THE MECHANISM OF HUMAN MONOCYTE ACTIVATION BY STAPHYLOCOCCAL TOXIC SHOCK SYNDROME TOXIN-1 (TSST-1)

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Abstract

Toxic shock syndrome (TSS) is a multisystem disease associated with staphylococcal TSS toxin-1 (TSST-1). TSST-1 and the related staphylococcal enterotoxins (SE) A, B, C, D, and E have a number of biological effects on human peripheral blood mononuclear cells (PBMC), including T lymphocyte mitogenicity and induction of IL-1 and TNF from human monocytes. The aim of this thesis is to examine the molecular mechanisms of human monocyte activation by TSST-1 and SE.

To gain insight into the mechanism of TSST-1 and SEA activation of PBMC, receptor binding assays were performed with ^{125}I-labeled toxins. Scatchard analyses revealed similar numbers of receptors and dissociation constants for TSST-1 and SEA on human PBMC and on purified monocytes. SEA, but not SEB, SEC, SED or SEE significantly inhibited binding of ^{125}I-TSST-1 to PBMC. Cross-competition between TSST-1 and SEA in binding assays suggested that they may be binding to overlapping epitopes on the same receptor. Affinity cross-linking of ^{125}I-labeled TSST-1 and SEA to human blood monocytes showed the presence of 2 membrane subunits consistent with the 35 kd alpha and 28 kd beta chains of human HLA-DR. The anti-HLA-DR mAb, L243, inhibited radiolabeled TSST-1 and SEA binding to human monocytes and neutralized monocyte-dependent T cell mitogenicity of both toxins, adding further support that HLA-DR is the major receptor. Based on these studies and those of others who demonstrated overlapping receptor epitopes for SEA and SEB (Fraser, Nature 339:221-223, 1989) and distinct epitopes for TSST-1 and SEB (Scholl et al., J. Immunol 143:2583-2588, 1989), we postulate that SEA occupies a binding site within HLA-DR that partially overlaps with both TSST-1 and SEB.

The role of protein phosphorylation in the activation of normal human monocytes by TSST-1 and SE was examined by two-dimensional gel electrophoresis. Examination of ^{32}P-orthophosphate-labeled monocytes showed that within 5 min, TSST-1 consistently stimulated the dephosphorylation of several phosphoproteins in a dose-dependent manner. In contrast, neither SEA nor SEB induced this dephosphorylation pattern, but instead, increased the phosphorylation of a

different set of proteins. Phosphorylation patterns induced by two other monocyte agonists, PMA and bacterial LPS, demonstrated little similarity to those induced by TSST-1. Moreover, using an anti-phosphotyrosine mAb, TSST-1 and SE were shown to stimulate the tyrosine-specific phosphorylation of several cytosolic proteins that were distinct from those induced by PMA. This suggests that tyrosine phosphorylation induced by TSST-1 or SEA is not mediated by activation of protein kinase C. Collectively, the data suggest that the early intracellular signal transduction pathways utilized by TSST-1, SE, LPS and PMA in monocytes are dissimilar despite common biological consequences such as lymphocyte mitogenesis and cytokine induction.

TSST-1 was also tested for its ability to induce the cytokines, IL-1 and TNF, from fractionated human PBMC. Highly purified monocytes alone or T lymphocytes alone did not produce IL-1ß or TNFα when incubated with TSST-1 for up to 72 h. However, TSST-1 added to a 1:1 ratio of monocytes and T lymphocytes resulted in significant extracellular TNFα and IL-1ß production at 24 h. The nature of the monocyte/T cell interaction did not involve IFN-τ but did require direct cell contact between metabolically active monocytes and T lymphocytes. Furthermore, TSST-1-mediated monocyte/T cell interaction also involved LFA-1 since mAbs to this adhesion molecule significantly reduced cytokine secretion.

Finally, the functional relevance of protein kinases in cytokine production by TSST-1-stimulated monocyte/T lymphocyte co-cultures was explored. IL-1ß secretion was suppressed by inhibitors of protein kinase C (H7), tyrosine kinases (genistein) and cAMP- and cGMP-dependent kinases (HA1004). In contrast, secretion of TNFα was blocked by only H7 and genistein, suggesting that induction of these two cytokines is differentially regulated.

