections, surgical wound infections, and vaginal cases not associated with menstruation (Bartlett et al., 1982; Reingold, 1982). Moreover, nonmenstrual TSS is also associated with the use of foreign bodies, such as nasal packing during surgery or barrier contraceptives (Barbour et al., 1984). It has also been shown that nonmenstrual TSS is associated with influenza, and recalcitrant erythematous desquamating syndrome (MacDonald et al., 1987;Cone et al., 1992). Influenza associated TSS appears to be a consequence of upper respiratory tract damage caused by initial viral infection and subsequent superinfection by toxigenic *S. aureus* (Murray et al., 1995). The case-fatality rate for influenza associated TSS is approximately 90% in children (Murray et al.,1995). Recalcitrant erythematous desquamating syndrome is a TSS-like illness that

occurs in AIDS patients and often results in patient's death (Cone et al., 1992; Schlievert et al., 1993).

The most striking aspect of TSS is the rapidity and severity with which the manifestation can present and progress in a previously healthy individual of any race, sex, and age. The estimated prevalence of TSS is 0.22 to 1.23 cases per 100,000 with a mortality rate of 3 to 7% and a recurrence rate of 15 to 22% (Chow, 1995). The absence of specific antibody to TSST-1 appears to be a major risk factor for acquiring the disease (Kotzin et al., 1993; Chow et al., 1995). Patients who develop TSS have no or low levels of detectable antibodies to TSST-1 (Chow et al., 1995). Moreover, recurrence of TSS is common among TSS patients (Chow et al., 1995; Murray et al., 1995). The high level release of IFNγ during the course of illness is thought to suppress the synthesis of protective antibodies, such that 85% of acute TSS patient remain susceptible to the illness upon recovery (Murray et al., 1995).

II. Streptococcal Toxic Shock Syndrome

A severe and often fatal TSS-like syndrome associated with both group A streptococci (GAS) and factors produced by these organisms has been reported with increasing frequency (Stevens et al., 1989). These infections have been termed streptococcal toxic shock syndrome (STSS) (Cone et al., 1987). The increased number of cases coincided with the reemergence of SPEA-producing streptococci, and it has been postulated that the TSS-like illness is caused by SPEA (Hauser et al., 1991). STSS is usually characterized by hypotension and two or more of the following: renal dysfunction, liver involvement, erythematous rash, necrosis of soft tissues, coagulopathy, and acute

respiratory distress syndrome (Breiman et al., 1993). Streptococcal TSS resembles staphylococcal TSS in many clinical characteristics except severity. Streptococcal TSS may be 30 to 50% fatal, as opposed to 5 to 15% fatality rate for staphylococcal TSS (Schlievert et al., 1995). Moreover, as many as 25% of streptococcal TSS patients have significant soft tissue destruction not seen in staphylococcal TSS (Schlievert et al., 1995).

Several risk factors are associated with the development of STSS. For example, STSS occurs frequently as a complication of chicken pox in children because chicken pox results in a transient state of immuno-suppression and causes skin leisons that increase susceptibility to infection (Bradley et al., 1991). Major risk factors in adults include prior use of nonsteroidal anti-inflammatory agents, penetrating and non-penetrating wounds, and genital infections in women (Schlievert et al., 1993; Murray et al., 1995).

To establish a case of STSS, GAS are typically isolated from an otherwise sterile site. Several studies have suggested that most STSS isolates produce SPEA or contain the gene for SPEA, compared with only 15% of non-STSS isolates (Hauser et al., 1991; Musser et al., 1991; Yu et al., 1989). Although recent evidence indicates that SPEC also contribute to the illness, the role of SPEB is rather unclear since both STSS and non-STSS isolates contain the SPEB gene and often produce SPEB (Schlievert et al., 1993). Furthermore, other groups of streptococci, including B, C, F, and G may also cause STSS (Schlievert et al., 1993).

III. Other diseases

There have been recent reports demonstrating that PTSAg secreting staphylococci and/or streptococci can be isolated from patients with guttate psoriasis and atopic

dermatitis at the time of disease exacerbation (Leung et al., 1995; Leung et al., 1993). The data implicating a role for superantigens are particularly convincing in guttate psoriasis. Guttate psoriasis is a chronic inflammatory skin disorder that is frequently associated with streptococci throat infection (Schlievert et al., 1993). Interestingly, all strains of streptococci isolated from patients with guttate psoriasis secreted SPEC, a PTSAg known to stimulate Vβ2+ T cells (Baker et al., 1993). Moreover, local proliferation of Vβ2+ T cells, which accounts up to 70% of the T cells, in the skin leisions but not the peripheral blood was noted in these patients (Lewis et al., 1993; Leung et al., 1995). These observations support the concept that guttate psoroasis is associated with superantigenic stimulation of T cells triggered by SPEs.

Numerous reports have also shown PTSAgs are associated with atopic dermatitis. *S. aureus* isolates from more than half of the patients with atopic dermatitis secrete SEA, SEB, and TSST-1 (Leung et al., 1993). These PTSAgs have been shown to induce histamine release from mast cells and amplify IgE-mediated allergic reactions, suggesting their involvement in the pathogenesis of the illness (Leung et al., 1993). Recently, it has been demonstrated that patch testing with staphylococcal toxins induces local eczematoid skin reactions (Strange et al., 1996). Although these data suggest that staphylococcal PTSAgs may play a role in atopic dermatitis, it is not clear whether they act via a superantigenic mechanism since TCR Vβ skewing has not yet been observed.

Several pieces of evidence suggest that PTSAgs may have the ability in initiating or exacerbating an autoimmune disorder. Unfortunately, in most instances of human diseases such as rheumatoid arthritis, the evidence is rather circumstantial and mainly based on the ability of PTSAgs to leave their footprints on the immune system by activating, anergizing,

or deleting specific T cell subsets (Paliard et al., 1991; Howell et al., 1991). However, the effect of PTSAgs on animal models of autoimmunity is more compelling (Kim et al., 1991; Soos et al., 1993). Thus, it is reasonable to predict that PTSAgs do play a role in the course of some autoimmune disorders in humans.

C. Potential Therapeutic Applications of Superantigens

Exploitation of the unique T-cell capabilities of superantigens for therapeutic application is an emerging area of interest. The ability of superantigens to selectively induce deletion or inactivation of specific T cells bearing the appropriate TCR has suggested a new approach for prevention of autoimmune disease. A number of animal studies, such as animal model of lupus and experimental autoimmune encephalomyelination (EAE), have indicated the capacity of superantigens to prevent the initiation of an autoimmune disorder. Indeed, Kim and his coworkers have demonstrated that treatment of SEB in MRL-lpr/lpr mice (which develop a lupus-like illness) before the onset of clinical disease reduced the levels of anti-DNA antibodies, Vβ8+ T cells, circulating immune complexes, proteinuria, lymph node hyperplasia, and vasculitis (Kim et al., 1991). In addition, Soos and his colleagues have found exciting results using their murine EAE model of human multiple sclerosis, in which mice were immunized with myelin basic protein (MBP) to induce a Vβ8.2+ T-cell-dependent response leading to demyelination. They showed that SEB administration prior to MBP immunization could protect PL/J mice from the development of EAE (Soos et al., 1993). Similar results were also obtained by treating Lewis rats with SEE (Rott et al., 1992). These data suggested

that superantigens could potentially be used for the prevention of diseases by selectively eliminating specific T-cell subpopulations.

Furthermore, investigators have also taken advantages of the extremely potent T-cell-activating properties of superantigens for potential treatment of malignant disease. An interesting approach in this area has utilized antibody targeting for the treatment of experimental tumors. These studies are based on the observation that T cells activated by superantigens exhibit potent cytotoxic activity against tumor cells (Hedlund et al., 1993, Kalland et al., 1993). Dohlsten et al. have constructed recombinant fusion proteins between SEA and tumor-specific MAb, which recognizes human colon carcinoma cells, in order to target SEA directly to the tumor site in the body (Dohlsten et al., 1994). Using tumor-bearing SCID mice as a model, this group of investigators demonstrated that injection of these fusion proteins resulted in tumor infiltration of T cells and significant reduction of the tumor load in these mice (Litton et al., 1996). Although further studies are required to address the efficacy and unanticipated difficulties of this approach, the principle of targeted T-cell activation by MAb-superantigen hybrid molecules could have potential applications in the treatment of a number of cancers that are currently unresponsive to conventional therapy.

Chapter 2

Materials and Methods

2.1. Purification of TSST-1 and SEs

Reagents and glassware used in the purification of TSST-1 were maintained pyrogen-free to prevent endotoxin contamination. All glasswares were baked at 180°C overnight and autoclaved prior to use. Reagents were prepared using pyrogen-free water from the Milli-Q pore system at all times. Highly purified recombinant TSST-1 was prepared from culture supernatant of *Staphylococcus aureus* as described previously (Kum et al., 1993). A single colony of strain was picked from a brain-heart infusion (BHI) agar plate and inoculated into 20 ml of pyrogen-free dialyzable BHI broth. Cultures were then incubated for 24 h (stationary phase) at 37°C with shaking (250 rpm in a controlled environment incubator). From each seed culture, 1ml amounts were inoculated into several 1000 ml flasks containing 250 ml BHI broth. After shaking for 24 h, culture medium was centrifuged at 15,000 x g for 30 min at 4°C (L8-8OM Ultracentrifuge; Beckman instruments, Palo Alto, CA) to remove bacterial cells. Supernatants were then concentrated 25-fold with an Amicon Spiral Cartridge concentrator (Model CH2PRS; Acrylic Reservoir RA2000; Spiral Cartridge S1 Y10) with a molecular weight cutoff of 10 kDa. The concentrated supernatants were filter-sterilized and stored at 4°C until purification.

TSST-1 was then purified from concentrated culture supernatant by a combination of preparative isoelectric focusing and chromatofusing as previously described (Kum et al., 1993). Briefly, 45 ml of concentrated culture supernatant were mixed with Pharmalyte (pH 6.7 – 7.7; Sigma Chemical Co., St. Louis) to give a final concentration of 2% (w/v). The mixture was focused at 12 W on a Rotofor cell (Bio-Rad Laboratories, Mississauga, Ontario) for 4 h at room temperature. Electrolytes in the anode and cathode chambers

were 0.1 M H_3PO_4 and 0.1 M NaOH respectively. The presence of TSST-1 in the fractions was determined by SDS PAGE followed by western blotting using in-house prepared anti-TSST-1 rabbit polyclonal antibody (Lot 2-1990). Fractions containing TSST-1 were pooled, dialyzed at 4°C against pyrogen-free water with 6 changes of water for 48 h and lyophilized. TSST-1 was reconstituted in 10 ml of 25 mM Tris-acetate buffer, pH 8.3, and applied to a 1.5 x 30 cm column (Pharmacia K15/30) of pH 6-8 gradient polybuffer exchanger (PBE 94; Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 25 mM Tris-acetate, pH 8.3. TSST-1 was eluted with 12 bed volumes of polybuffer 96-acetate, pH 6.0 (Pharmacia Fine Chemicals, Uppsala, Sweden) at a flow rate of 36 ml/h. Fractions positive for TSST-1 as determined by SDS PAGE followed by western blotting (as above) were pooled and dialyzed against several changes of pyrogen-free water at 4°C and lyophilized. Toxin purity was accessed by SDS-PAGE followed by silver staining.

SEA and SEB were purchased from Toxin Technology (Sarasota, FL) and further purified by chromatofocusing using a pH 6 to 8 and a pH 6 to 9 gradient polybuffer exchanger respectively (PBE 94; Pharmacia Fine Chemicals, Uppsala, Sweden). 2 mg of SEA was reconstituted in 5 ml of TRIS-acetate buffer (25 mM; pH 8.3) and applied to a column (K15/30, 1.5 by 30 cm; Pharmacia) containing 80 ml of PBE 94 equilibrated with TRIS-acetate buffer. Elution of SEA was accomplished with polybuffer 96-acetate (pH 6.0; Pharmacia) at a flow rate of 36 ml/h. 10 mg of SEB was reconstituted with 5 ml of Ethanolamine-acetate buffer (25 mM; pH 9.4) and applied to a similar column containing 80 ml of PBE 94 equilibrated with Ethanolamine-acetate buffer. Elution of SEB was accomplished in the same way as SEA. Fractions were assayed for SEA and SEB by ELISA, and those containing SEA and SEB were pooled separately, dialyzed against

several changes of pyrogen-free water for 72 h at 4°C, and lyophilized. Purity of the purified toxins was assessed by running on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining. Each toxin preparation contained a homogeneous protein band with a size identical to the established molecular weight for the toxin.

2.2. Silver Staining and Western Blot

To assess the purity of the purified toxins, silver staining and western blot immunostaining after SDS-PAGE were performed. SDS-PAGE was performed using a Protean XII apparatus (16 cm x 18 cm x 1.5 mm slab gel, Bio-Rad Laboratories, Mississauga, Ontario). Samples were loaded onto a 4% stacking and 14% resolving gel prepared by standard procedures (Sambrook et al., 1989). The gel was then undergo silver staining using the silver staining kit (Bio-Rad) after electrophoretic separation of the samples according to manufacturer's instruction (Bio-Rad). On the other hand, immunoblot analysis was performed as previously described (Burnette, 1981). Following SDS-PAGE, proteins were transferred to a 0.45 μm nitrocellulose membrane at 150 mM for 45 min using a Semi-Dry Electroblotter A (Ancos Dimension Laboratories Inc., Mississauga, Ontario, Canada) with transblot buffer (25 mM Tris-HCL, 192 mM glycine, 20% methanol; pH 8.3). The nitrocellulose was incubated in 5% skim milk for 1 h at room temperature to block non-specific sites followed by overnight incubation in a 1: 100 dilution of polyclonal rabbit anti-serum raised against TSST-1 from S. aureus MN8 (Lot 2-1990) (Rosten et al., 1989) or 1 μg/ml of rabbit anti-serum to SEA (Lot 4-1993; Toxin Technology, Sarasota, FLA) or 1 μg/ml of rabbit anti-serum to SEB (Lot 3-1992; Toxin

Technology, Sarasota, FLA) or 1μg/ml of murine anti-TSST-1 MAb 5 (described below) in 5% skim milk (Difco). Following three 2-min washes with 20 mM Tris-buffered saline (pH 7.5) containing 1 % Tween-20 (TBS-T), the nitrocellose was rocked with either biotinylated goat-anti-rabbit immunoglobulin G (1:1000; Gibco/BRL) or biotinylated goat-anti-mouse immunoglobulin G (1:1000; Gibco/BRL), depending on the primary antibody used, in TRIS-buffered saline containing 0.5% bovine serum albumin (TBS-BSA) for approximately 2 h at room temperature. After washing with TBS-T, streptavidin-horseradish peroxidase conjugate (1:1000; Gibco/BRL) in TBS-BSA was added and the nitrocellose was incubated for another 30 min at room temperature. Following a final washing step with TBS-T, color development was achieved with 4-chloronapthol as described by the manufacturer (Gibco/BRL).

2.3. Quantitation of TSST-1 and SEs by Immunoassay

TSST-1 was quantitated by a non-competitive enzyme-linked immunosorbent assay (ELISA) procedure similar to the method previously described (Rosten et al., 1987). Purified rabbit polyclonal anti-TSST-1 antibodies (1 μg/ml) in 0.05 M bicarbonate-carbonate buffer (pH 9.6) was coated onto microtitre plates (0.1 ml/well) (ImmulonI, Dynatech Laboratories, Chantilly, VA) overnight at room temperature. Unbound antibodies were removed by three 2-min washes with PBS containing 0.05% Tween-20 (PBS-T). A TSST-1 standard (Toxin Technology) serially diluted in PBS-T and the samples were added in 0.1 ml volumes to the wells. After incubation at 37°C for 90 min, wells were washed with PBS-T, and 0.1 ml of biotinylated anti-TSST-1 (prepared using the Protein Biotinylation System (Gibco/BRL) following the manufacturer's instructions;

Lot 1-1995) diluted 1: 2000 in PBS-T was added to the wells. The ELISA plate was incubated for another 90 min at 37°C and was followed by three 2-min washes with PBS-T. Streptavidin-alkaline phosphatase (Gibco/BRL; diluted 1:2000 in PBS-T) was added (0.1 ml/well) and plates were incubated for 20 min at 37°C. Detection was performed as previously described (Rosten et al., 1987) using p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO). Absorbances were measured at 410 nm using a Dynatech MR5000 microplate reader (Dynatech Laboratories). The sensitivity limit of the ELISA was estimated to be 0.5 ng/ml of TSST-1.

SEA and SEB was quantitated by non-competitive ELISA procedure. Affinity purified anti-SEA or anti-SEB antibodies (Toxin Technology) (2 μg/ml) in 0.05 M bicarbonate-carbonate buffer (pH 9.6) were coated overnight at room temperature. Unbound antibodies were removed by three two-min washes with PBS-T. SEA or SEB standard (Toxin Technology), serially diluted from 128 to 0.5 ng/ml in PBS-T, were added in 0.1 ml volumes to the wells and were incubated for 90 min at 37°C. After washes with PBS-T, 0.1 ml of alkaline phosphatase conjugated anti-SEA (prepared by the method of Nakamura et al., 1986; Lot2-1993) or alkaline phosphatase conjugated anti-SEB (prepared by the method of Nakamura et al., 1986; Lot1-1994) diluted 1:250 was added to each well followed by incubation for another 90 min at 37°C. Finally, wells were washed with PBS-T and incubated with 0.1 ml of 1 mg/ml solution of p-nitrophenyl phosphate (Sigma) followed by measurement of absorbance (O.D.) at 410 nm. The sensitivity of the immunoassay was 1 ng/ml and 0.5 ng/ml of SEA and SEB respectively.

2.4. Preparation of Monoclonal Antibodies

IgG1 isotypic control was purchased from R&D Systems (Minneapolis). Anti-TSST-1 murine MAbs 5 and 6 were produced by hybridomas (5 & 6) generated by Kum W. W. S (University of British Columbia). The hybridomas were expanded in RPMI 1640 medium containing 15% heat inactivated fetal bovine serum (HyClone Laboratories, Inc. Utah). Then, they were injected i.p. (5×10^6 cells in 0.5 ml saline) into female BALB/c mice (8 weeks old) (Charles River Biological Laboratory, Montreal, Quebec, Canada) which were primed with 0.5 ml incomplete freund's adjuvant (Sigma) 3 days previously to generate ascites. MAbs from ascitic fluids were precipitated by addition of an equal volume of a saturated solution of ammonium sulfate. The precipitate was redissolved in PBS and the antibodies were further purified by protein G chromatography using the MAb Trap kit (Pharmacia Fine Chemicals) according to the manufacturer's instruction.