In conclusion, our data are consistent with a superantigen role for both TSST-1 and SE and indicate that TSS pathogenesis occurs as a result of TSST-1 interaction with both monocytes and T lymphocytes. Further studies focusing on the mechanism of cell activation by this toxin will not only enhance our knowledge of superantigens in general, but will also aid in our understanding of other bacterial toxin-mediated diseases.

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List Of Abbreviations

APC	Antigen Presenting Cell
BSA	Bovine serum albumin
BHI	Brain heart infusion broth
biotin-X-NHS	Biotinyl-Ε-amino caproic acid-N-hydroxysuccinimide ester
°C	Degrees celsius
CDC	Center for Disease Control
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CPM	Counts per minute
DNA	Deoxyribonucleic acid
DSS	Disuccinimidyl suberate
EDTA	Ethylene diaminetetra acetic acid
E. coli	Escherichia coli
ELISA	Enzyme-Linked Immunosorbent Assay
ET	Electrophoretic type
ETA	Streptococcal erythrogenic toxin A
ExFT	Exfoliative toxin
FBS	Fetal bovine serum
fg/ml	Fentogram per millilitre
H7	[1-(5-isoquinolinylsulfonyl)-2-methylpiperazine
HA1004	[N-(2-guanidinoethyl)-5-isoquinolinesulfonamide
HEPES	N-2-hydroxyethylpiperazine
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
ICAM-1	Intercellular adhesion molecule-1
ICAM-2	Intercellular adhesion molecule-2
kb	Kilobases
IFN-τ	Interferon-gamma
kd	Kilodalton
K_d	Dissociation constant
LFA-1	Lymphocyte Function-Associated antigen
LPS	Lipopolysaccharide
MAM	M. arthriditis mitogen
mAb	Monoclonal antibody
mRNA	Messenger ribonucleic acid
min	Minute
Mo	Monocytes
MHCII	Major histocompatibility class II antigen
MTT	(3-[4,5-dimethylthiazol-2, 5-dipheny tetrazolium bromide
MLV-LPS	Multilamellar liposomes lipopolysaccharide
ng/ml	Nanogram per millitre
nM	Nanomolar
O.D.	Optical density
PBMC	Peripheral blood mononuclear cells
^{32}P	Phosphorus-32
PAEC	Porcine aortic endothelial cells
PBS	Phosphate buffered saline
PEC	Pyrogenic exotoxin C
pI	Isoelectric point
pM	Picomolar
pg/ml	Picogram per millilitre
PMA	Phorbol-12-myristate-13-acetate
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
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
S. pyogenes	Streptococcal pyogenes
SPEA	Streptococcal pyrogenic toxin A
SPEB	Streptococcal pyrogenic toxin B
SPEC	Streptococcal pyrogenic toxin C
S. aureus	Staphylococcus aureus
TCR	T cell antigen receptor
TNF	Tumor necrosis factor
TSS	Toxic shock syndrome
TSST-1	Toxic shock syndrome toxin-1
V	Volts
V_β	Beta chain of variable region of T cell antigen receptor

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Chapter 1

Introduction

1.1 History And Epidemiology Of TSS

In 1978, Todd and his associates introduced the term toxic shock syndrome (TSS) to describe a unique illness in seven children, four girls and three boys, aged 8 to 17. The disease consisted of the acute onset of "high fever, headache, confusion, conjunctival hyperemia, a scarlatiniform rash, subcutaneous edema, vomiting, watery diarrhea, oliguria, and a propensity to acute renal failure, hepatic abnormalities, disseminated intravascular coagulation, and severe prolonged shock" (Todd et al., 1978). During the convalescent phase of the disease, a typical desquamation of trunk and extremities as well as the palms and soles was observed. Cultures taken prior to antibiotic therapy revealed infection with Staphylococcus aureus (Todd et al., 1978). This initial report of Todd et al. (1978) received little attention until mid-1980 when numerous cases resembling TSS among young menstruating women were reported to the Centers for Disease Control (CDC). This subsequently led to the development of the case definition for TSS (Table I) by the CDC to provide uniformity of diagnosis and to facilitate epidemiologic studies. By April, 1982, 1660 cases of TSS were reported to the CDC, 96% involving women (CDC, 1982). A striking association was found between TSS, tampon use at menstruation, and cervical or vaginal S. aureus infection. In one case-controlled study of 52 women with TSS, all TSS patients had used tampons during menstruation compared with 44 of 52 age-matched control women who did not. No significant differences in tampon brand, frequency of tampon change, type of contraceptive use or frequency of sexual intercourse were noted (Shands et al., 1980). Analysis of additional TSS patients showed that 62 of 64 (96%) were infected with a vaginal or cervical isolate of S. aureus compared with 7 of 71 (10%) control individuals. The demonstration of S. aureus isolates in menstruating women with TSS was also confirmed in other studies

Table I

Case Definition For TSS

1. Fever: (temperature > 38.9°C)

2. Rash (diffuse macular erythroderma)

3. Desquamation, 1-2 weeks after onset of illness, particularly of palms and soles

4. Hypotension (systolic blood pressure < 90 mm Hg for adults or < 5th percentile by age for children < 16 years of age, or orthostatic syncope)

5. Involvement of three or more of the following organ systems:
   A. Gastrointestinal (vomiting or diarrhea at onset of illness)
   B. Muscular (severe myalgia or elevated creatine phosphokinase level > 2 x upper limit of normal)
   C. Mucous membrane (vaginal, oropharyngeal, or conjunctival hyeremia)
   D. Renal (Blood urea nitrogen or serum creatinine at twice the upper limit of normal, or > 5 white blood cells per high power field-in the absence of a urinary tract infection)
   E. Hepatic (total bilirubin, serum glutamic oxaloacetic transaminase or serum glutamic pyruvic transaminase at least twice the upper limit of normal)
   F. Hematologic (platelets < 100,000/mm^3)
   G. Central nervous system (disorientation or alterations in neurologic signs when fever and hypotension are absent)

6. Negative results of the following tests, if obtained:
   A. Blood, throat, or cerebrospinal fluid cultures
   B. Serologic tests for Rocky Mountain spotted fever, leptospirosis, or measles

From Chesney et al., 1984.

(Davis et al., 1980; Reingold et al., 1982a). Although TSS was linked to many tampon brands, epidemiologic studies showed that "Rely" tampons (Procter and Gamble Co.) posed the greatest risk and this led to its subsequent withdrawal from the market (CDC, 1980).

Epidemiologically, at its peak in 1980, the incidence of TSS ranged from 3 to 15 cases per 100,000 menstruating women (Davis et al., 1980; Kehrberg et al., 1981; Todd et al., 1985). Since this period, the incidence of the disease has been declining either due to less frequent case reporting or due to changing habits in tampon use. Of the total number of TSS cases reported to CDC between 1979 to 1983, 96% involved women, and of this, 90% were associated with menstruation (CDC, 1983). The age range of female patients was 1 to 80 years, with a mean of 22.9 years. Approximately 97% of the cases occurred in white non-Hispanics. Recurrent rates of disease were high (30%) while the case-fatality ratio was approximately 3 to 5% (CDC, 1983).

Although frequently associated with menstruation, TSS has also been reported in a wide variety of medical and surgical conditions. Nonmenstrual TSS results mainly from infections with S. aureus (Bartlett et al., 1982; Green and LaPeter, 1982; Reingold et al., 1982b). Moreover, nonmenstrual TSS appears to be highly associated with the use of foreign bodies such as nasal packings during surgery (Barbour et al., 1984) or barrier contraceptives (Schwartz et al., 1989). Although lower in incidence compared with its menstrual counterpart, nonmenstrual TSS has been slowly increasing, accounting for 27% of total TSS cases in 1984, up from 7% in 1980 (CDC, 1984). Despite the fact that the clinical features are similar, there are differences between menstrual and nonmenstrual TSS. Nonmenstrual TSS patients tend to be older and the disease occurs in both sexes. Moreover, in nonmenstrual TSS cases, a higher proportion of non-Whites (11%) are affected in contrast to menstrual TSS cases (2%) (Reingold et al., 1982b). Finally, unlike menstrual TSS patients who share many common features (eg. young women, menstruation, tampon user, etc.), nonmenstrual TSS patients are a less homogeneous group with varying underlying conditions (K.C. Kain, M. Schulzer and

A.W. Chow, manuscript submitted). As will be discussed later, the staphylococcal toxins mediating the two types of TSS are not entirely identical.