2.5. Characterization of Monoclonal Antibodies

To measure the relative titre of each MAbs, a range of MAb concentration were tested by ELISA for reactivity with various toxins. 1 μg/ml of toxins in 0.05 M bicarbonate-carbonate buffer (pH 9.6) were coated overnight at room temperature. Unbound toxins were removed by three two-min washes with PBS-T. PBS containing 3% of BSA (PBS-BSA) in 0.1 ml volumes was added to the wells and incubated for 90 min at 37°C to block any non-specific sites. After washes with PBS-T, MAb5 or MAb6 or IgG1 diluted in PBS-BSA or PBS-BSA alone was added in 0.1 ml volumes into the triplicate wells and plates were incubated for 90 min at 37°C. After washes with PBS-T, 0.1 ml of alkaline phosphatase conjugated goat anti-mouse IgG antibodies (R&D systems) diluted

1:1000 was added to each well followed by incubation for another 90 min at 37°C. Finally, wells were washed with PBS-T and incubated with 0.1 ml of 1 mg/ml solution of p-nitrophenyl phosphate (Sigma) followed by measurement of the absorbance (O.D.) at 410 nm. The titre was arbitrarily designated as the lowest concentration of MAb that gave an A_{410} of greater than or equal to 3 times background. For negative controls, toxins or MAbs were omitted from the wells.

2.6. Purification of Human peripheral blood mononuclear cells (PBMC)

Fresh human peripheral blood mononuclear cells (PBMC) were obtained from normal healthy donors and purified by Ficoll-histopaque 1.077 (Sigma) buoyant density gradient centrifugation as previously described (See et al., 1992). Cells at the interface were washed five times with Hank's balanced salt solution and diluted in growth medium [RPMI 1640 medium supplemented with 10 % fetal calf serum (heat inactivated at 56°C for 30 min), 2mM L-glutamine (GIBCO/BRL, Burlington, Ontario, Canada), and 20 μg/ml polymyxin B sulfate (Sigma)].

2.7. Mitogenicity assays

Mitogenicity induced by toxins was assessed using human blood mononuclear cells (PBMC) as indicators of proliferation. Incorporation of [^{3}H]thymidine into PBMC cellular DNA was used as an index of proliferation in a 3-day assay as previously described (Kum et al., 1993). 3×10^5 cells were cultured in 200 μl volumes with various concentrations of TSST-1 or SEB in round bottom 96-well tissue culture plates (Falcon Labware; Becton-Dickinson Canada Inc., Mississauga, Ontario, Canada) for 3 days at 37°C in 5% CO_2. For

MAb neutralization studies, various concentration of MAbs diluted in growth medium were pre-incubated with TSST-1 or SEB (100 pM of TSST-1 and 1pM of SEB; these doses are within the linear range of their respective dose-response curve) for 2 h at 37°C prior to the addition to the cells so as to promote antigen-antibody binding. At 48 h, cells were pulsed with 1 μCi of [^{3}H]-thymidine (5 Ci per mmol; Amersham, Arlington Heights, Ontario, Canada) and harvested 18 h later onto glass-fiber filter paper using an automatic harvester (Skatron, Sterling, Va, Norway). All samples were studied in triplicates and were counted in a liquid scintillation counter (LS1800, Beckman, Mississauga, Ontario, Canada). Controls included PBMC alone, PBMC plus MAb, and PBMC plus toxins.

2.8. Cytokine assays

3 x 10^5 cells were cultured in 200 μl volumes with various concentrations of TSST-1 or SEB in flat bottom 96-well tissue culture plates (Falcon Labware; Becton-Dickinson Canada Inc., Mississauga, Ontario, Canada) for 24h at 37°C in 5% CO_2. Again, for MAb neutralization studies, MAbs were prepared as described above except that 100 pM of TSST-1 and 10 pM of SEB were used (these doses are within the linear range of the dose-response curve). Culture supernatants were then collected, microfuged at 800 X g for 5 min, and frozen at -70°C until analysis. The presence of TNFα in stimulated culture supernatants was assayed by ELISA previously established in our laboratory (Kum et al., 1993; See et al., 1992). Goat anti-human TNFα (R&D Systems, Minneapolis, MN) at 2 μg/ml in 0.05 M bicarbonate-carbonate buffer, pH 9.6, was coated in 96-well ELISA plates overnight at 20°C (100 μl per well). Unbound antibodies were removed by three 2-min washes with PBS-T, followed by one wash with PBS alone. Human recombinant TNFα

(R & D Systems), serially diluted from 4000 to 17.5 pg/ml in PBS with 3% BSA, and samples were added in triplicates 100 µl volumes to respective wells and incubated at 37°C for 90 min. After washing with PBS-T, 100 µl of biotinylated goat anti-human TNFα antibody (Lot 2-1996; prepared using the protein biotinylation system (GIBCO/BRL, Burlington, Ontario)) diluted 1:2000 in PBS-BSA were added to the wells and the plates were incubated for 90 min. Plates were washed with PBS-T and incubated with 100 µl streptavidin alkaline-phosphatase (Sigma) (diluted 1: 2000 in PBS-BSA) for 20 min at 37°C. Wells were then washed 5 times with TBS, pH 7.5. An ELISA amplification system (Gibco/BRL) was then used to increase the sensitivity of the assay. Briefly, 50 µL of the BRL substrate was added into each well and incubated at room temperature for 15 min in the dark. Without removing the substrate solution, 50 µl of the BRL amplifier reagent was added and the plate was incubated for another 15 min in the dark. The reaction was stopped by the addition of 50 µl of 0.3 M H_2SO_4 and the O. D. at 495nm was measured using Dynatech MR 5000 microplate reader (Dynatech Laboratories). The sensitivity limit of the TNFα assay was 35 pg/ml.

2.9. Animal studies

D-galactosamine sensitized BALB/c mice were used to determine the neutralizing effect of monoclonal antibodies against SEB challenge as described previously (Miethke et al., 1992). Eight week old female BALB/c mice (20 g) (Charles River Biological Laboratory, Montreal, Quebec, Canada) were sensitized by i.p. injection of 20 mg D-galactosamine (Sigma) 1 hour prior to administration i.p. of a mixture of toxins and monoclonal antibodies. The mixture contained a concentration, predetermined to cause

90% lethality (Fig. 10), of either SEB or TSST-1 in the presence (5 X molar excess) or absence of monoclonal antibodies and were preincubated at 37°C for 2 hours. Death was recorded over a period of 72 hours. Mice injected with TSST-1 and MAb5 act as the positive control whereas mice injected with SEB alone serves as the negative control.

2.10. Synthesis of support-coupled peptides

In an attempt to map the cross-reactive epitopes on TSST-1 and SEB, overlapping peptides were synthesized by the method of Geysen et al. (Geysen et al., 1988) using the recommendations supplied in the commercially available epitope mapping kit (multipin peptide synthesis kit; Chiron Technologies, Clayton, Australia). Peptides were synthesized on polyethylene rods which have been radiation grafted (Geysen et al., 1984). This provides a reactive surface on the otherwise inert plastic, consisting of hydroxyl groups. These groups are then esterified with Fmoc glycine. The rods are then acetylated to "cap" any unreacted hydroxyl groups and so as to prevent them from reacting in the synthesis. The fmoc (9-fluorenylmethyloxycarbonyl) protecting group is removed and the free amine is coupled to Fmoc-β-alanine in a peptide bond. Again, the rods are acetylated to cap any unprotected, unreacted amine that may remain. This is the surface on the pins as supplied. All amino acids used in this study had their α-amino group protected with the 9-fluorenylmethyloxycarbonyl (Fmoc) group. The following side chain protecting groups were used: t-butyl ether (Bu^t) for serine, threonine and tyrosine; t-butyl ester (Obu^t) for aspartic acid and glutamic acid; t-butoxycarbonyl (Boc) for lysine, histidine and tryptophan; 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for arginine; and trityl (Trt) for cysteine. Protected amino acids were activated with diisopropylcarbodiimide (DIC)

(Sigma)/1-hydroxybenzotriazole (HOBt) (Chiron technologies). Synthesis of peptides was accomplished by repetitive cycles of Fmoc-protection, washing and amino acid coupling, adding one amino acid residue per cycle according to the manufacturer's instruction. After completing the synthesis of the desired peptides, the final Fmoc-deprotection group was removed and the terminal amino group can be capped by acetylation. Removal of side chain protecting groups was effected by trifluoroacetic acid (Fisher).

Two types of systematic peptide sets were synthesized. The first, referred to as a "scan", consisted of all possible overlapping peptides of a given length homologous with amino acid sequences of TSST-1 or SEB. For a protein with a linear sequence of N residues, there are thus (N-10) possible decapeptides. The second type of systematic peptides set, referred to as "nest set" or "window/window + 1 analysis set", comprises of all the possible linear tri-, tetra-, penta-, hexa-, hepta-, and octapeptides homologous with a given length of sequence.

2.11. . ELISA Testing

Support-coupled peptides were precoated with a pre-incubation mixture containing 10% ovalbumin (Sigma grade II) / 10% bovine serum albumin (Sigma fraction V)/ 1% Tween 20 (Sigma) in phosphate buffered saline (PBS) for 1 h at room temperature to block nonspecific absorption of antibodies. Overnight incubation at 4°C in a limited dilution (1 μg/ml) of MAb5 in the precubation mixture was followed by four washes in 0.5% Tween 20/PBS. Reaction for 1 h at room temperature with a 1:2500 dilution of goat anti-mouse IgG alkaline phosphatase conjugate in the pre-incubation mixture was again followed by extensive washing with Tween 20/PBS to remove excess conjugate. The presence of

antibody was detected by reaction with a freshly prepared developing solution (1 mg/ml solution of p-nitrophenyl phosphate (Sigma)) and the color produced was read in a Dynatech MR 5000 microplate reader (Dynatech Laboratories) at 410 nm. Color development was stopped when the absorbance of the most highly colored enzyme substrate solution reached an O.D. of approximately 2.3. This precaution was taken to reduce the possibility of non-linearity of the relationship between the amount of enzyme conjugate bound and ELISA absorbance. Prior to retesting, bound antibody was removed from the peptide by sonication in a disruption buffer containing 1% sodium dodecyl sulfate (Sigma), 0.1% 2-mercaptoethanol (Fisher), and 0.1 M sodium phosphate, pH 7.2. The rods, still bearing the covalently attached peptide, were then rinsed in water and methanol and used again or dried for storage.

For ELISA analysis, the large number of the peptides effectively acts as negative controls in the test. The observation of antibody binding to one rod but not to adjacent rods is already good evidence for the specificity of the test when peptides on adjacent rods differ in sequence by only one or two amino acids at their termini. The background level can thus be defined as the mean absorbance values for the set of peptides giving the lowest 25% of signals. The epitope was defined as the group of peptides with the highest significant mean signals that were greater than three standard deviation of the mean absorbance of all the peptides.

2.12. Inhibition of SEB-induced Mitogenesis by Synthetic Peptides

Peptides were prepared by NAPS Units, University of British Columbia. A scrambled peptide was made as a negative control. To assess the ability of peptides to

inhibit SEB-induced proliferation, peptides were added to the wells containing 3 x 10^5 cells human PBMC in round bottom 96-well tissue culture plates (Falcon Labware; Becton-Dickinson Canada Inc., Mississauga, Ontario, Canada). Then, 1 h later, SEB was added at a final concentration of 1 pM and incubated for 3 days at 37°C in 5% CO_2. At 48 h, cells were pulsed with 1 μCi of [^{3}H]-thymidine (5 Ci per mmol; Amersham, Arlington Heights, Ontario, Canada) and harvested 18 h later onto glass-fiber filter paper using an automatic harvester (Skatron, Sterling, Va, Norway). All samples were studied in duplicates and were counted in a liquid scintillation counter (LS1800, Beckman, Mississauga, Ontario, Canada).

2.13. Inhibition of SEB-Induced TNFα Production by Synthetic Peptides

To assess the ability of peptides to inhibit SEB-induced TNFα production, peptides were added to the wells containing 3 x 10^5 cells human PBMC in flat bottom 96-well tissue culture plates (Falcon Labware; Becton-Dickinson Canada Inc., Mississauga, Ontario, Canada). Then, 1 h later, SEB was added at a final concentration of 10 pM and incubated for 24h at 37°C in 5% CO_2. Culture supernatants were then collected, microfuged at 800 X g for 5 min, and frozen at -70°C until analysis. The presence of TNFα in stimulated culture supernatants was assayed by ELISA described previously.

2.14. Statistical Analysis

In vitro neutralization data and peptide inhibition data were analyzed by ANOVA. Data from in vivo neutralization studies were analyzed by the Fisher's exact test (1-tailed).

Differences were considered significant if the probability of a null hypothesis was less than 5 % ($P < 0.05$).

Chapter 3

Cross Neutralization of Toxic Shock Syndrome Toxin-1 and Staphylococcal Enterotoxin B by Anti-TSST-1 Monoclonal Antibodies

INTRODUCTION

Staphylococcal and streptococcal toxins, including toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxins (SEs) serotypes A, B, C1-3, D, E, G, H, and streptococcal pyrogenic toxins (SPEs) serotypes A, B, C, are known as pyrogenic toxin superantigens (PTSAgs) which cause profound disturbances on the homeostasis of the immune system (Schlievert et al., 1995, Bohach et al., 1990; Alouf et al., 1991). Their pathological effects range from an acute, but self-limiting, food poisoning caused by SEs, to a life-threatening toxic shock syndrome that can be caused by most PTSAgs (Kotzin et al., 1993; Tranter et al., 1995; Schlievert et al., 1995). Toxic shock syndrome (TSS) is characterized by fever, rash, desquamation of skin, widespread capillary leak, hypotension, shock, and multi-organ failure (Chow, 1993; Chesney et al., 1989). While the incidence of menstrual TSS associated with the use of tampons has declined from the peak in the 1980s, nonmenstrual TSS is becoming more prevalent in recent years and streptococcal toxic shock has emerged as a significant public health threat (Stevens, 1997; Chow, 1993). Furthermore, PTSAgs have also been implicated in other chronic immune-mediated diseases, such as rheumatoid arthritis and Kawasaki disease (Kotzin et al., 1993; Tranter et al., 1995).

The molecular and physiological events associated with acute TSS are well documented. PTSAgs bind to major histocompatibility complex (MHC) class II molecules outside of the peptide groove and, unlike conventional peptide antigens, require no prior processing for presentation to T cells (Marrack and Kappler, 1990; Jardetzky et al., 1994). Massive proliferation of T cells bearing specific Vβ elements on their receptors leads to uncontrolled release of cytokines, including interleukin (IL)-1α, -1β, -2, -4, -6, –10,

interferon (IFN)-γ, tumor necrosis factor (TNF)-α and β (Herman et al., 1991; Parsonnet, 1989; Miethke et al., 1993). It is this latter superantigenic property which underly the pathogenesis of TSS (Kain et al., 1993).

Biochemically, all PTSAgs are small polypeptides, 22-30 KDa, with a neutral to basic isoelectric point (Blomster-Hautamaa et al., 1986; Bergdoll, 1983; Schlievert et al., 1990). They are generally resistant to acid, heat, and protease (Bohach et al., 1990). In addition to similar biochemical properties, the PTSAgs share many immunobiological activities, such as the ability to induce fever, to enhance host susceptibility to endotoxin shock, and to stimulate T cell proliferation via a Vβ specific manner (Marrack and Kappler, 1990; Bohach et al., 1990). Further, sequence alignment indicates that a great deal of similarity exists among the SEs. PTSAgs can be divided into two groups based on sequence comparison (Ulrich et al., 1995; Ulrich et al., 1998). Group 1 contains SEB, SEC 1-3, SPEA and SPEC. Group 2 contains SEA , SED , and SEE (and probably SEH, only 38% identical to SEE, its closest relative). In contrast, TSST-1 and SPEB are hardly homologous to any of the members in the PTSAgs family. Furthermore, PTSAgs have classically been regarded as being antigenically distinct (Bohach et al., 1988; Fleischer, 1994). Historically, the SE antigenic heterogeneity actually provides the criteria for differentiating SEs into serotypes when they were classified. Except for the cross-reactivity between SEA and SEE and among SEC1, SEC2, and SEC3, serological assays do not indicate any other cross-reactivity among PTSAgs (Bergdoll et al., 1971; Avena et al., 1967; Reiser et al., 1984). Using more sensitive assays such as immunoblotting and coprecipitation, cross-reactivity among SPEA, SEB, and SEC1 with anti-sera against purified SEs has been demonstrated (Hynes et al., 1987). Others have identified

monoclonal antibodies which can cross-react with SEA, SEB, SEC, SED, and SEE in ELISA (Meyer et al., 1984; Shinagawa et al., 1991). Furthermore, Bohach et al showed that monoclonal antibodies against SPEA and SEC1 could neutralize mitogenicity induced by homologous and heterologous toxins (SPEA, SEC1, and SEB) (Bohach et al., 1988). However, none of the identified monoclonal antibodies against PTSAgs have demonstrated cross-reactivity between TSST-1 and the SEs or SPEs.

Initial efforts towards understanding the structural basis for common biological activities between TSST-1 and SEs have involved secondary structural predictions derived from techniques such as circular dichroism (CD) and tryptophan quenching. Despite the differences in antigenic properties and primary amino acid sequence, both SEs and TSST-1 exhibit a similar pattern of secondary structure, low α-helical content together with a high content of β structure, as indicated by CD analysis (Singh et al., 1988; Singh and Betley, 1989; Singh et al., 1988). Thus, the functional similarity of TSST-1 with other SEs may depend on active sites maintained at the level of secondary and tertiary structures. The recently reported crystal structures of SEB (Swaminathan et al., 1992), as well as TSST-1 (Prasad et al.,1993; Acharya et al., 1994; Papageorgiou et al., 1995)), SEA (Schad et al., 1995), SEC2 (Papageorgiou et al., 1995), and SED (Sundstrom et al., 1996) supported this hypothesis. Although there is considerable primary sequence dissimilarity between TSST-1 and the SEs, the crystallographic data reveals strikingly similar structural architectures among these PTSAgs. TSST-1 and other characterized PTSAgs exhibit a similar two-domain fold, a C-terminal β-grasp motif (domain A) and a characteristic N-terminal claw-like β barrel (domain B), with a long diagonal α-helix separating the two domains. Although some differences can be noted when these structures are superimposed, TSST-1

and other SEs share remarkably similar secondary and tertiary structures (Schad et al., 1994).