1.2 Risk Factors For TSS

After a number of TSS cases had been reported to the CDC, investigators began case-control studies to identify specific factors that might be associated with increased risk of developing TSS, some of which are listed in Table II. These studies led to the recognition that tampon use during menstruation was a major risk factor (Davis et al., 1980; Shands et al., 1980). Women who use tampons have been estimated as having a 20-fold higher risk of developing TSS compared with non-tampon users (Smith and Jacobson, 1986). Furthermore, the risk of developing TSS is 3-fold higher in those who use super-absorbent tampons rather than regular tampons (Smith and Jacobson, 1986). In one study, the absorbency of the tampon was found to be the best predictor for the risk of developing TSS (Osterholm et al., 1982). Superabsorbent tampons made of polyester foam embedded with a carboxymethylcellulose product were found to be highly associated with TSS (Kass and Parsonnet, 1987). To date, the precise role of tampons in TSS has not been ascertained although several hypotheses have been put forth (reviewed by Kass and Parsonnet, 1987). The most plausible explanation is that tampons increase the partial pressure of oxygen in an otherwise anaerobic vagina, conditions favorable to the growth of S. aureus and its production of extracellular toxins (Mahoney Jr, 1988).

Although tampons were identified as a major risk factor, the incidence of TSS (3 to 15 cases per 100,000 menstruating women) was still lower than that predicted for the number of women who used tampons. The demonstration of vaginal or cervical isolates of S. aureus in virtually all cases of menstrual TSS was an important step towards understanding the pathogenesis of TSS (Davis et al., 1980; Shands et al., 1980). The isolation of this microorganism in all nonmenstrual TSS cases also provided solid evidence that S. aureus infection was a major etiologic agent (Reingold, 1982b). It has been assumed that women who develop menstrual TSS

Table II

Risk Factors For TSS

High
. Absence of antibody to TSST-1
. Infection with TSST-1-producing strain of S. aureus
. Menstruating female less than 35 years of age
. Continuous use of superabsorbent tampons
. Nasal surgery with packing
Moderate
. Regular absorbency tampons used regularly during menstruation
. Alternating use of tampons and pads
. Contraceptive sponge
Low
. Contraceptive diaphragm
. Intrauterine contraceptive device
. Surgical wound infections
. Early postpartum state

Note: Table modified from Smith and Jacobson, 1986

have been colonized with S. aureus before the onset of menstruation (Davis et al., 1982a). Studies also show that vaginal colonization with S. aureus in healthy control women is only about 10% (Davis et al., 1982a). Even more important than the isolation of S. aureus in TSS patients is the demonstration of their production of the exoprotein, TSS toxin-1 (TSST-1). Nearly 100% of S. aureus isolates from menstrual TSS patients and a large proportion of nonmenstrual TSS S. aureus isolates produce this toxin (Bergdoll et al., 1981; Schlievert et al., 1981). As will be evident later on, TSST-1 is now believed to be responsible for most of the symptoms of TSS.

The other important risk factor for developing TSS is the absence of serum antibodies to TSST-1. Most TSS patients lack or have low levels of circulating antibodies to TSST-1. This is in contrast to the wide prevalence and high titers of anti-TSST-1 in healthy controls (91% of the normal population) (Bonventre et al., 1984; Notermans et al., 1983). The occurrence of TSST-1 antibodies in sera of healthy individuals suggests that exposure to TSST-1-producing S. aureus strains is quite common. Thus, the low titers of anti-TSST-1 in TSS patients may reflect the lack of prior exposure to TSST-1-producing S. aureus in these individuals or possibly, a number of exposures is necessary to induce an antibody response (Stolz et al.,1985). However, these individuals may also have immunological defects (possibly induced by TSST-1 itself) since some fail to seroconvert to TSST-1 even after one or multiple episodes of this disease (Rosten et al., 1988; Stolz et al., 1985).

1.3 Clinical Spectrum Of TSS

There is no laboratory test available for the diagnosis of TSS. Diagnosis of this disease is based on the clincal findings as outlined by the CDC (Table I). The major features of TSS are multisystem organ damage and massive capillary vasodilation with intravascular fluid loss (Chesney et al., 1984). Patients generally experience fever, myalgia, headache, vomiting and diarrhea. Hypotension, a diffuse sunburn-like erythroderma and a strawberry-like tongue are

also characteristic features of TSS. Treatment of TSS consists of aggressive fluid replacement and antistaphylococcal antibiotic therapy to remove the offending microorganism. Once the initial life-threatening complications of TSS have passed, the subsequent course of the disease is towards rapid resolution. The fever drops within 48 to 72 hours of onset, the erythroderma disappears, and the myalgia resolves after a week. During convalescence, a tell-tale desquamation, particularly of the palms and soles is evident. Death, although rare, occurs mainly as a result of refractory cardiac arrhythmias, irreversible respiratory failure, and bleeding due to coagulation defects (Chesney et al., 1984). The recurrence rate is about 30%, but is decreased with antistaphylococcal antibiotic therapy and the discontinuation of tampon use (Davis et al., 1982b).

1.4 TSS-Associated S. Aureus Isolates

The association of S. aureus with TSS is now well-established with the isolation of these microorganisms in both menstrual and nonmenstrual TSS cases. However, there are some phenotypic and genotypic differences between TSS- and non-TSS-associated S. aureus isolates as reviewed by See and Chow (1989). TSS-associated S. aureus isolates are more sensitive to group I phage types, particularly phage type 29 alone or in combination with phage 52, compared with non-TSS strains (Altemeier et al., 1982). A large number of TSS-associated S. aureus isolates also remain nontypable (30% to 40%) (Chow et al., 1984). TSS isolates also demonstrate resistance to penicillin, arsenate, and cadmium but are susceptible to mercury (Kreiswirth et al., 1982). The determinants for these resistances are assumed to be chromosomally mediated since TSS isolates are less likely to carry plasmids than other control isolates (Kreiswirth et al., 1982; Todd et al., 1984). In addition, TSS-associated S. aureus isolates produce less hemolysis on sheep blood agar compared with control isolates (Chow et al., 1983; Todd et al., 1984). However, the reduced hemolytic activity is only unique to vaginal TSS isolates. Non-genital TSS-associated isolates do not differ significantly from nongenital control isolates (Chow et al., 1983). An

explanation for the decreased hemolytic activity in vaginal isolates of TSS strains may be the result of a specific down-regulation of the α-hemolysin gene in these isolates (Clyne et al., 1988). TSS-associated S. aureus isolates have been documented to possess more proteolytic activity compared with control isolates (Barbour, 1981; See and Chow, 1989). Differences in the type of proteases produced by TSST-1-positive S. aureus isolates and TSST-1-negative S. aureus isolates have also been reported (Lawellin et al., 1989). Furthermore, vaginal TSS isolates make less lipase and nuclease enzymes than control isolates, which may explain their tendency to colonize the genital tract as opposed to the skin or nasal mucosa (Schlievert et al., 1982a).

Perhaps the most relevant phenotypic difference between TSS-associated S. aureus isolates and control isolates is the ability of the former to produce exotoxins. TSST-1, the exoprotein strongly implicated in TSS, is produced by more than 95% of S. aureus isolates associated with menstrual TSS and by 60% to 75% of S. aureus associated with nonmenstrual TSS (Bergdoll et al., 1981; Garbe et al., 1985; Schlievert et al., 1981). This is in contrast to other clinical isolates in which TSST-1 is produced in only 5% to 15% of isolates (See and Chow, 1989). Aside from TSST-1, TSS S. aureus isolates are more likely to produce one or more of the enterotoxins (A, B, C, D, and E) as well as epidermal toxin (Crass and Bergdoll, 1986; Kapral, 1982; Todd et al., 1984). The role of the enterotoxins in TSS pathogenesis will be discussed later.

Genotypically, Kreiswirth et al. (1982) have described TSST-1 production by TSS-associated S. aureus as a variable genetic trait because only 20% of randomly tested clinical S. aureus isolates have the *tst* structural gene for the toxin. The *tst* gene has been found on the S. aureus chromosome and is not encoded by plasmid or transmitted by lysogenic conversion (Kreiswirth et al., 1989). It has also been shown that the *tst* gene and its flanking sequences are present only in TSST-1-positive strains and absent in TSST-1-negative strains. Additionally, genotypic variability has been shown to exist among TSST-1-producing isolates since *tst* and its flanking sequences have been found within different chromosomal loci in different isolates, suggesting that the *tst* gene may be mobile