Recently, our laboratory has developed a neutralizing murine anti-TSST-1 monoclonal antibody (MAb5) which demonstrated significant cross-reactivity with SEB in ELISA. This finding suggested that cross-reactive epitopes are likely present on TSST-1 and SEB. Since previous studies have shown that MAb5 can neutralize various TSST-1 induced superantigenic activities both *in vitro* and *in vivo*, in this study, we want to evaluate if MAb5 can cross-neutralize similar functional activities induced by SEB. Our experimental approach included (i) an assessment of the reactivity of anti-TSST-1 MAb5 with various toxins in an ELISA, (ii) a determination of the ability of MAb5 to cross-neutralize SEB-induced mitogenesis and TNFα secretion in vitro, and (iii) an evaluation of the ability of MAb5 to neutralize the lethal effect of SEB in vivo.

RESULTS

Cross-reactivity of MAb5 with SEA and SEB.

Purified anti-TSST-1 monoclonal antibody, MAb5, was tested for its immunological reactivity to representative PTSAgs from the three sequence homology groups by ELISA and western blotting. Previously, the MAb5 had been isotyped and was determined to be of IgG1 subclass. Table VI shows the results of antibody titers of MAb5 against TSST-1, SEA, and SEB in ELISA. MAb5 reacted strongly with TSST-1, cross-reacted with SEB, and reacted weakly with SEA. The baseline titre of IgG1 isotypic control was consistently 100 μg/ml or more against all toxins. However, cross-reactions were not seen in the western immunoblot. The reactions between MAb5 to the homologous toxin, TSST-1, was strong and easily observed but no reactions between MAb5 to any of the heterologous toxins were seen.

Inhibition of SEB-Induced TNFα Secretion by MAb5.

Dose response study of SEB-induced TNFα secretion from Human PBMC was carried out to determine the dose of SEB used in the subsequent experiments. 10 pM of SEB, within the linear range of the dose response curve, was used for cytokine neutralization studies (Fig. 7). Serial dilutions of MAb5 and MAb6 were studied for their ability to inhibit SEB-induced TNFα production in human PBMC from 3 donors (Fig. 8). MAb5 significantly ($P < 0.05$) inhibited SEB-induced production of TNFα in a dose-dependent manner, whereas anti-TSST-1 MAb6, which serves as an isotypic control, had no effect.

Table IV.

Characterization of Anti-TSST-1 MAb5: reactivity in ELISA and Western Blot

Superantigens	ELISA titre[b] (μg/ml) Mab5	IgG1[c]	Western Blotting[d] with Mab5
TSST-1[a]	0.03	> 100	+++++
SEB	1.09	> 100	-
SEA	> 35	> 100	-

[a] Toxin was coated at 0.1 μg.
[b] Titre was designated as the lowest concentration that gave an A_{410} of 3 times background.
[c] IgG1 is the isotypic control.
[d] Relative intensities of the signals in Western Blot are indicated: - , no reaction; +++++, very strong reaction.

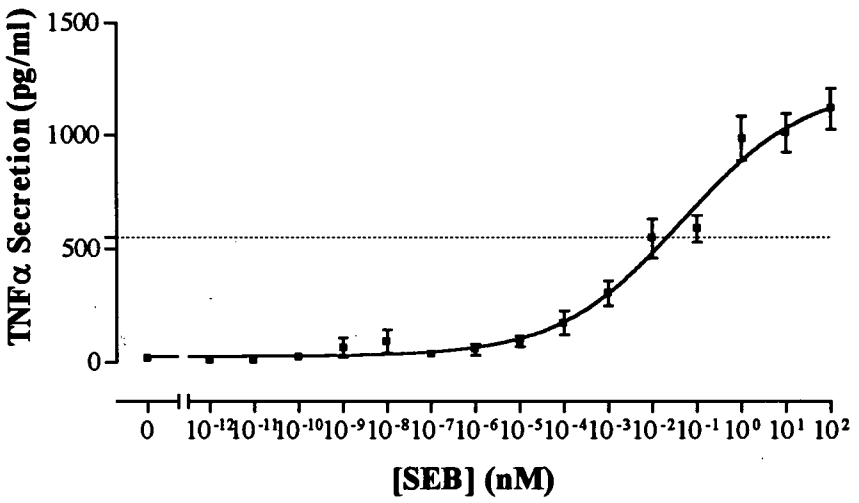


Figure 7. Dose response of SEB-induced TNFα secretion of human PBMC. $3X10^5$ cells were incubated with increasing concentration of SEB for 18 h at 37°C. Supernatants were then harvested and the concentration of TNFα in the supernatant was measured by TNFα assay. Results represent the mean ± SEM for triplicate measurement in 4 different donors.

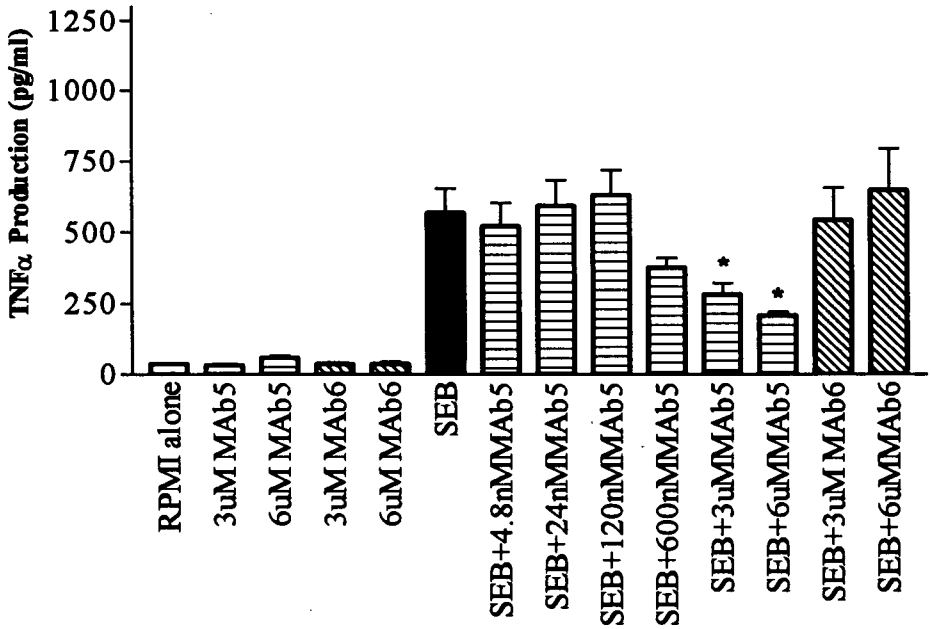


Figure 8. Effect of anti-TSST-1 MAb5 on SEB-induced TNFα secretion from Human PBMC. Results represent the mean ± SEM for triplicate measurement in 3 donors. * represents statistically significant inhibition ($p < 0.05$; 1-way ANOVA).

Inhibition of SEB-Induced Mitogenesis by MAb5.

Dose response study of SEB-induced human PBMC mitogenesis was carried out and 1pM SEB, within the linear range of the dose response curve, was used in the inhibition study (Fig. 9). Serial dilutions of MAb5 were tested for their ability to neutralize SEB-induced mitogenesis of human PBMC from 4 different donors (Fig. 10). MAb5 was able to inhibit SEB-induced mitogenesis significantly ($P < 0.05$) in a dose dependent manner. Again, MAb6 showed no effect.

Animal studies.

The in vivo effect of MAb5 was examined for its ability to neutralize SEB-induced lethality in the D-galactosamine sensitized BALB/c mouse model of lethal shock (Table VII). A predetermined dose of SEB LD_{90} was injected i.p. with or without MAbs one hour after D-galactosamine sensitization (Fig. 11). 15 animals per group were studied and death after 72 hours was the end point. At the end of the 72 hour period, 73% mortality (11 of 15) was observed in mice challenged with SEB. This was not significantly different from the mortality of 87% (13 of 15) in the presence of MAb5, or the mortality of 80% (12 of 15) in the presence of MAb6. Both MAb5 and MAb6 were unable to neutralize the lethal effect of SEB in D-galactosamine sensitized mice at the concentrations studied ($P > 0.05$, 1-tailed, Fisher's exact test).

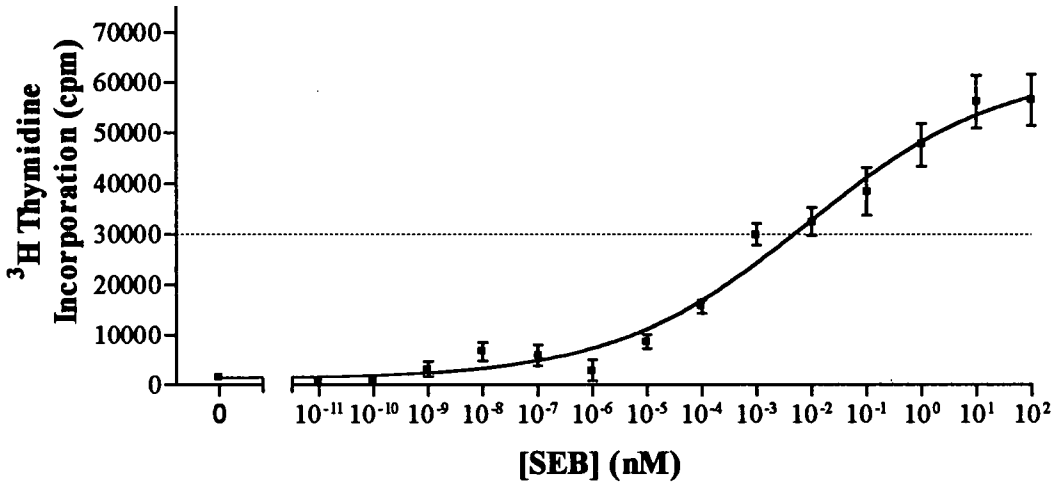


Figure 9. Dose response of SEB-induced mitogenesis of human PBMC. Results represent the mean ± SEM for triplicate measurement in 4 donors.

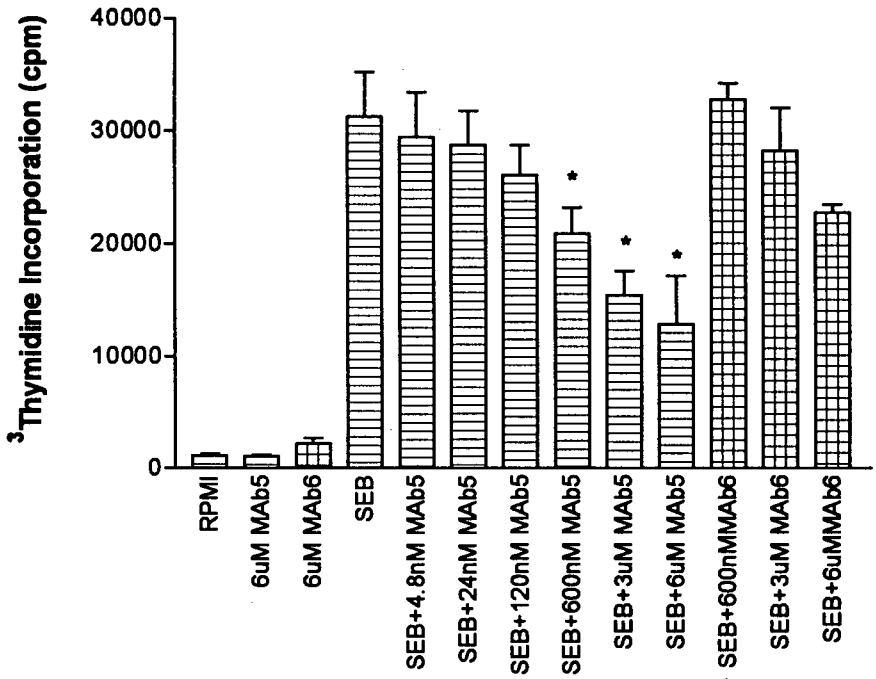


Figure 10. Effect of anti-TSST-1 MAb5 on SEB-induced mitogenesis of human PBMC. Results represent the mean ± SEM for triplicate measurements in 4 donors. * represents statistically significant inhibition ($p < 0.05$; 1-way ANOVA).

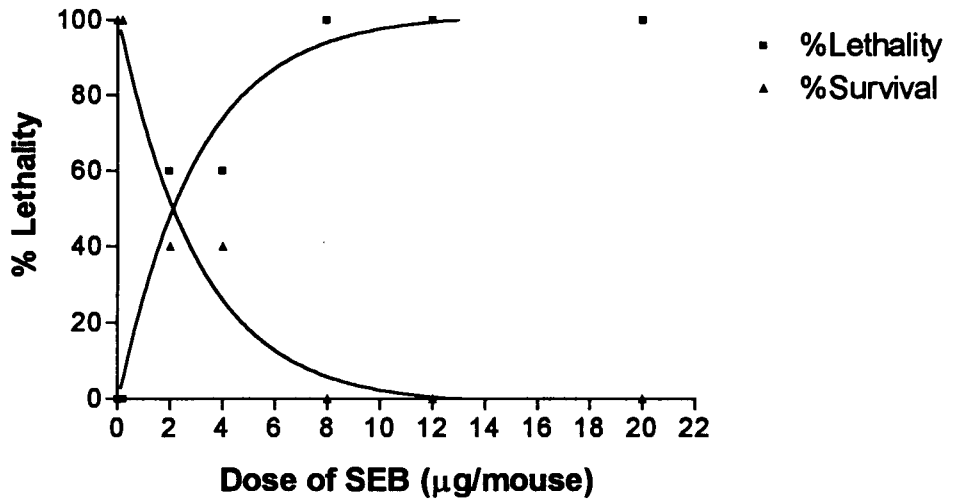


Figure 11. Dose response of SEB-induced lethality in D-galactosamine sensitized murine model.

Table VII.

Neutralization studies of Mab5 on SEB-induced lethality in D-galactosamine sensitized murine model.

Group	Dead/Total
Saline control	0/5
SEB alone	11/15
SEB + Mab5	13/15
SEB + Mab6	12/15
TSST-1 alone	4/5
TSST-1 + Mab5	0/5

DISCUSSION

In order to evaluate whether shared antigenic determinants exist on TSST-1 and SEB, a murine anti-TSST-1 neutralizing monoclonal antibody, MAb5, was assessed for its ability to cross-react with a panel of staphylococcal superantigens. The ELISA results indicated that MAb5 was capable of cross-reacting with SEB, suggesting that TSST-1 and SEB share at least one cross-reactive epitope, which was recognized by our MAb5. However, it is likely that this cross-reactivity was of lower affinity since 35 fold more MAb5 was required to demonstrate reactivity to SEB by ELISA compared to TSST-1 in ELISA. It is interesting to note that this cross-reaction is not detectable in western blot. This might be expected if the recognized determinant is a discontinuous conformational epitope which consists of linearly distant residues brought together by folding of the polypeptide chain. The denaturing condition in SDS-PAGE might disrupt the specific conformation of the epitope required for recognition by MAb5, and thus no longer recognizable in western blot. However, solid phase ELISA (used in this study) has the same problem as western blot since coating of toxins on the ELISA plates might also alter the conformation of the molecules. It is well documented that solid phase ELISA preferentially detects MAbs that recognize sequential epitopes and may not be effective in detecting MAbs that recognize conformational epitope (Brennand et al., 1986; Friguet et al., 1984; Vaidya et al., 1985; Fieseretal, 1987). An alternative possibility is that the cross-reactive epitope is inaccessible to MAb5 in western blot, or that western blot is not sensitive enough to detect this low affinity cross-reaction. Even though the nature of the recognized SEB eptiope, whether continuous or conformational, is not distinguishable at

this point, our ELISA data strongly suggested that MAb5 recognizes cross-reactive epitope on SEB.

Anti-TSST-1 MAb5 could neutralize the superantigenic activity, including mitogenicity and TNFα release, induced by both TSST-1 (Fig. 12 and Fig. 13) and SEB (Fig. 8 and Fig. 10). However, MAb5 was less effective at neutralizing SEB. Approximately, 1000 fold more MAb5 was required to significantly inhibit both SEB-induced mitogenesis and TNFα release than that needed for the neutralization of TSST-1. In addition, MAb5 can only partially neutralize SEB-induced activity. In contrast, MAb5 caused nearly complete neutralization of TSST-1. The ineffectiveness of MAb5 in completely neutralizing SEB-induced activity in vitro is expected and can be attributed to its lower affinity to SEB than to TSST-1 as reflected in the ELISA assay. However, the in vitro data strongly suggest that TSST-1 and SEB share cross-neutralizing epitopes.

Previously, our laboratory has demonstrated the neutralizing effect of MAb5 on TSST-1-induced lethality (LD90) in D-galactosamine mice using a dose of TSST-1: MAb5 molar ratio of 1:5. In order to evaluate if MAb5 can also neutralize SEB-induced lethality in vivo, the same molar ratio of SEB (LD90) and MAb5 was used in this pilot study. It is not surprising to observe that MAb5 was not able to neutralize SEB-induced lethality *in vivo* in this study, since 1000 times more MAb5 was required to significantly cross-neutralize SEB-induced activity in vitro. Based on the in vitro data, it seems logical to predict that a much higher dose of MAb5 might be needed to demonstrate any cross-neutralizing effect in SEB-challenged mice, although the precise mechanism for SEB-induced toxicity and the protective mechanism of MAb5 in the murine model are not fully

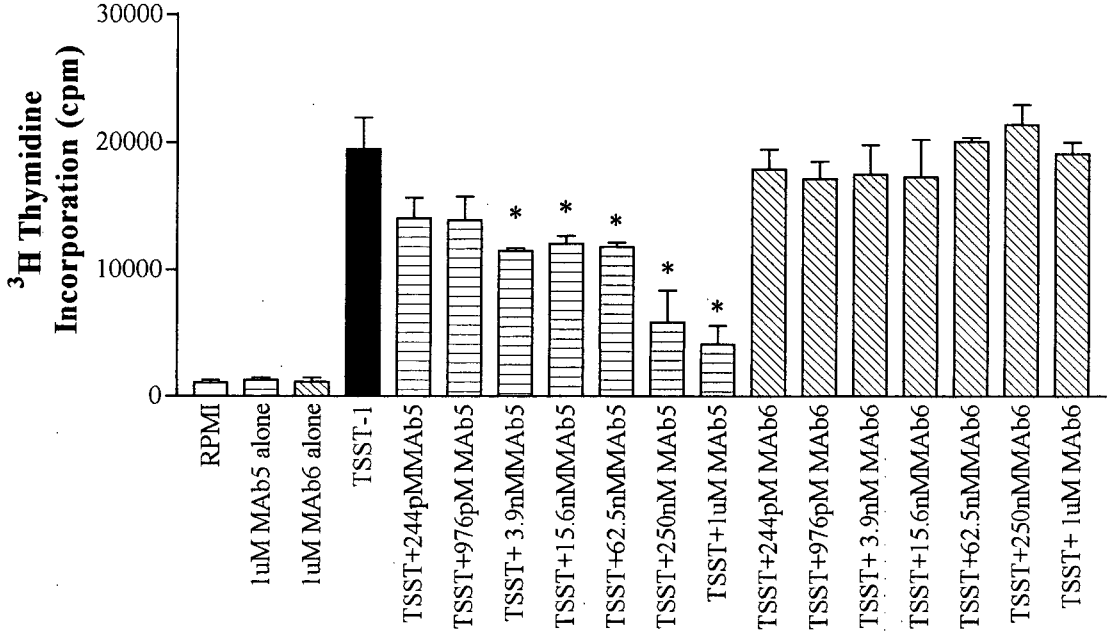


Figure 12. Effect of anti-TSST-1 MAb5 on TSST-1-induced mitogenesis of human PBMC. Results represent the mean ± SEM of triplicate measurement in 2 donors. * represents statistically significant inhibition ($p < 0.05$; one-way ANOVA).

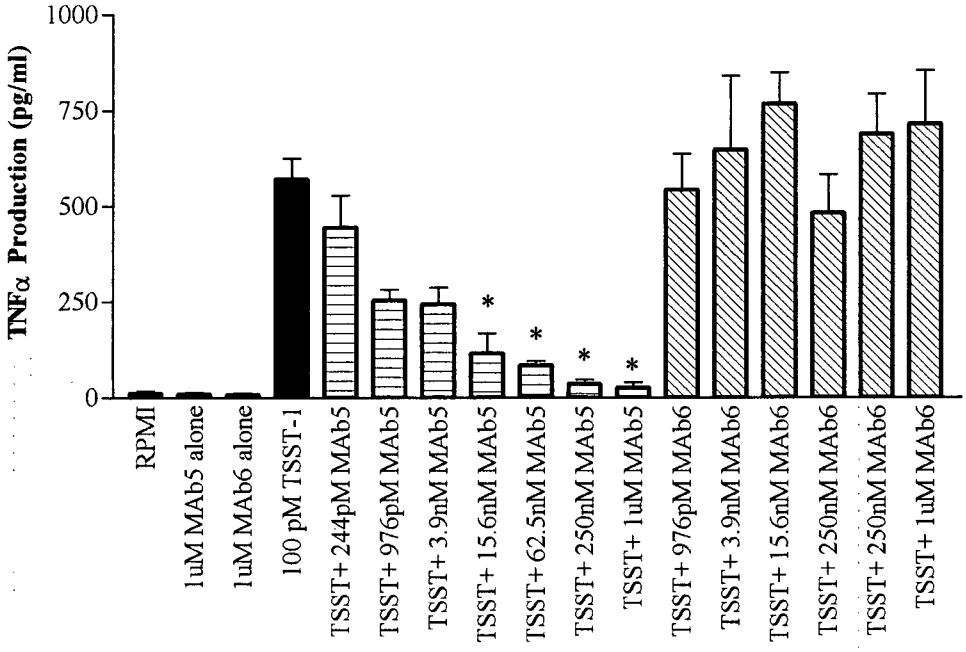


Figure 13. Effect of anti-TSST-1 MAb5 on TSST-1-induced TNFα secretion from human PBMC. Results represent the mean ± SEM of triplicate measurement in 2 donors. * represents statistically significant inhibition ($p < 0.05$; one-way ANOVA).

understood. Thus, there are insufficient data to conclude that MAb5 is not able to cross-neutralize SEB-induced lethality in vivo. A dose response experiment should be done to further evaluate the cross-neutralizing effect of MAb5 in vivo.

The results obtained with our MAb studies showed that determinants on SEB and TSST-1 are located at or near a functionally important site on the toxins required for mitogenicity and cytokine production, two biological properties shared by all staphylococcal superantigens. Although it is generally known that TSST-1 exhibits minimal sequence homology with SEs, amino acid sequence alignment analysis reveal that the PTSAgs possess 4 uniformly conserved consensus sequences (Hoffmann et al., 1994). Fig. 14 illustrates the 4 regions with large degree of homology between the amino acid sequences of PTSAgs family. It is possible that these conserved regions or motifs adjacent to these regions are recognized by MAb5 and are important functional domains for mitogenicity, TNFα release and possibly other shared immunobiological properties of these PTSAgs. However, no conclusive evidence has been shown that all these conserved regions are responsible for superantigenic activities of all PTSAgs and the exact location of our recognized cross-reactive epitopes are not known. Alternatively, the motifs that direct all these common biological activities might be derived from the folding of PTSAgs, and these conformational epitopes might be found in other parts of the molecules. Thus, definitive studies are necessary to determine the nature and location of these cross-reactive determinants.

Realizing that PTSAgs elicit superantigenicity through binding to the major histocompatibility complex (MHC) class II molecules and interacting with the specific Vβ region of the T cell receptor (TCR), it is presumed that MAb5 neutralized SEB-induced

Region 1

Toxin	Residue #	
SEA	79	K Y K G K K V D L Y G
SEB	76	K Y K D K Y V D V F G
SEC1	76	K Y K D E V V D V Y G
SEC2	76	K Y K D E V V D V Y G
SEC3	76	K Y K D E V V D V Y G
SED	74	H F K S K N V D V Y P
SEE	76	K Y K G K K V D L Y G
SPEA	70	L F K D K N V D I Y G
SPEC	63	F K R D D H V D V F G
TSST-1	56	F T K G E K V D L N T

Region 3

Toxin	Residue#	
SEA	147	K K N V T V Q E L D L Q A R R Y L
SEB	152	K K K V T A Q E L D Y L T R H Y L
SEC1	151	K K S V T A Q E L D I K A R N F L
SEC2	151	K K S V T A Q E L D I K A R N F L
SEC3	151	K K S V T A Q E L D I K A R N F L
SED	142	K K N V T V Q E L D A Q A R R Y L
SEE	144	K K E V T V Q E L D L Q A R H Y L
SPEA	137	K K V T A Q E L D Y K V R K Y L
SPEC	124	K D I V T F Q E I D F K I R K Y L
TSST-1	121	K K - - - - Q - L - I
TSST-1	129	L D F E I R H Q L

Region2

Toxin	Residue #	
SEA	106	C Y G G V T L H D N N
SEB	113	C Y G G V T E H N G N
SEC1	110	C Y G G I T K H E G N
SEC2	110	C Y G G I T K H E G N
SEC3	110	C Y G G I T K H E G N
SED	101	C T Y G G V T P H E G N
SEE	103	C Y G G V T L H D N N
SPEA	98	C I Y G G V T N H E G N
SPEC	85	Y I Y G G I T P A Q N N
TSST-1	83	F Q I S G V T N T E K L

Region4

Toxin	Residue#	
SEA	209	L L R I Y R D N K T I N S E
SEB	213	Y L Y N D N K V D S K
SEC1	213	Y L Y N D N K T V D S K
SEC2	213	Y L Y N D N K T V D S K
SEC3	213	Y L Y N D N K T V D S K
SED	204	Q L R I Y S D N K T L S T E
SEE	206	L L R I Y R D N K T I N S E
SPEA	197	Y L I Y K D N E T L D S N
SPEC	184	I F A K Y K D N R I I N K
TSST-1	179	P P I N I D E I K T I E A E

Figure 14. Conserved regions of primary structure among sequences in the PTSAg family. Residue # refers to the primary sequence position of the first (N-terminal) conserved residue in each region.
Taken from Hoffmann et al., 1994.

superantigenic activity by binding to the toxin and blocking these interactions (Hamad et al., 1994; Shimonkevitz et al., 1996). However, further studies are needed to deduce the precise mechanism involved in the cross-neutralizing activity exhibited by MAb5.

The report published recently by Ulrich and his coworkers, while this manuscript was in preparation, demonstrated that antibodies induced by SEB mutant vaccines cross-reacted with heterologous PTSAgs, including TSST-1, SEA, and SEC1 (Ulrich et al., 1998). They further showed that this vaccine protected both mice and rhesus monkeys from lethal toxic shock when they were challenged with these toxins (Ulrich et al., 1998). Interestingly, these cross-reactive antibodies also had a low titre for TSST-1 and confer partial protection when the animals were challenged with TSST-1 (Ulrich et al., 1998). However, the precise locations of these cross-reactive antigenic epitopes were not known. Our observations in this study substantiate the fact that TSST-1 and SEB do share common cross-reactive epitopes that are important for superantigenic activities.

Chapter 4

Identification of Cross-Neutralizing Epitopes on Toxic Shock Syndrome Toxin-1 and Staphylococcal Enterotoxin B by Epitope Peptide Mapping and Synthetic Peptide Inhibition Studies.

INTRODUCTION

Staphylococcal and streptococcal pyrogenic superantigens (PTSAgs) are exotoxins that are associated with a variety of immune-mediated diseases, including food poisoning, toxic shock syndrome (TSS), and other chronic immune disorders, due to their profound effects upon the immune systems (Kotzin et al., 1993; Schlievert et al., 1993). PTSAgs are characterized by their ability to bind major histocompatibility (MHC) class II receptors on antigen presenting cells (APCs) and T cell receptors (TCR) which express particular Vβ elements (Herman et al., 1989; Fields et al., 1996; Schlievert et al., 1997). These interactions lead to massive proliferation of T cells and excessive release of proinflammatory cytokines (Hackett wt al., 1993; Grossman et al., 1992). The latter superantigenic properties are thought to be responsible for the pathogenesis of TSS (Parsonnet, 1989).

One of the ways to control PTSAgs-associated disease is vaccination. Formaldehyde inactivated toxoid vaccine had been developed in an attempt to prevent TSS. However, the vaccination approach to controlling SAg-associated disease is complicated by the diversity of PTSAgs produced by clinical isolates. PTSAgs can be divided into sequence homology groups consisting of (1) SEA, SED, and SEE; (2) SEB, SEC1, SEC2, SEC3, streptococcal superantigen (SSA), SPEA, and SPEC; (3) TSST-1 (Ulrich et al., 1995; Ulrich et al., 1998). It would not be practical to develop specific vaccines for each of these PTSAgs. Despite the considerable sequence divergence among these PTSAgs, they exhibit strikingly similar protein folds and structural architectures (Swaminathan et al., 1992; Schad et al., 1995, Sundstrom et al., 1996, Acharya et al., 1994;

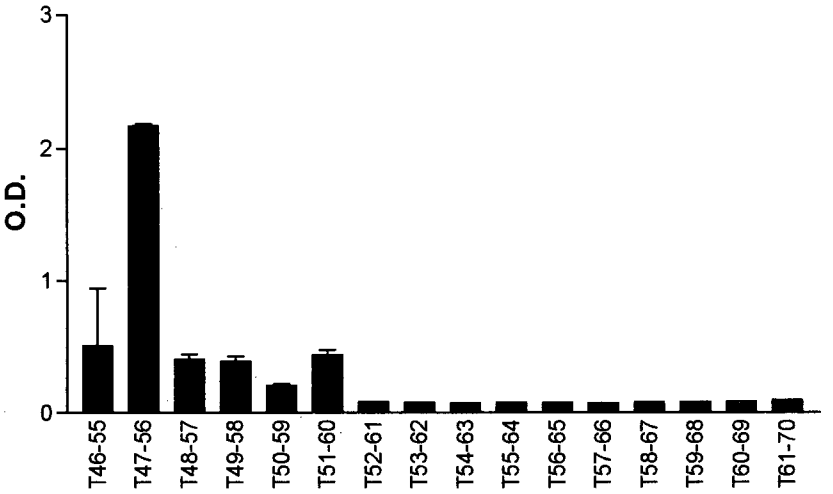
Papageorgiou et al., 1995). Recently, Ulrich et al. demonstrated that cross-reactive antibodies against most PTSAgs could be induced by immunizing mouse with just one mutant PTSAg vaccine and thereby suggested the presence of shared neutralizing epitopes among these toxins (Ulrich et al., 1998). Yet, the precise locations of these antigenic determinants are not known. This information, however, is essential for developing a common, cross-reactive vaccine. Therefore, before an effective vaccine can be developed, it is important to have more information about critical recognition sites that can induce cross-reactive antibodies for neutralization against the whole panel of PTSAgs. To this end, much effort had been focused on the mapping of shared antigenic determinants of the SEs within the sequence homology groups, especially SEB and SEC1 (Spero and Morlock, 1979; Thompson et al., 1984, Turner et al., 1992). Little is known about shared antigenic determinant across different sequence homology groups. Previously, our laboratory had developed an anti-TSST-1 monoclonal antibody, MAb5, which was able to neutralize TSST-1, as well as SEB-induced superantigenic activities in vitro. In the current study, by mapping the neutralizing domains on TSST-1 and SEB recognized by MAb5, we were able to describe two topographically different epitopes in TSST-1 and SEB in which functional activities were located.

RESULTS

Determination of a TSST-1 Epitope recognized by MAb5. In order to precisely identify the epitope on TSST-1 defined by MAb5, overlapping decapeptides spanning a small portion of TSST-1 sequence (T46-70) were made since this region has been previously characterized as containing epitope(s) binding to MAb5 (Kum et al., unpublished data). The amino acid sequence of TSST-1 had been deduced from the nucleotide sequence of TSST-1 (Lee et al., 1992). The results obtained for the synthesized decapeptides when tested by ELISA with two different antibodies, anti-TSST-1 MAb5 and isotypic control IgG1, were shown in Fig.15a and Fig. 15b. Using limited dilution of MAb5 (1 μg/ml), scan (a) in Fig.15 showed that MAb5 reacted strongly with one single epitope, ^{47}FPSPYYSPAF^{56}. The isotypic control, IgG1, did not react with any of the peptides as shown in Fig. 15b, thereby demonstrating the specificity of the reaction with MAb5.

In order to identify the minimum TSST-1 antigenic sequence in contact with MAb5, MAb5 was reacted with multiple sets of overlapping peptides, which differ only in length, spanning the TSST-1 sequence ^{47}FPSPYYSPAF^{56}. This multiple length scanning or "window" approach, analogous to viewing the protein through multiple windows of different width, identifies the location of linear epitopes and define their boundaries or ends of minimal antibody-binding sequence (Geysen et al., 1987). It can be seen in Fig. 16. that none of the tri- or tetrapeptides in this region reacted with MAb5, but one pentapeptide, ^{52}YSPAF^{56} did. Window/window+1 analysis applied to the penta- and

(a)



(b)

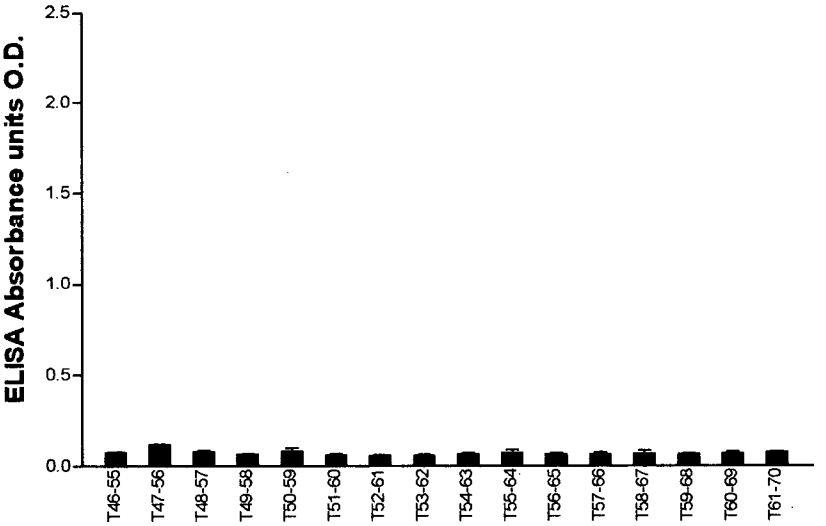


Figure 15. Scan of TSST-1 (46-70) decapeptides with (a) anti-TSST-1 MAb5; (b) isotypic control IgG1.