(Kreiswirth et al., 1989). Interestingly, the *tst* gene appears to be closely linked to tryptophan auxotrophy (Chu et al.,1985). Chu et al. (1988) have mapped the *tst* gene in a $tst^{+}trp^{-}$ and a $tst^{+}trp^{+}$ isolate. The mapping data revealed that in the trp^{-} isolate, *tst* is in or near the tryptophan operon, while in the trp^{+} isolate, *tst* is in or near the *tyr B* operon. Thus, *tst* has been located in at least two different sites. Kreiswirth et al. (1989) have observed additional correlations between genotype and phenotype among TSS-associated S. aureus. Southern blots were prepared from *ClaI*-digested whole cell DNA from TSS-associated S. aureus isolates followed by hybridization with a *tst*-specific probe. Several distinctive *ClaI*-hybridization patterns were observed; isolates with similar phenotypic characteristics (eg. enterotxin production, heavy-metal susceptibility, or tryptophan auxotrophy) shared a common blotting pattern. These results suggest that isolates demonstrating common *tst* blotting patterns may be clonal in origin (Kreiswirth et al., 1989).

The production of TSST-1 and several other exoproteins by S. aureus is regulated by the accessory gene regulator (*agr*), defined by transposon insertion which affects the synthesis of these exoproteins (Recsei et al., 1986). Mutations of the *agr* product (241 amino acids in size) blocks post-exponential phase synthesis of the following staphylococcal proteins: serine protease, nuclease, lipase, fibrinolysin, α-hemolysin, ß-hemolysin, δ-hemolysin, staphylococcal enterotoxin B, and TSST-1, whereas production of other exoproteins such as protein A and coagulase is increased (Peng et al., 1988; Recsei et al., 1986). Thus, the *agr* appears to act as a toggle metabolic switch; at stationary phase, the synthesis of certain S. aureus proteins are shut down to allow the synthesis of new exponential phase proteins required for growth and cell division in the new growth cycle (Peng et al., 1988).

Although phenotypic and genotypic differences have been noted for TSS- and non-TSS-associated S. aureus isolates, there is also evidence of a distinction between TSS-associated genital and nongenital TSS isolates. Chow and Bartlett (1985) have reported that nongenital TSS-associated S. aureus differed significantly from genital isolates in hemolytic activity, phage distribution,

and TSST-1 production. Garbe et al. (1985) additionally noted differences in TSST-1 production between menstrual and nonmenstrual TSS S. aureus isolates (98% versus 62%, respectively). More recently, the phylogenetic relation among TSS-associated S. aureus strains have been examined by multilocus enzyme electrophoresis with 18 metabolic enzymes (Musser et al., 1990). A single clone, designated as electrophoretic type (ET) 41, accounted for 88% of TSS cases with a female urogenital focus and 53% of TSS cases involving nonurogenital infections. Furthermore, our own laboratory studies show that urogenital TSS isolates of this clone were less hemolytic and more likely to co-produce both TSST-1 and SEA than other ETs derived from the same site (Chang et al., 1990). The clonality of menstrual TSS-associated S. aureus isolates may explain why the phenotypic characteristics of these strains are so similar (Musser et al., 1990; See and Chow, 1989). Two hypotheses have been put forth to explain the predominance of ET 41 in the majority of menstrual TSS cases (Musser et al., 1990). It is possible that in the cervicovaginal milieu, ET 41 isolates may regulate the expression of the *tst* gene differently than that of isolates of other clones. Alternatively, ET 41 strains may possess phenotypic characteristics that allow them to thrive better in the human vagina than other ET types (Musser et al., 1990). Although further studies are required to resolve this issue, the clonality of urogenital TSS S. aureus isolates adds to the contention that menstrual TSS is a more homogeneous disease than its nonmenstrual TSS counterpart.