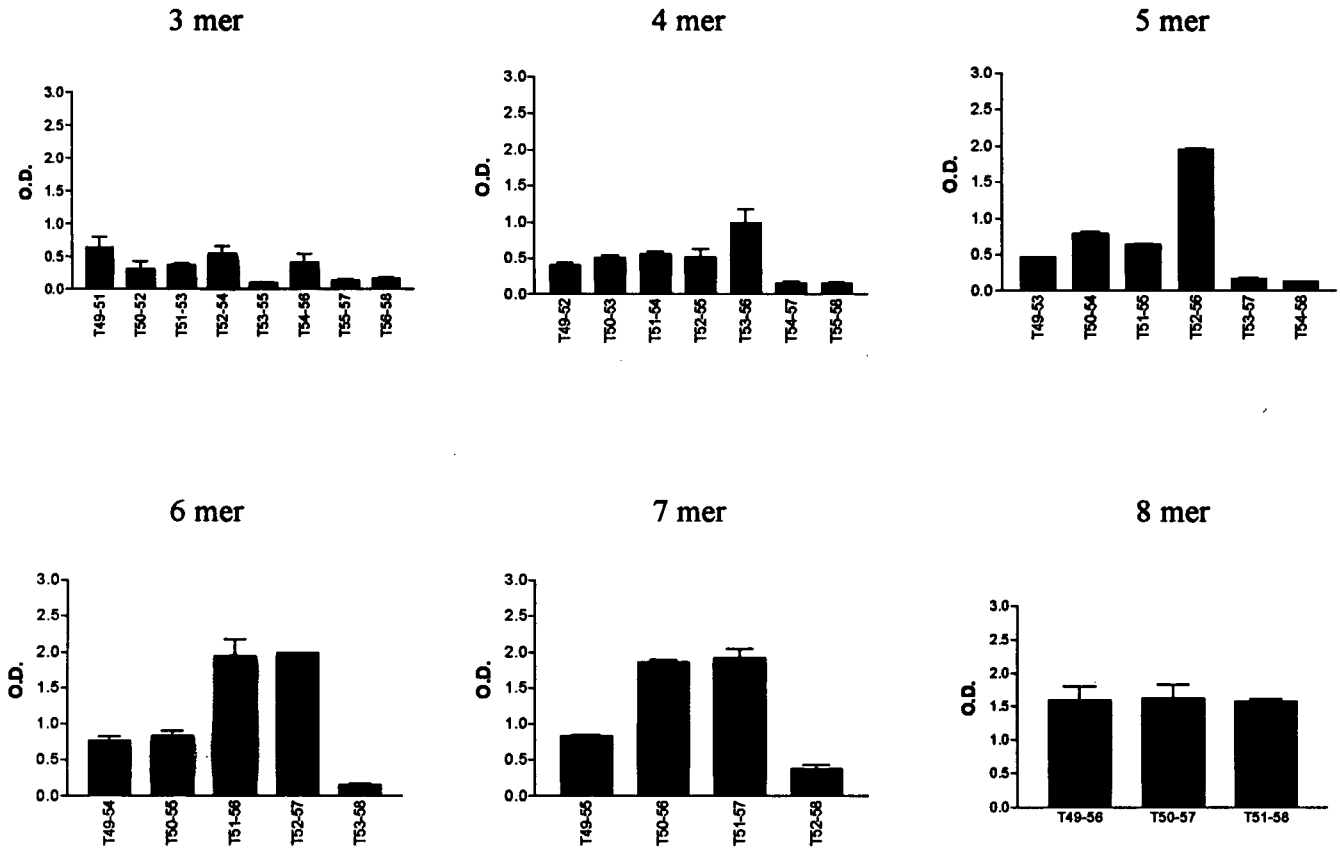


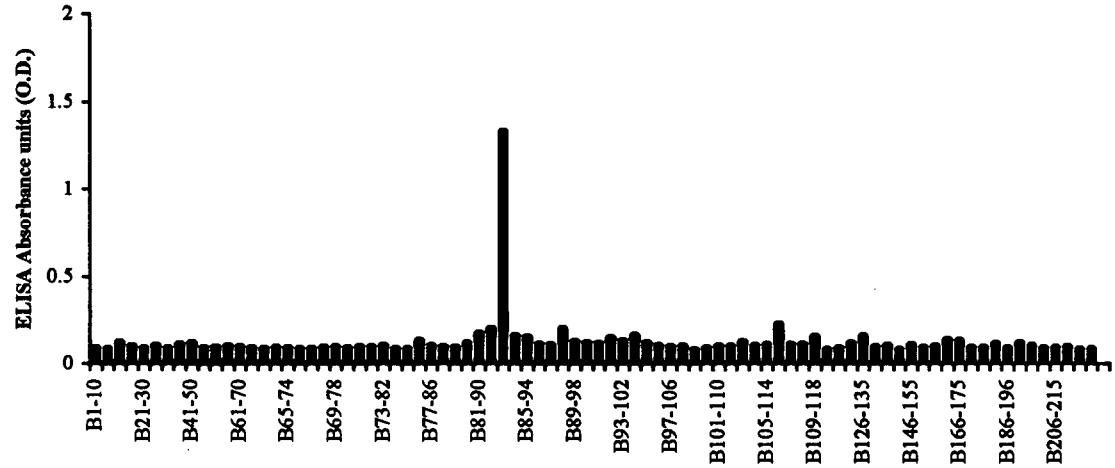
Figure 16. Window/ window + 1 analysis on TSST-1 epitope T(47-56).

hexapeptide set demonstrates that the omission of the N-terminal Y51 from ^{51}YYSPAF^{56} or C-terminal T57 of ^{52}YSPAFT^{57} did not reduce the reactivity of pentapeptide ^{52}YSPAF^{56}, suggesting that Y51 and T57 were not directly involved in antibody binding. However, analysis of the tetra- and pentapeptide set demonstrates that further omission of N-terminal Y52 or C-terminal F56 abolished the reactivity of tetrapeptide ^{53}SPAF^{56} and ^{52}YSPA^{55}. Taken altogether, these results suggest the presence of a single sequential epitope comprising the five residues ^{52}YSPAF^{56}, represents the minimum epitope needed for recognition by MAb5.

Determination of a SEB epitope recognized by MAb5. To reveal MAb5's cross-reactive epitope on SEB, the initial attempt was to synthesize a series of overlapping decapeptides corresponding to SEB (residues 62-118), since secondary structural alignment of TSST-1 and SEB revealed that TSST-1 (41-75) aligned with SEB (63-89) (Acharya et al., 1994). Using a limited concentration of MAb5, ELISA testing indicated that MAb5 also recognized one single peptide, ^{83}DVFGANYYYQ^{92} with a good signal to background ratio (Fig. 17a). Again, the isotypic control IgG1 did not react with any of the peptides, indicating the specificity of the reaction with MAb5 (Fig. 17b).

In order to further characterize this SEB epitope, ^{83}DVFGANYYYQ^{92}, peptides corresponding to the complete set of all the possible linear octa-, hepta-, hexa-, and pentapeptide homologous with the native sequence were synthesized. Significant binding of MAb5 to pentapeptides ^{84}VFGAN^{88} and ^{85}FGANY^{89} was still observed when the peptide target was sequentially reduced from octapeptides to pentapeptides, suggesting that the sequential epitope defined by MAb5 on SEB comprises fewer than 5 residues, likely within the tetrapeptide ^{85}FGAN^{88} (Fig. 18).

(a)



(b)

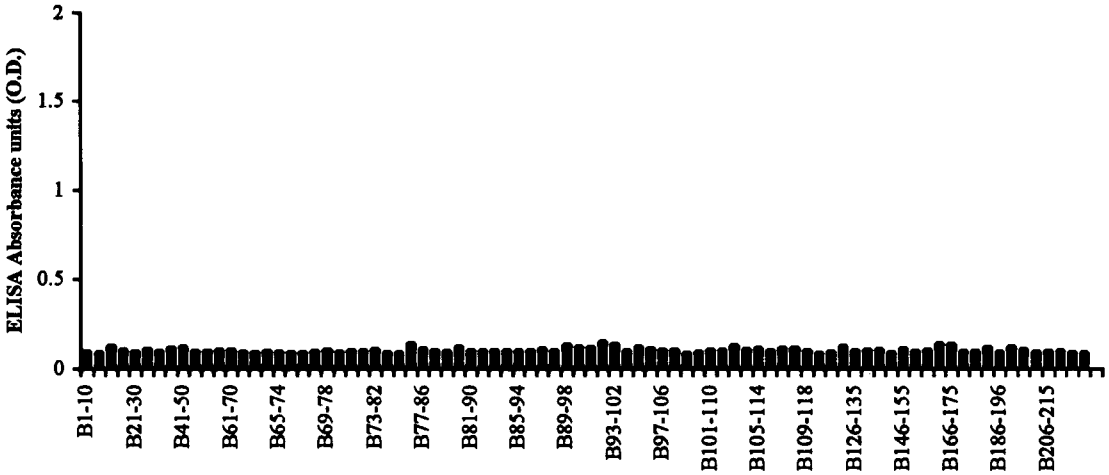


Figure 17. Scans of SEB (1-239) decapeptides with (a) anti-TSST-1 MAb5; (b) isotypic control, IgG1.

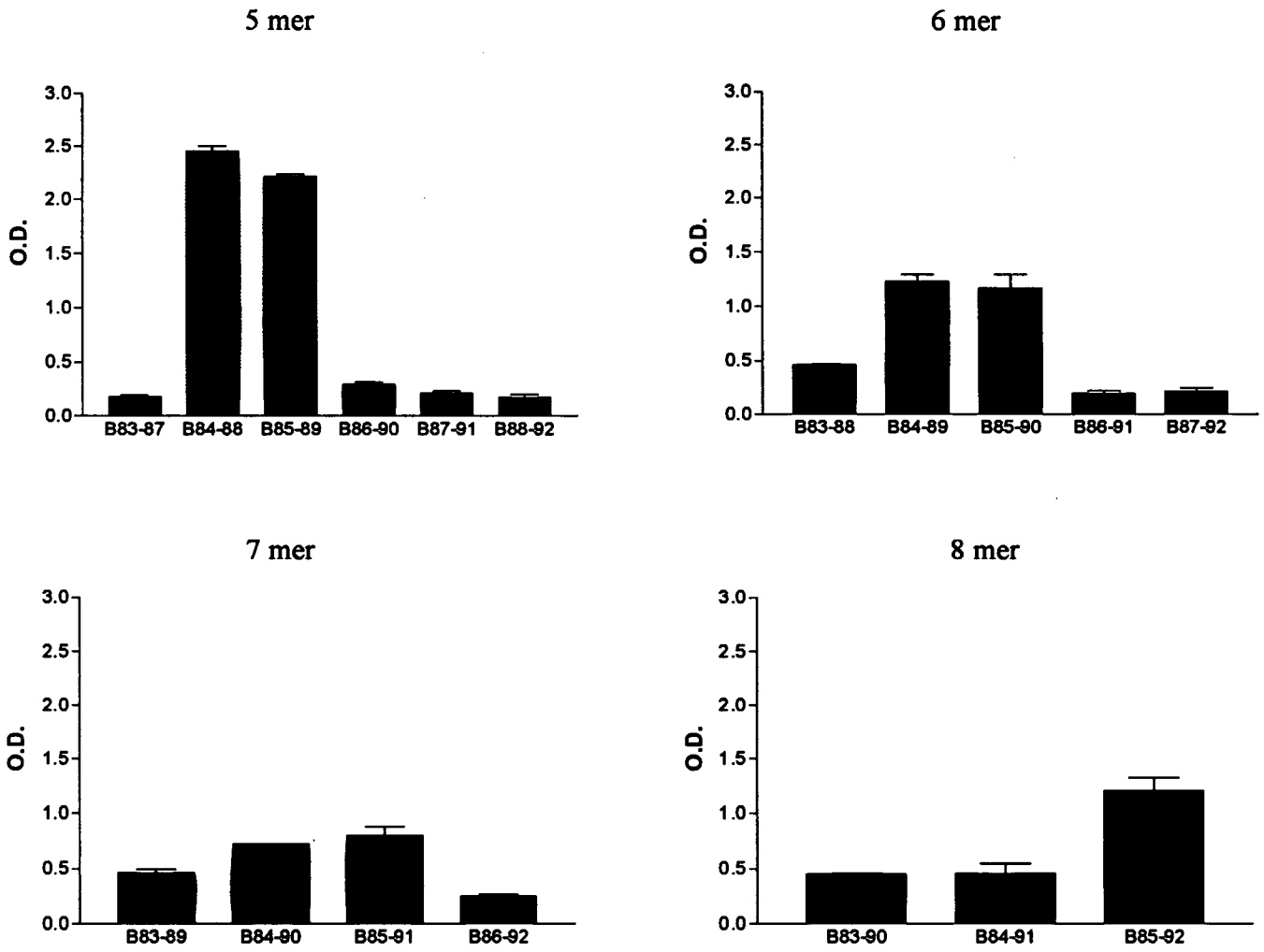


Figure 18. Window/window + 1 analysis on SEB epitope (83-92)

Peptide inhibition studies. In an attempt to relate the mapped epitopes with function, synthetic peptide corresponding to SEB (residues 83-92), ^{83}DVFGANYYYQ^{92}, was tested for its ability to inhibit SEB-induced superantigenic activities. First of all, peptides were tested for stimulation of proliferation and TNFα production. Neither SEB peptide nor the scrambled control peptide showed any increase over control values. Next, these peptides were tested for their ability to inhibit SEB-induced superantigenic activities. SEB(83-92) significantly ($p < 0.05$) inhibited both SEB-induced mitogenesis and TNFα production in a dose dependent manner while the scrambled peptide had no effect (Fig. 19 and Fig. 20). SEB(83-92) was effective at 1.5 mM for inhibition of both SEB-induced mitogenesis and TNFα production.

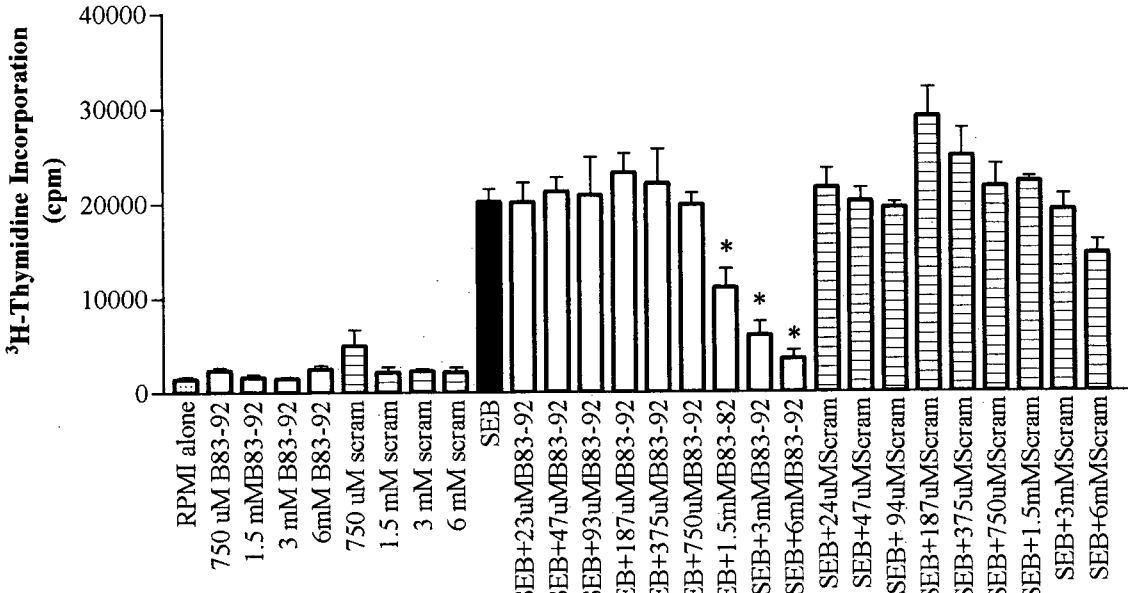


Figure 19. Effect of SEB peptide (83-92) on SEB-induced mitogenesis of human PBMC. B83-92 represents SEB peptide (83-92); Scram represents scrambled control peptide. Results represent the mean ± SEM for duplicate measurement in 3 donors. * represents statistically significant inhibition ($p < 0.05$; one-way ANOVA).

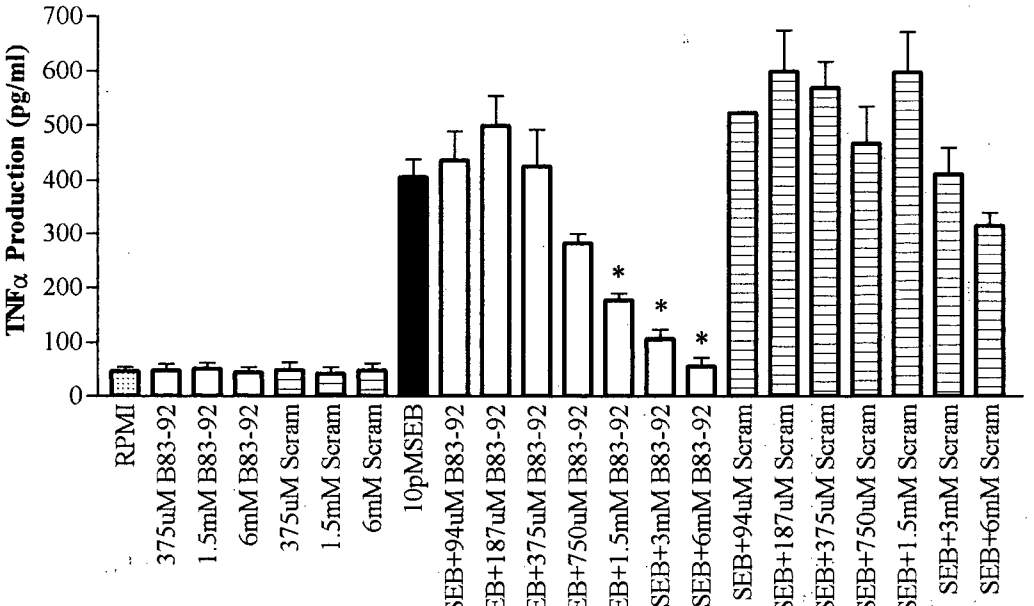


Figure 20. Effect of SEB peptide (83-92) on SEB-induced TNFα secretion of human PBMC. B83-92 represents SEB peptide (83-92); Scram represents scrambled control peptide. Results represent the mean ± SEM for duplicate measurement in 3 donors. * represents statistically significant inhibition ($p < 0.05$; one-way ANOVA).

DISCUSSION

The anti-TSST-1 MAb5 was initially of interest because it not only cross-reacted with SEB by ELISA, but also neutralized both TSST-1- and SEB-induced superantigenic activities, suggesting the presence of cross-reactive functional epitopes between these two toxins (see chapter 2). The present study describes the epitope mapping of MAb5 against TSST-1 and SEB sequences. Peptide scanning, together with window/window+1 analysis revealed that MAb5 preferentially reacted strongly with peptides corresponding to TSST-1 ^{52}YSPAF^{56}, SEB ^{84}VFGAN^{88}, and SEB ^{85}FGANY^{89}, suggesting that regions TSST-1 (52-56) and SEB (85-88) are binding sites of MAb5. These apparently conserved epitopes on TSST-1 and SEB correspond to region 1 in Fig. 14. Although the sequences of these peptides were not identical, they were composed of similar residues including alanine (A), tyrosine (Y) and phenylalanine (F). When TSST-1 ^{52}YSPAF^{56} is superimposed onto SEB ^{84}VFGAN^{88} and SEB ^{85}FGANY^{89}, the r.m.s. (raw mean square) differences in α-carbon positions for these 5 residues are 1.4Å and 1.3Å respectively. These structural motifs are all extended structures and share a very similar backbone conformation (Fig. 21). When the structural motif of TSST-1 ^{52}YSPAF^{56} and SEB ^{85}FGANY^{89} are compared, it seems that the first and last residues of the epitopes can be conservatively substituted with aromatic residues. These observations suggest that MAb5 might have a relatively low degree of specificity and is able to bind several comparatively loosely related peptides. Although TSST-1 epitope ^{52}YSPAF^{56} and SEB epitope ^{84}VFGANY^{89} are topologically located at different regions of the molecules according to the crystal structures of TSST-1 and SEB, both regions are very solvent exposed and thus immunoaccessible. When the α-

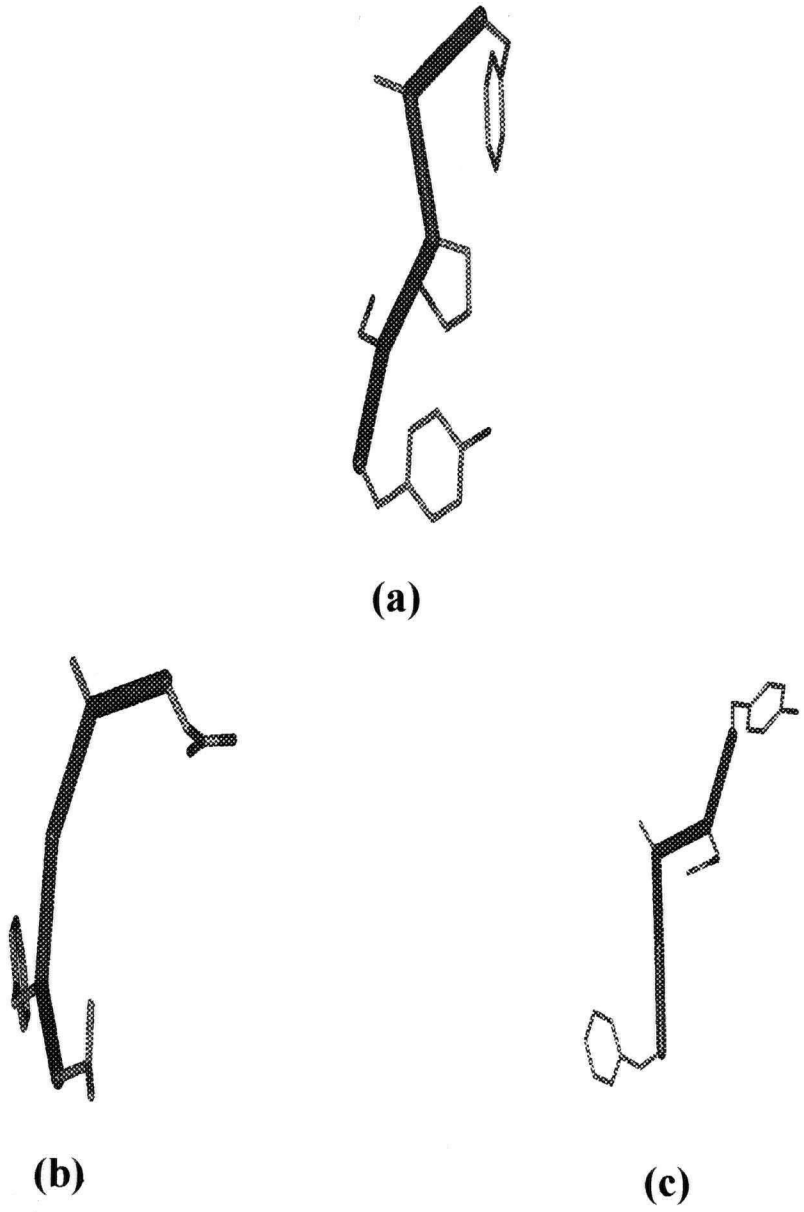


Figure 21. Structural motif of (a) TSST-1 ^{52}YSPAF^{56}, (b) SEB ^{84}VFGAN^{88}, and (c) SEB ^{85}FGANY^{89}.

carbons of SEB were superimposed with TSST-1 using the computer-assisted protein modeling program O (Alwyn Jones and Morten Kjeldgaard, version 6.2.2, 1994), SEB (84-89) was shown to align with TSST-1 (64-69). However, TSST-1 epitope recognized by MAb5 was mapped to TSST-1 (52-56) instead. One possible explanation for these discordant observations may relate to the stereochemical properties of these peptides. However, these were not apparent from examining the crystal structures. It is important to note that these peptides are only mimicking the native protein and their lack of reactivity with a particular antibody does not necessarily exclude the actual presence of epitopes within that region of the molecule, which could be recognized by the antibody. On the other hand, binding of antibody to peptides may be possible due to partial recognition. Thus, caution must be taken in interpreting the results of epitope mapping with peptides. In addition, the approach used in this study can be expected to detect only continuous (sequential), not discontinuous (conformational) epitopes (Benjamin et al., 1984). It is believed that the complete antigenic structure of apparently linear epitopes might embody distant residues (Cason et al., 1989). These may be either distant residues directly in contact with the antibody forming part of a discontinuous epitope, or neighboring regions contributing to the overall secondary and tertiary structure of the motif recognized by an antibody (Cason et al., 1989). These flanking regions might play an important role in facilitating the antigen-antibody binding, and such phenomenon would be missed in studies that rely solely upon peptide mapping data to define MAb epitope. As a result, the binding sites of MAb5 on SEB and TSST-1 identified in the present study might not be their complete epitopes. Furthermore, epitope mapping can only provide information about possible locations of epitopes; the functional nature of the epitope may not be revealed.

The exact mechanism of inhibition of TSST-1 and SEB-induced superantigenic activities by MAb5 presented previously (chapter 3) is unclear. However, based on previous findings with other specific MAbs that neutralized PTSAgs-induced superantigenic activities, it appears that the inhibition is accomplished through blocking the toxins from binding to either the MHC class II receptors or the TCRs (Shimonkevitz et al., 1996; Hamad et al., 1994). The TSST-1 epitope, ^{52}YSPAF^{56}, recognized by MAb5 was mapped to β3/β4 loop at the base of domain B of TSST-1, a region topologically adjacent to the identified MHC class II binding loop centered around L30 and G31 (Kim et al., 1994, Ulrich et al., 1998). Previous findings that MAb5 inhibited binding of ^{125}I-TSST-1 to human monocytes agrees with this observation (Kum et al., unpublished data). Thus, MAb5 was thought to inhibit TSST-1-induced superantigenic activities through sterically hindering the binding of TSST-1 to MHC class II molecules.

The three-dimensional model based on the crystal structure of SEB would place our identified SEB epitope ^{85}FGAN^{88} at the interdomain cleft of SEB. This region has been shown to associate with both TCR binding (Y90, Y91) and MHC class II binding (Y89, Q92, Y94, and S96) (Swaminathan et al., 1992; Li et al., 1998; Papageorgiou et al., 1998). We suspect that MAb5 inhibited SEB-induced superantigenic activities by hampering the toxin from binding to both receptors specifically. Our finding that the synthetic peptide corresponding to SEB (83-92), ^{83}DVFGANYYYQ^{92}, inhibited SEB-superantigenic activities concurred with this interpretation. Similar inhibition of SEB-induced proliferation was reported for a peptide of SEB (residues 61-92) but not an overlapping peptide (41-70), suggesting that the functional region involved amino acid residues between 70-90 (Jett et al., 1994). Our findings confirmed this prediction. The present

study did not evaluate the ability of SEB peptide (83-92) to block SEB from binding to MHC class II molecules. However, similar experiments have been done with SEB peptide (81-92) and SEB (90-114), showing that the former lacks the ability to block SEB binding to MHC class II molecules but the latter is highly efficient in inhibiting binding (Komisar et al., 1994). Taken together, it seems that the epitope SEB (84-89) contributes to TCR-binding, but since it is adjacent to the MHC class II binding region, MAb5 may actually block SEB from MHC class II binding as well through steric hindrance.

In summary, the regions TSST-1 (52-56) and SEB (84-89) are recognized by MAb5. Since MAb5 can cross-neutralize both TSST-1- and SEB-induced superantigenic activities, these MAb5 binding sites are worthy for further detailed investigation. If the peptides identified are proven to be important immunogenic epitopes, they could provide useful tools for the creation of polyvalent vaccines effective for the prevention of both TSST-1 and SEB associated diseases.

Chapter 5

Summary of Results and Conclusions

The major objective of this research was to study the cross-neutralizing epitopes of SEB and TSST-1. In addition to shared biochemical and immunobiological properties, these toxins shared strikingly similar structural architecture. It is very likely that they would also share antigenic determinants. However, none of the identified MAbs against PTSAgs demonstrated cross-reactivity between TSST-1 and SEB. Previously, our laboratory developed an anti-TSST-1 MAb, MAb5, which demonstrated cross-reactivity between TSST-1 and SEB in ELISA, suggesting that these two toxins do share antigenic determinants. These observations provided the framework of the research thesis in which possible cross-neutralizing epitopes shared by TSST-1 and SEB were evaluated and the location and function of these epitopes were also examined.

A. Summary of findings

1. MAb5 was found to partially cross-inhibit SEB-induced superantigenic activities, including mitogenesis and TNFα secretion, in vitro.
2. MAb5 recognized a linear TSST-1 peptide of 5 amino acids (^{52}YSPAF^{56}), as well as two linear SEB peptides of 5 amino acids (^{84}VFGAN^{88}) and (^{85}FGANY^{89}). The data suggest that the epitopes recognized by MAb5 on TSST-1 and SEB might be TSST-1 (52-56) and SEB (85-88).
3. Peptide corresponding to SEB (83-92), was found to inhibit SEB-induced superantigenic activities in vitro, suggesting that MAb5 inhibit SEB activities through blocking a functional motif of the molecule.
4. Our data, together with previous findings in the literature including crystallographic studies, mutational studies, and peptide studies, suggests that a possible mechanism

for MAb5 inhibition of SEB-induced activities is through blocking SEB from interacting with the TCR.

B. Conclusion

Over the years, a great deal of attention has been focused on the mapping of shared and unique antigenic determinants of the SEs within the sequence homology groups, especially SEB and SEC1 (Spero and Morlock, 1979; Thompson et al., 1984, Turner et al., 1992). Attempts using antibodies generated by immunization with synthetic peptides containing highly conserved stretches of SE primary sequence identified a region that induce neutralizing antibodies against both SEB and SEC1. Peptides corresponding to the residues 130-160 of SEB, or an overlapping region of SEC1 (residues 148-162), induced antibodies that neutralized T cell mitogenicity of both toxins (Hoffmann et al., 1994; Jett et al., 1994). However, comparatively, information of shared neutralizing epitopes across the sequence homology groups of PTSAgs is lacking. My thesis addresses this area of research by evaluating and localizing possible cross-neutralizing epitopes across sequence homology groups of PTSAgs, particularly TSST-1 and SEB. Very recently, while this manuscript was under preparation, Ulrich et al. demonstrated that cross-reactive antibodies against most PTSAgs could be induced by immunization with just one mutant PTSAgs vaccine and thereby suggested the presence of shared neutralizing epitopes among these PTSAgs (Ulrich et al., 1998). Yet the precise locations of these antigenic determinants are not known. This information, however is essential for developing a TSS vaccine. My research results not only substantiate the recent findings of Ulrich et al., but also attempt to address some of these important issues. It is my hope that my findings would generate

interest in this area of research, and such investigations will continue in the future.

References

Abe, J., T. Takeda, Y. Watanabe, H. Nakao, N. Kobayashi, D. Y. M. Leung, and T. Kohsaka. 1993. Evidence for superantigen production by *Yersinia pseudotuberculosis*. J. Immunol. 151, 4183-4188.

Abrahmsen, L., M. Dohlsten, S. Segren, P. Bjork, E. Jonsson, and T. Kalland. 1995. Characterization of two distinct sites MHC class II binding sites in the superantigen staphylococcal enterotoxin A. EMBO. J. 14:2978-2986.

Acharya, K. R., E. F. Passalacqua, E. Y. Jones, K. Harlos, D. I. Stuart, R. D. Brehm, and H. S. Tranter. 1994. Structural basis of superantigen action inferred from crystal structure of toxic shock syndrome toxin-1. Nature, 367:94-97.

Akolkar, P. N., B. Gulwani-Akolkar, N. Chirmule, et al. 1995. The HIV glycoprotein gp160 has superantigen-like properties. Clin. Immunol. Immunopathol. 76, 255-265.

Alouf, J. E., H. Knoll, and W. Kohler. 1991. The family of mitogenic, shock-inducing and superantigenic toxins from *Staphylococci* and *Streptococci*, p. 559-598. *In* J. E. Alouf and J. H. Freer (ed.), Sourcebook of Bacterial Protein Toxins. Academic Press. London.

Avena, R. M., and M. S. Bergdoll. 1967. Purification and some physiochemical properties of enterotoxin C, Staphylococcus aureus strain 361. Biochemistry. 6:1474-1480.

Baker, B. S., S. Bokth, A Powles, J. J. Garioch, H. Lewish, H. Valdimarsson, and L. Fly. 1993. Group A streptococcal antigen-specific T lymphocytes in guttate psoriatic leisons. Br. J. Dermatol. 128:493-499.

Barbour, S. D., D. M. Shlaes, and S. R. Guertin. 1984. Toxic shock syndrome associated with nasal packing: analogy to tampon-associated illness. Pediatrics 73:163-167.

Bartlett, P., A. L. Reingold, D. R. Graham, B. B. Dan, D. S. Selinger, G. W. Tank, and K. A. Wichterman. 1982. Toxic shock syndrome associated with surgical wound infections. JAMA 247:1448-1450.

Benjamin, D. C., J. A. Berzofsky, I. J. East, F. R. N. Gurd, C. Hannum, S. J. Leach, E. Margiliash, J. G. Michael, A. Miller, E. M. Prager, M. Reichlin, E. E. Sercarz, S. J. Smith-Gill, P. E. Todd, and A. C. Wilson. 1984. The antigenic structure of proteins: a reappraisal. A. Rev. Immun. 2:67-101.

Bergdoll, M. S., C. R. Borja, R. N. Robbins, and K. F. Weiss. 1971. Identification of enterotoxin E. Infect. Immun. 4:593-595.

Bergdoll, M. S., B. A. Crass, R. F. Reiser, R. N. Robbins, and J. P. Davis. 1981. A new staphylococcal enterotoxin, enterotoxin F, associated with toxic shock syndrome Staphylococcus aureus isolates. Lancet. 1:1017-1021.

Bergdoll, M. S. 1983. Enterotoxins, p.559. In: C. S. F. Easmon and C. Adlam. (ed.), Staphylococci and staphylococcal infections. Academic Press. Inc. New York.

Bergdoll, M. S., and P. M. Schlievert. 1984. Toxic-shock syndrome toxin. Lancet. 2:691.

Betley, M. J., D. W. Borst, and L. B. Regassa. 1992. Staphylococcal enterotoxins, toxic shock syndrome toxin, and streptococcal pyrogenic toxins: A comparative study of their molecular biology. Chem. Immunol. 55:1-35.

Blanco, L., E. M. Choi, K. Connolly, M. R. Thompson, and P. F. Bonventre. 1990. Mutants of staphylococcal toxic shock syndrome toxin-1: mitogenicity, and recognition by a neutralizing monoclonal antibody. Infect. Immun. 58: 3020-3028.

Blomster-Hautamaa, D. A., B. N. Kreiswirth, R. P. Novick, and P. M. Schlievert. 1986. Resolution of highly purified toxic-shock syndrome toxin-1 into two distinct proteins by isoelectric focusing. Biochemistry. 25:554-559.

Bohach, G. A., C. J. Hovde, J. P. Handley, and P. M. Schlievert. 1988. Cross-neutralization of staphylococcal and streptococcal pyrogenic toxins by monoclonal and polyclonal antibodies. Infect. Immun. 56(2):400-404.

Bohach, G. A., D. J. Fast, R. D. Nelson, and P. M. Schlievert. 1990. Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illness. Crit. Rev. Microbiol. 17:251-272.

Bonventure, P. F., H. Heeg, C. D. Edward III, and C. M. Cullen. 1995. A mutation at histidine residue 135 of toxic shock syndrome toxin yields an immunogenic protein with minimal toxicity. Infect. Immun. 63:509-515.

Bradley, J. S., P. M. Schlievert, and P. B. Sheehan. 1991. Streptococcal toxic shock-like syndrome as a complication of primary varicella. Pediatr. Infect. Dis. J. 10:77-78.

Breiman, R. F., J. P. Davis, R. R. Facklam, B. M. Gray, C. W. Hoge, E. L. Kaplan, E. A. Mortimer, P. M. Schlievert, B. Schwartz, D. L. Stevens, and J. K. Todd. 1993. Defining severe invasive streptococcal infections: rationale and consensus definition. JAMA. 269: 390-391.

Brennand, D. M., M. J. Danson, and D. W. Hough. 1986. A comparison of ELISA screening methods for the production of monoclonal antibodies against soluble protein antigens. J. Immunol. Methods 93:9.

Burnette W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112:195.

Cason, J., D. Patel, J. Naylor, D. Lunney, P. S. Shepherd, J. M. Best, and D. J. McCance. 1989. Identification of Immunogenic regions of the major coat protein of human papillomavirus type 16 that contain type-restricted epitopes. J. gen. Virol. 70:2973-2987.

Chesney, P. J. 1989. Clinical aspects and spectrum of illness of toxic shock syndrome. Revs. Infect. Dis. 11(Suppl):S1-S7.

Chintagumpala, M. M., J. A. Mollick, and R. R. Rich. Staphylococcal toxins bind to different sites on HLA-DR. 1991. Staphylococcal toxins bind to different sites on the HLA-DR. J. Immunol. 147:3876-3881.

Choi, Y., J. A. Lafferty, J. R. Clements, J. K. Todd, E. W. Gelfand, J. Kappler, P. Marrack, and B. L. Kotzin. 1990. Selective expansion of T cells expressing Vβ2 in toxic shock syndrome. J. Exp. Med. 172:981-984.

Chow, A. W. 1993. Toxic shock syndrome. P. 1010-1011. In The 5 Minute Clinical Consult. Lea and Febiger, London.

Chow, A. W. 1995. Toxic shock syndrome. Med. N. Amer. P. 140-148.

Cone, L. A., D. R. Woodard, P. M. Schlievert, and G. S. Tomory. 1987. Clinical and bacteriologic observations of a toxic shock-like syndrome due to streptococcus pyrogenes. N. Engl. J. Med. 317:146-149.

Cone, L. A., D. R. Woodard, R. G. Byrd, K. Schulz, S. M. Kopp, and P. M. Schlievert. 1992. A recalcitrant, erythematous, desquamating disorder associated with toxin-producing staphylococci in patients with AIDS. J. Infect. Dis. 165: 638-642.

Dadaglio, G., S. Garcia, L. Montagnier, and M-L. Gougeon. 1994. Selective anergy of Vb8+ T cells in human immunodeficiency virus-infected individuals. J. Exp. Med. 179, 413-424.

Dahlsten, M., L. Abrahmsen, P. Bjork, P. A. Lando, G. Hedlund, G. Forsberg, T. Brodin, N. R. Gascoigne, C. Forberg, and P. Lind. 1994. Monoclonal antibody-superantigen fusion proteins: tumor specific agents for T cell based tumor therapy. Proc. Natl. Acad. Sci. USA. 91:8945-8949.

Dellabona, P., J. Peccoud, J. Kappler, P. Marrack, C. Benoist, and D. Mathis. 1990. Superantigens interact with MHC class II molecules outside of the antigen groove. Cell. 62, 1115-1121.

Deresiewicz, R. L., J. Wood, M. Chan, R. W. Finberg, and D. L. Kasper. 1994. Mutations affecting the activity of toxic shock syndrome toxin-1. Biochemistry. 33:12844-12851.

Deringer, J. R., R. J. Ely, C. V. Stauffacher, G. A. Bohach. 1996. Subtype-specific interactions of type C staphylococcal enterotoxins with the T cell receptors. Mol. Microbiol. 22(3):523-534.

Dick, G. F., and G. H. Dick. 1924. A skin test for susceptibility to scarlet fever. JAMA. 82:256-266.

Dobrescu, D., B. Ursea, M. Pope, A. S. Asch, and D. N. Posnett. 1995. Enhanced HIV-1 replication in Vb12 T cells due to human cytomegalovirus in monocytes: evidence for a putative herpesvirus superantigen. 1995. Cell 82, 753-763.

Drake, C. G., and B. L. Kotzin. 1992. Superantigens: Biology, immunology, and potential role in disease. J. clin. Immunol. 12, 149-162.

Dyson, P. J., A. M. Knight, S. Fairchild, E. Simpson, and K. Tomonari. 1991. Genes encoding ligands for deletion of V beta 11 T cells cosegregate with mammary tumor virus genomes. Nature 349, 531-532.

Earhart, C. A., D. T. Mitchell, D. L. Murray, D. M. Pinheiro, M. Matsumura, P. M. Schlievert, and D. H. Ohlendorf. 1998. Structures of 5 mutants of toxic shock syndrome toxin-1 with reduced biological activity. Biochemistry. 37(20):7194-7202.

Fast, D. J., P. M. Schlievert, and R. D. Nelson. 1989. Toxic shock syndrome-associated staphylococcal and streptococcal pyrogenic toxins are potent inducers of tumor necrosis factor production. Infect. Immun. 57:291-294.

Festenstein, H. 1973. Immunogenetic and biological aspects of in vitro lymphocyte allotransformation (MLR) in the mouse. Transplant. Rev. 15, 62-88.

Fields, B. A., E. L. Malchiodi, H. Li, X. Ysern, C. V. Stauffacher, P. M. Schlievert, K. Karjalainen, and R. A. Muriuzza. 1996. Crystal structure of a T cell receptor β chain complexed with superantigens. Nature. 384:188-192.

Fieser, T. M., J. A. Tainer, H. M. Geysen, R. A. Houghten, and R. A. Lerner. 1987. Of protein flexibility and peptide conformation on reactivity of monoclonal anti-peptide antibodies with a protein α-helix. Proc. Natl. Acad. Sci. USA. 84:8568-8572.

Fleischer, B., and H. Schrezenmeier. 1988. T cell stimulation by staphylococcal enterotoxins. Clonally variable response and requirement for major histocompatibility complex class II molecules on accessory or target cells. J. Exp. Med. 167, 1697-1707.

Fleischer, B. 1989. Bacterial toxins as probes for the T-cell antigen receptor. Immunol. Today 10, 262-264.

Fleischer, B. Superantigens. Review article. APMIS. 102:3-12. 1994.

Frankel, W. N., C. Rudy, J. M. Coffin, and B. T. Huber. 1991. Linkage of MIs genes to endogenous mammary tumor viruses of inbred mice. Nature 349, 526-528.

Fraser, J. D. 1989. High affinity binding of staphylococcal enterotoxin A and B to HLA-DR. Nature. 339:221-223.

Fraser, J. D., and K. R. Hudson. 1993. Superantigens-remnants of a past process? Res. Immunol. 144(3):188-93; discussion 214-222.

Fraser, J. D., R. G. Urban, J. L. Strominger, and H. Robinson. 1992. Zinc regulates the function of two superantigens. Proc. Natl. Acad. Sci. USA. 89:5507-5511.

Friguet, B. L. Djavadi-Ohaniance, and M. E. Goldberg. 1984. Some monoclonal antibodies raised with a native protein bind preferentially to the denatured antigen. Mol. Immunol. 21:673.

Geysen, H. M., S. J. Rodda, T. J. Mason, G. Tribbick, and P. G. Schoofs. 1987. Strategies of epitope analysis using peptide synthesis. J. Immunol. Methods. 102:259-274.

Grossman, D., J. G. Lamphear, J. A. Mollick, M. J. Betley, and R. R. Rich. 1992. Dual role for class II histocompatibility complex molecules in staphylococcal enterotoxin-induced cytokine production and in vivo toxicity. Infect Immun. 60:5190-5196.

Hackett, S. P., and D. L. Stevens. 1993. Superantigens associated with staphylococcal and streptococcal toxic shock syndrome are potent inducers of tumor necrosis factor β synthesis. J. Infect. Dis. 168:232-235.

Hamad, A. R. A., A. Herman, P. Marrack, and J. W. Kappler. 1994. Monoclonal antibodies defining functional sites on the toxin superantigen staphylococcal enterotoxin B. J. Exp. Med. 180:615-621.

Hanna, E. E., and D. W. Watson. 1973. Enhanced immune responses after immunosuppression by streptococcal pyrogenic exotoxin. Invest. Immun. 7:1009-1011.

Harris, T. O., D. Grossman, J. W. Kappler, P. Marrack, R. R. Rich, M. J. Betley. 1993. Lack of complete correlation between emetic and T cell stimulatory activities of Staphylococcal enterotoxins. Infect. Immun. 63:3175-3183.

Harris, T. O., and M. J. Betley. 1995. Biological activities of staphylococcal enterotoxin type A mutants with N-terminal substitutions. Infect. Immun. 63:2133-2140.

Hauser, A. R., D. L. Stevens, E. L. Kaplan, and P. M. Schlievert. 1991. Molecular analysis of pyrogenic exotoxins from streptococcus pyrogenes isolates associated with toxic shock –like syndrome. J. Clin. Microbiol. 29:1562-1567.

Hayball, R. D., J. H. Robinson, R. E. O'Hehir, A., Verhoef, J. R. Lamb, and R. A. Lake. 1994. Identification of two binding sites of staphylococcal enterotoxin B that confer specificity for TCR V beta gene products. Int. Immunol. 6(2):199-211.

He, X., J. Goronzy, and C. Weyand. 1992. Selective induction of rheumatoid factors by superantigens and human helper T cells. J. Clin. Invest. 89:673-680.

Hedlund, G., M. Dohlsten, C. Petersson, and T. Kalland. 1993. Superantigen-based tumor therapy: in vivo activation of cytotoxic T cells. Cancer. Immunol. Immunother. 36:89-93.

Herman, A., G. Croteau, R. P. Sekaly, J. Kappler, and P. Marrack. 1990. HLA-DR alleles differ in their ability to present staphylococcal enterotoxins to T cells. J. Exp. Med. 172:709-717.

Herman, A., Kappler, J. W., Marrack, P. and A. M. Puller. 1991. Superantigens-mechanism of T cell stimulation and role in immune responses. Annu. Rev. Immunol. 9, 745-772.

Herman, A. G., N. Labrecque, J. Thibodeau, P. Marrack, J. W. Kappler, and R. P. Sekaly. 1991. Identification of the staphylococcal enterotoxin A superantigen binding site in the β-1 domain of the human histocompatibility antigen HLA-DR. Proc. Natl. Acad. Sci. U.S.A. 88:9954-9958.

Herrmann, T., R. S. Accolla, and H. R. MacDonald. 1989. Different staphylococcal enterotoxins bind preferentially to distinct major histocompatibility complex class II isotypes. Eur. J. Immunol. 19:2171-2174.

Hofer, M. F., M. R. Lester, P. M. Schlievert, and D. Y. M. Leung. 1995. Effect of bacterial toxins on IgE synthesis. Clin. Exp. Allergy. 25:1218-1227.

Hofer, E. F., K. Newell, R. Duke, P. M. Schlievert, J. H. Freed, and D. Y. M. Leung. 1996. Differential effects of staphylococcal toxic shock syndrome toxin-1 on B cell apoptosis. Proc. Natl. Acad. Sci. USA. 93:5425-5430.

Hoffman, M. L., L. M. Jablonski, K. K. Crum, S. P. Hackett, Y-I. Chi, C. V. Stauffacher, D. L. Stevens, and G. A. Bohach. 1994. Prediction of T cell receptor and major histocompatibility complex binding sites on staphylococcal enterotoxin C1. Infect. Immun. 62:3396-3407.

Holmberg, S. D., and P. A. Blake. 1984. Staphylococcal food poisoning in the united states. New facts and old misconceptions. JAMA. 251(4):487-489.

Hovde,C. J., J. C. Marr, M. L. Hoffmann, S. P. Hackett, Y. I. Chi, K. K. Crum, D. L. Stevens, C. V. Stauffacher, G. A. Bohach. 1994. Investigation of the role of the disulfide bond in the activity and structure of staphylococcal enterotoxin C1. Mol. Microbiol. 13(5):897-909.

Howell, M. D., J. P. Diveley, K. A. Lundeen, A. Esty, S. T. Winters, D. J. Carlo, and S. W. Brostoff. 1991. Limited T cell-receptor β-chain heterogeneity among interleukin-2 receptor –positive synovial T cells suggests a role for superantigen in rheumatoid arthritis. Proc. Natl. Acad. Sci. USA. 88:10921-10925.

Hudson, K. R., H. Robinson, and J. D. Fraser. 1993. Two adajacent residues in staphylococcal enterotoxin A and E determine T cell receptor Vβ specificity. J. Exp. Med. 177(1):175-184.

Hudson, K. R., R. E. Tiedemann, R. G. Urban, S. C. Lowe, J. L. Strominger, and J. D. Fraser. 1995. Staphylococcal enterotoxin A has two cooperative sites on major histocompatibility complex class-II. J. Exp. Med. 189:711-720.

Hurley, J. M., R. Shimonketivz, A., Hanagan, K. Enney, E. Boen, S. Malmstrom, B. L. Kotzin, and M. Matsumura. 1995. Identification of class II histocompatibility complex and T cell receptor binding sites in the superantigen toxic shock syndrome toxin-1. J. Exp. Med. 181(6):2229-2235.

Hynes, W. L., C. R. Weeks, J. J. Iandolo, and J. J. Ferretti. 1987. Immunologic cross-reactivity of type-A streptococcal exotoxin (erythrogenic toxin) and staphylococcal enterotoxins B and C1. Infect. Immun. 55:837-838.

Janeway, C. A., Jr., J. Yagi, P. J. Conrad, M. E. Katz, B. Jones, S. Vroegop, and S. Buxser. 1989. T-cell responses to MIs and to bacterial proteins that mimic its behavior. Immunol. Rev. 107, 61-88.

Jardetzky, T. S., J. H. Brown, J. C. Gorga, L. J. Stern, R. G. Urban, Y-I. Chi, C. V. Stauffacher, J. L. Strominger, and D. C. Wiley. 1994. Three-dimensional structure of a human class II histocompatibility molecule complexed with superantigen. Nature. (Lond) 368:711-718.

Jett, M., R. Neil, C. Welch, T. Boyle, E. Bernton, D. Hoover, G. Lowell, R. E. Hunt, S. Chatterjee, and P. Gemski. 1994. Identification of staphylococcal enterotoxin B sequences important for induction of lymphocyte proliferation by using synthetic peptide fragments of the toxin. Infect. Immun. 62:3408-3415.

Jones, C. L., amd S. A. Khan. 1986. Nucleotide sequence of the enterotoxin B gene from Staphylococcus aureus. J. Bacteriol. 166:29-33.

Kain, K. C., M. Schulzer, and A. W. Chow. 1993. Clinical spectrum of nonmenstrual toxic shock syndrome (TSS): comparison with menstrual TSS by multivariate discriminant analyses. Clin. Infect. Dis. 16(1):100-106.

Kalland, T., M. Dohlsten, L. Abrahmsen, G. Hedlund, P. Bjork, P. A. Lando, A. Sundstedt, E. Akerblom, and P. Lind. 1993. Targeting of superantigens. Cell. Biophy. 22:147-164.

Kappler, J. W., U. Staerz, J. White, and P. Marrack. 1988. Self-tolerance eliminates T cells specific for MIs-modified products of the major histocompatibility complex. Nature (London) 332, 35-40.

Kappler, J. W., A. Herman, J. Clements, and P. Marrack. 1992. Mutations defining functional regions of the superantigen staphylococcal enterotoxin B. J. Exp. Med. 175:387-396.

Karp, D. R., and E. O. Long. 1992. Identification of HLA-DR1 β chain residues critical for binding staphylococcal enterotoxin A and E. J. Exp. Med. 175:387-396.

Kim, C., K. A. Siminovitch, and A. Ochi. 1991. Reduction of lupus nephritis in MRL/Ipr mice by a bacterial superantigen treatment. J. Exp. Med. 174:1431-1437.

Kim, J., R. G. Urban, J. L. Strominger, and D. C. Wiley. 1994. Toxic shock syndrome toxin-1 complexed with a class II major histocompatibility molecule HLA-DR1. Science. 266:1870-1874.

Kim, Y. B., and D. W. Watson. 1970. A purified group A streptococcal pyrogenic exotoxin: physiochemical and biological properties including the enhancement of susceptibility to endotoxin lethal shock. J. Exp. Med. 131:611.

Komisar, J. L., S. Small-Harris, and J. Tseng. 1994. Localization of binding sites of staphylococcal enterotoxin B (SEB), a superantigen, for HLA-DR1 by inhibition with synthetic peptides of SEB. Infect. Immun. 62:4775-4780.

Kotzin, B. L., D. Y. M. Leung, J. Kappler, and P. Marrack. 1993. Superantigens and their potential role in human disease. Adv. Immunol. 54, 99-166.

Kum, W. W. S., K. B. Laupland, R. H. See, and A. W. Chow. 1993. Improved purification and biologic activities of staphylococcal toxic shock syndrome toxin-1. J. Clin. Microbiol. 31:2654-2660.

Kum, W. W. S., J. A. O. Wood, and A. W. Chow. 1996. A mutation at glycine residue 31 of toxic shock syndrome toxin-1 defines a functional site critical for major histocompatibility complex class II binding and superantigenic activity. J. Infect. Dis. 174:1261-1270.

Lafon, M., M. Lafage, A. Martinez-Arends, R. Ramirez, F. Vuillier, D. Charron, V. Lotteau, and D. Scott-Algara. 1992. Evidence in humans of a viral superantigen. Nature 358, 507-509.

Lafon, M., D. Scott-Algara, P. N. Marche, P-A. Cazenave, and E. Jouvin-Marche. 1994. Neonatal deletion and selective expansion of mouse T cells by exposure to rabies virus nucleocapsid superantigen. 1994. J. Exp. Med. 180, 1207-1215.

Larkin, S. M., D. N. William, M. T. Osterholm, R. W. Tofte, and Z. Posalaky. 1982. Toxic shock syndrome: clinical, laboratory, and pathological findings in nine fatal cases. Ann. Intern. Med. 96:858-864.

Lee, P. K., B. N. Kreiswirth, J. R. Deringer, S. J. Projan, W. Eisner, B. L. Smith, E. Carlson, R. P. Novick, P. M. Schlievert. 1992. Nucleotide sequence and biological properties of toxic shock syndrome toxin-1 from ovine and bovine-associated Staphylococcus aureus. J. Infect. Dis. 165:1056-1063.

Leung, D. Y., R. Harbeck, P. Bina, R. F. Reiser, E. Yang, D. A. Norris, J. M. Hanifin, and H. A. Sampson. 1993. Presence of IgE antibodies to staphylococcal exotoxins on the skin of patients with atopic dermatitis: evidence for a new group of allergens. J. Clin. Invest. 92:1374-1380.

Leung, D. Y., J. B. Travers, R. Giorno, D. A. Norris, R. Skinner, J. Aelion, L. V. Kazemi, M. H. Kim, A. E. Trumble, and M. Kotb. 1995. Evidence for a streptococcal superantigen-driven process in acute guttate psoriasis. J. Clin. Invest. 96:2106-2112.

Lewis, H. M., B. S. Baker, S. Bokth, A. V. Powles, J. J. Garioch, H. Valdimarsson, and L. Fly. 1993. Restricted T cell-receptor V beta gene usage in the skin of patients with guttate and chronic plaque psoriasis. 1993. Br. J. Dermatol. 129:514-520.

Li, H., A. Llera, D. Tsuchiya, L. Leder, X. Ysern, P. M. Schlievert, K. Karjalainen, and R. A. Mariuzza. 1998. Three dimensional structure of the complex between a T cell receptor beta chain and the superantigen staphylococcal enterotoxin B. Immunity. 9(6):807-816.

Litton, M. J., M. Dohlsten, P. A. Lando, T. Kalland, L. Ohlsson, J. Andersson, and U. Andersson. 1996. Antibody-targeted superantigen therapy induces tumor-infiltrating lymphocytes, excessive cytokine production, and apoptosis in human colon carcinoma. Eur. J. Immunol. 26:1-9.

MacDonald, K. L., M. T. Osterholm, C. W. Hedberg, C. G. Schrock, G. F. Peterson, J. M. Jentzen, S. A. Leonard, and P. M. Schlievert. 1987. Toxic shock syndrome. A newly recognized complication of influenza and influenza-like illness. JAMA. 257:1053-1058.

MacDonald, H. R., R. Schneider, R. K. Lee, R. C. Howe, H. Acha-Orbea, H. Festenstein, R. M. Zinkernagel, and H. Hengartner. 1988. T-cell receptor Vβ use predicts reactivity and tolerance to Misa-encoded antigens. Nature (London) 332, 40-45.

MacDonald H, S. Bashchieri, and R. Lee. 1991. Clonal Expansion precedes anergy and death of Vβ8+ peripheral T cells responding to staphylococcal enterotoxin B in vivo. Eur. J immunol. 21:1963-1966.

Marrack, P., and J. Kappler. 1990. The staphylococcal enterotoxins and their relatives. Science 248, 705-711.

Meyer, R. F., L. Miller, R. W. Bennett, and J. D. Macmillan. 1984. Development of a monoclonal antibody capable of interacting with five serotypes of Staphylococcus aureus enterotoxin. Appl. Environ. Microbiol. 47:283-287.

Miethke, T., K. Duschek, C. Wahl, K. Heeg, and H. Wagner. 1993. Pathogenesis of the toxic shock syndrome: T cell mediated lethal shock caused by the superantigen TSST-1. Eur J Immunol. 23: 1494-1500.

Mollick, J. A., R. G. Cook, and R. R. Rich. 1989. Class II MHC molecules are specific receptors for staphylococcal enterotoxin A. Science. 244:817-820.

Mollick, J. A., M. M. Chintagumpala, R. G. Cook, and R. R. Rich. 1991. Staphylococcal exotoxin activation of T cells. Role of exotoxin-MHC class II binding affinity and class II isotypes. J. Immunol. 146:463-468.

Mollick, J. A., R. L. MaMasters, D. Grossman, and R. R. Rich. 1993. Localization of a site on bacterial superantigens that determines T cell receptor β chain specificity. J. Exp. Med. 177:283-293.

Murray, D. L., G. S. Prasad, C. A. Earhart, B. A. B. Leonard, B. N. Kreiswirth, R. P. Novick, D. H. Ohlendorf, and P. M. Schlievert. 1994. Immunobiological and biochemical properties of mutants of toxic shock syndrome toxin-1. J. Immunol. 152:87-95.

Murray, D. L., D. H. Ohlendorf, and P. M. Schlievert. 1995. Staphylococcal and streptococcal superantigens: Their role in Human Diseases. ASM News. 61(5):229-235.

Murray, D. L., C. A. Earhart, D. T. Mitchell, D. H. Ohlendorf, R. P. Novick, and P. M. Schlievert. 1996. Localization of biologically important regions on toxic shock syndrome toxin-1. Infect. Immun. 64:371-374.

Musser, J. M., A. R. Hauser, M. Kim, P. M. Schlievert, K. Nelson, and R. K. Selander. 1991. Streptococcus pyrogenes causing toxic shock-like syndrome and other invasive diseases:clonal diversity and pyrogenic exotoxin expression. Proc. Natl. Acad. Sci. USA. 88:2688-2672.

Nakamura, R. M., A. Vollew, and D. E. Bidwell. 1986. Enzyme immunoassays: heterogenous and homogeneous systems, p. 27.1-27.20. In D. M. Weir (ed.), Volume 1: Immunochemistry. Blackwell Scientific Publications. Palo Alto, CA.

Overington, J. P. 1992. Comparison of three-dimensional structures of homologous proteins. Curr. Opin. Struct. Biol. 2:394-401.

Paliard, X., S. G. West, J. A. Lafferty, J. R. Clements, J. W. Kappler, P. Marrack, and B. L. Kotzin. 1991. Evidence for the effects of a superantigen in rheumatoid arthritis. Science. 253:325-328.

Papageorgiou, A. C., K. R. Acharya, R. Shapiro, E. F. Passalacqa, R. D. Brehm, and H. S. Tranter. 1995. Crystal structure of the superantigen staphylococcal enterotoxin C2 from staphylococcus aureus reveals a zinc-binding site. Structure. 3:769-779.

Papageorgiou, A. C., R. D. Brehm, D. D. Leonidas, H. S. Tranter, and K. R. Acharya. 1996. The refined crystal structure of toxic shock syndrome toxin-1 at 2.07 A resolution. J. Mol. Biol. 260:553-569.

Papageorgiou, A. C., and K. R. Acharya. 1997. Superantigens as immunomodulators: recent structural insights. Structure. 5(8):991-996.

Papageorgiou, A. C., S. T. Howard, and K. R. Acharya. 1998. Crystal structure of microbiol superantigen staphylococcal enterotoxin B at 1.5 a Resolution: Implications for superantigen recognition by MHC class II molecules and T cell receptors. J. Mol. Biol. 277:61-79.

Parsonnet, J. 1989. Mediators in the pathogenesis of toxic shock syndrome: overview. Rev Infect Dis;11(suppl. 1):S263-S269.

Peavy, D. L., W. H. Adler, and R. T. Smith. 1970. The mitogenic effects of endotoxin and staphylococcal enterotoxin B on mouse spleen cells and human peripheral lymphocytes. J. Immunol. 105, 1453-1458.

Prasad, G. S., C. A. Earhart, D. L. Murray, R. P. Novick, P. M. Schlievert, and D. H. Ohlendorf. 1993. Structure of toxic shock syndrome toxin-1. Biochemistry. 32:13761-13766.

Pullen, A. M., P. Marrack, and J. Kappler. 1988. The T-cell repertoire is heavily influenced by tolerance to polymorphic self-antigens. Nature 335, 796-801.

Reingold, A. L., N. T. Hargrett, B. B. Dan, K. N. Shands, B. Y. Strickland, and C. V. Broome. 1982. Nonmenstrual toxic shock syndrome. Ann. Int. Med. 96:871-874.

Reiser, R. F., R. N. Robbins, A. L. Noleto, G. P. Khoe, and M. S. Bergdoll. 1984. Identification, purification and some physiochemical properties of staphylococcal enterotoxin C_3. Infect. Immun. 45:625-630.

Rodda, S. J., H. M. Geysen, T. J. Mason, and P. G. Schoofs. 1986. The antibody response to myoglobin-I. Systemic synthesis of Myoglobin peptides reveals location and substructure of species-dependent continuous antigenic determinants. Mol. Immun. 23(6):603-610.

Rosten, P. M., K. H. Bartlett, and A. W. Chow. 1987. Detection and quantitation of toxic shock syndrome toxin-1 in vitro and in vivo by noncompetitive enzyme-linked immunosorbent assay. J. Clin. Microbiol. 25:327-332.

Rosten, P. M., K. H. Barlett, and A. W. Chow. 1989. Purification and purity assessment of toxic shock syndrome toxin-1. Rev. Infect. Dis. 11(Suppl):S110-116.

Rott, O., H. Wekerle, and B. Fleischer. 1992. Protection from experimental allergic encephalomyelitis by application of a bacterial superantigen. Int. Immunol. 4:347-353.

Roussell, A., B. F. Anderson, H. M. Baker, J. D. Fraser, and E. N. Baker. 1997. Crystal structure of the streptococcal superantigen SPE-C: dimerization and zinc binding suggest a novel mode of interaction with MHC class II molecules. Nature Struct. Biol. 4:635-643.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning, a laboratory manial. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Sprong Harbor, N. Y.

Schad, E. M., I. Zaitseva, V. N. Zaitseva, M. Dohlsten, T. Kalland, P. M. Schlievert, D. H. Ohlendorf, and L. A. Svensson. 1995. Crystal structure of the superantigen staphylococcal enterotoxin type A. EMBO. J. 14:3292-3301.

Schlievert, P. M., and D. W. Watson. 1979. Biogenic amine involvement in ptrogenicity and enhancement of lethal endotoxin shock by group A streptococcal pyrogenic exotoxin. Proc. Soc. Esp. Biol. Med. 162:269-274.

Schlievert, P. M., K. N. Shands, B. B. Dan, G. P. Schmid, and R. D. Nishimura. 1981. Identification and characterization of an exotoxin from Staphylococcus aureus associated toxic-shock syndrome. J. Infect. Dis. 143:509-516.

Schlievert, P. M. 1982. Enhancement of host susceptibility to lethal endotoxin shock by staphylococcal pyrogenic exotoxin type C. Infect. Immun. 36:123-128.

Schlievert, P. M. 1986. Staphylococcal enterotoxin B and toxic shock syndrome toxin-1 are significantly associated with non-menstrual TSS. Lancet. 1:1149-1150.

Schlievert, P. M., G. A. Bohach, C. J. Hovde, B. N. Kreiswirth, and R. P. Novick. 1990. Molecular studies of toxic shock syndrome-associated staphylococcal and streptococcal toxins, p.311. In: R. P. Novick. (ed.), Molecular biology of the staphylococci. VCH Publishers. Inc.

Schlievert, P. M. 1993. Role of superantigens in human disease. J. Infect. Dis. 167:997-1002.

Schlievert, P. M., G. A. Bohach, D. H. Ohlendorf, C. V. Stauffacher, D. Y. M. Leung, D. L. Murray, C. A. Earhart, L. M. Jablonski, M. L. Hoffmann, and Y. I. Chi. 1995. Molecular structure of staphylococcus and streptococcus superantigens. J. Clin. Immun. 15(6):4S-10S.

Schlievert, P. M. 1997. Searching for superantigens. Immunol. Invest. 26(1&2), 283-290.

Scholl, P. R., A. Diez, R. S. Geha. 1989. Staphylococcal enterotoxin B and toxic shock syndrome toxin-1 bind distinct sites on HLA-DR and HLA-DQ molecules. J. Immunol. 143:2583-2588.

Scholl, P. R., A. Diez, R. Karr, R. P. Sekaly, J. Trowsdale, and R. S. Geha. 1990. Effect of isotypes and allelic polymorphism on the binding of staphylococcal exotoxins to MHC class II molecules. J. Immunol. 144:226-230.

See, R. H., G. Krystal, and A. W. Chow. 1990. Binding competition of toxic shock syndrome toxin-1 and other staphylococcal exoproteins for receptors on human peripheral blood mononuclear cells. Infect. Immun. 58:2392-2399.

See, R. H., and A. W. Chow. 1992. Staphylococcal toxic shock syndrome toxin-1-induced tumor necrosis factor alpha and interleukin-1β secretion by human peripheral blood monocytes and T lymphocytes is differentially suppressed by protein kinase inhibitors. Infect. Immun. 60:3456-3459.

See, R. H., W. W. S. Kum, A. H. Chang, S. H. Goh, and A. W. Chow. 1992. Induction of tumor necrosis factor and interleukin-1 by purified staphylococcal toxic shock syndrome toxin-1 requires the presence of both monocytes and T lymphocytes. Infect. Immun. 60:2612-2618.

Shimonkevitz, R., B. E. Boen, S. Malmstorm, E. Brown, J. M. Hurley, B. L. Kotzin, and M. Matsumura. 1996. Dilineation by use of specific monoclonal antibodies of the T cell receptor and the major histocompatibility complex interaction sites on the superantigen toxic shock syndrome toxin-1. Infect. Immun. 64(4):1133-1139.

Shinagawa, K., T. Kanazawa, N. Matsusaka, S. Sugii, and K. Nagata. 1991. Murine monoclonal antibodies reactive with staphylococcal enterotoxins A, B, C2, D, E. FEMS. Microbiol. Lett. 64(1):35-39.

Singh, B. R., M. L. Evenson, and M. S. Bergdoll. 1988. Structural analysis of staphylococcal enterotoxins B and C1 using circular dichroism and fluorescence spectroscopy. Biochemistry. 27:8735-8741.

Singh, B. R., N. P. Kokan-Moore, and M. S. Bergdoll. 1988. Molecular topography of toxic shock syndrome toxin-1 as revealed by spectroscopic studies. Biochemistry. 27:8730-8735.

Singh, B. R., and M. J. Betley. 1989. Comparative structural analysis of staphylococcal enterotoxins A and E. J. Biol. Chem. 264:4404-4411.

Soos, M. J., J. Schiffenbauer, and H. M. Johnson. 1993. Treatment of PL/J mice with the superantigen, staphylococcal enterotoxin B, prevents development of experimental allergic encephalomyelitis. J. Neyroimmunol. 43:39-44.

Soos, J. M. and H. M. Johnson. 1994. Multiple binding sites on the superantigen, staphylococcal enterotoxin B, imparts versatility in binding to MHC class II molecules. Biochem. Biophys. Res. Commun. 201:596-602.

Spero, L., and B. A. Morlock. 1979. Cross-reactivity between tryptic polypeptides of staphylococcal enterotoxins B and C1. J. Immunol. 122:1285-1289.

Stevens, D. L., M. H. Tanner, J. Winship, R. Swarts, K. M. Ries, P. M. Schlievert, and E. Kaplan. 1989. Severe group A streptococcal infections associated with a toxic shock-like syndrome and scarlet fever toxin A. N. Eng., J. Med. 321:1-7.

Stevens, D. L. 1997. Streptococcal toxic shock syndrome, p. 481-501. In D. Y. M. Leung, B. T. Huber, P. M. Schlievert (ed.), Superantigens. Marcel Dekker. New York.

Strange, P., L. Skov, S. Lisby, P. L. Nielsen, and O. Baadsgaard. 1996. Staphylococcal enterotoxin B applied on intact normal and intact atopic skin induces dermatitis. Arch. Dermatol. 132:27-33.

Stone, R. L., and P. M. Schlievert. 1987. Evidence for the involvement of endotoxin in toxic shock syndrome. J. Infect. Dis. 155:682-689.

Stuart, P. M., and J. P. Woodland. 1992. *Yersinia enterocolitica* produces superantigenic activity. J. Immunol. 148, 225-233.

Sundstrom, M., D. L. Abrahmsen, P. Antonsson, K. Mehindate, W. Mourad, and M. Dohlsten. 1996. The crystal structure of staphylococcal enterotoxin D reveals Zn^{2+}-mediated homodimerization. EMBO. J. 15:6832-6840.

Sutkowski, N, T. Palkama, C. Ciurli, R-P. Sekaly, D. A. Thorley-Lawson, and B. T. Huber. 1996. An Epstein Barr virus-associated superantigen. J. Exp. Med.

Swaminathan, S., W. Furey, J. Pletcher, and M. Sax. 1992. Crystal structure of staphylococcal enterotoxin B, a superantigen. Nature. 359:801-806.

Thibodeau, J., M. Dohlsten, I. Cloutier, P. M. Lavoie, P. Bjork, F. Michel, C. Leveille, W. Mourad, T. Kalland, and R. P. Sekaly. 1997. Molecular characterization and role in T cell activation of Staphylococcal enterotoxin A binding to the HLA-DRα chain. J. Immunol. 158:3698-3704.

Thibodeau, J., I. Cloutier, P. M. Lavoie, N. Labrecque, W. Mourad, T. Jardetzky, and R. P. Sekaly. 1994. Subsets of HLA-DR1 molecules defined by SEB and TSST-1 binding. Science. 266:1874-1878.

Thompson, N. E., M. J. Ketterhagen, and M. S. Bergdoll. 1984. Monoclonal antibodies to staphylococcal enterotoxins B and C: cross-reactivity and localization of epitopes on tryptic fragments. Infect. Immun. 45:281-285.

Todd, J., and M. Fishaut. 1978. Toxic-shock syndrome associated with phage group-I staphylococci. Lancet. 2:1116-1118.

Tomai, M., M. Kotb, G. Majumdar, and E.H. Beachy. 1990. Superantigenicity of streptococcal M protein. J. Exp. Med. 172, 359-362.

Tranter, H. S., R. D. Brehm and K. R. Acaharya, 1995. Molecular topology is important for the function of staphylococcal superantigens, p. 5-23. *In* J. Thibodeau & R. Sekaly (ed.), Bacterial Superantigens: Structure, Function and Therapeutic Potential. Springer-Verlag. Heidelberg. Germany.

Turner, T. N., C. L. Smith, and G. A. Bohach. 1992. Residues 20, 22, and 26 determines subtype specificities of staphylococcal enterotoxins C1 and C2. Infect Immun. 57:23-28.

Ulrich, R. G., S. Bavari, and M. A. Olson. 1995. Staphylococcal enterotoxin A and B share a common structural motif for binding class II major histocompatibility complex molecules. Nature. Struct. Biol. 2:554-560.

Ulrich, R. G., M. A. Olson, and S. Bavari. 1998. Development of engineered vaccines effective against structurally related bacterial superantigens. Vaccine. 16(19):1857-1864.

Vaidya, H. C., D. N. Dietzler, and J. H. Ladenson. 1985. Inadequacy of traditional ELISA for screening hybridoma supernatants for murine monoclonal antibodies. Hybridoma. 4:271.

Woodland, D. L., M.P. Happ, K. J. Gollob., and E. Palmer. 1991. An endogenous retrovirus mediating deletion of αβ T cells? Nature 349, 529-530.

Watson, D. W. 1959. Host-parasite factors in group A streptococcal infections: pyrogenic and other effects on immunologic distinct exotoxins related to scarlet fever toxins. J. Exp. Med. 111:255-283.

Wen, R., G. A. Cole, S. Surman, M. A. Blackman, and D. L. Woodland. 1996. Major histocompatibility complex class II-associated peptides control the presentation of bacterial superantigens to T cells. J. Exp. Med. 183:1083-1092.

White, J., A. Herman, A. M. Pullen, R. Kubo, J. W. Kappler, and P. Marrack. 1989. The Vβ-specific superantigen staphylococcal enterotoxin B: Stimulation of mature T cells and clonal deletion in neonatal mice. Cell 56, 27-35.

Yagi, J., J. Baron, S. Buxser, and C. A. Janeway, Jr. 1990. Bacterial proteins that mediate the association of a defined subset of T cell receptor: CD4 complexes with class II MHC. J. Immunol. 144:892-901.

Yu, C. E., and J. J. Ferretti. 1989. Molecular epidemiologic analysis of the type A streptococcal exotoxin (erythrogenic toxin) gene (speA) in clinical Streptococcus pyrogenes strains. Infect. Immun. 57: 3715-3719.