1.5 Evidence For The Involvement Of TSST-1 And Other Staphylococcal Toxins In The Pathogenesis Of TSS

The initial identification of S. aureus as the offending microoganism in both menstrual and nonmenstrual TSS cases prompted an investigation into its extracellular toxins to identify a causative agent which might be responsible for the disease. There was general consensus that TSS was likely a toxin-mediated disease for the following reasons: 1) the multisystemic nature of TSS, 2) the

absence of bacteremia and 3) the lack of tissue invasion observed with TSS-associated S. aureus isolates. In 1981, two groups independently isolated an exotoxin from TSS S. aureus isolates thought to be the etiologic agent in the pathogenesis of the disease. Pyrogenic exotoxin C (PEC), isolated by Schlievert et al. (1981), was found to be produced in nearly 100% of menstrual TSS S. aureus isolates. Furthermore, PEC was found to cause fever and to induce many of the clinical features of TSS in experimental animals (Schlievert et al., 1981; Schlievert, 1982). Staphylococcal enterotoxin F (SEF) was initially described by Bergdoll et al. (1981) because of its emetic properties in monkeys. They also found that 94% of TSS S. aureus isolates produce this toxin compared with only 5% of control isolates. Subsequent biochemical and immunological studies indicated that the two toxins were the same (Bonventre et al., 1983) and at an international symposium for TSS in 1984, the toxin was renamed toxic shock syndrome toxin-1 (TSST-1).

Aside from the fact that TSST-1 is produced by virtually all menstrual TSS S. aureus isolates (Bergdoll et al., 1981; Schlievert et al., 1981) and by a majority of nonmenstrual isolates (Garbe et al., 1985), there is now strong evidence implicating this toxin as the etiologic agent in TSS. Initial challenge studies in rodents using purified TSST-1 were unsuccessful since these animals are resistant to the effects of the toxin (Quimby and Nguyen, 1985; Peavy et al., 1970). However, in rabbits, with the exception of the rash, desquamation, and vomiting (rabbits are incapable of vomiting), the administration of purified TSST-1 subcutaneously, intravenously, or intravaginally results in symptoms similar to those of humans (see Table III) (Melish et al., 1989a; Parsonnet et al., 1987; Reeves et al., 1986; Schlievert, 1982). Infection of rabbits with a TSST-1-positive S. aureus strain also results in significant lethality compared with non-TSST-1-producing control strains (Arko et al., 1984; Melish et al., 1989a; Scott et al., 1983). Rasheed et al. (1985) showed that transfer of the TSST-1 trait from a TSST-1-positive to a non-TSS S. aureus strain by a bacteriophage resulted in a TSS-like illness and death when rabbits were infected with the recombinant strain. Rabbits challenged with the recombinant strain

Table III

Comparison Of TSS Features In Humans and Rabbits

Symptom	Human	Rabbit
. Fever	+	+
. Rash + desquamation	+	only skin redness
. Hyperemia of mucous membranes	+	conjunctival hyperemia
. Vomiting and diarrhea	+	no vomiting, diarrhea
. Hypotension	+	+
. ↑ blood urea nitrogen,	+	+
. ↑ bilirubin, aspartate aminotransferase	+	+
. ↑ creatine phosphokinase	+	+
. ↑ lactate dehydrogenase	+	+
. ↑ albumin	+	+
. Hypocalcemia	+	+
. Thrombocytopenia, lymphopenia	+	+
. Erythrophagocytosis in spleen and lymph node	+	+
. Periportal inflammation and fatty changes in liver	+	+
. Pulmonary congestion and edema	+	+
. Acute renal tubular necrosis	+	+
. Myocardial damage	+	+
. Mucosal separation (esophagus)	+	+
. Ulceration of vaginal epithelium	+	-

Note: Table from de Azavedo et al. (1989).

developed fever, severe hypotension, conjunctival hyperemia, erythroderma and respiratory distress before succumbing to the disease. Histopathological studies showed marked congestion in all tissues, edema and various degrees of inflammation. Except for a mild liver triaditis seen among rabbits inoculated with the TSST-1-negative recipient strain, none of the controls showed the extensive congestion or histopathological changes described for rabbits receiving the TSST-1-producing S. aureus strain (Rasheed et al., 1985). Using a rabbit uterine model for TSS, de Azavedo et al. (1985a) also reported that a S. aureus strain harboring a plasmid carrying the cloned *tst* gene induced more death than isogenic TSST-1-negative strains or TSST-1-negative strains carrying a plasmid with a deletion of the *tst* gene (de Azavedo et al., 1985a). Their results indicate that TSST-1 produced in vivo is able to penetrate the epithelial mucosal barrier of the reproductive tract to reach the systemic circulation. Finally, rabbits developed a TSS-like illness when inserted with a vaginal tampon containing TSST-1-producing S. aureus and simulated menses (defribinated rabbit blood) (Chow et al., 1990; Melish et al., 1989a).

Further indication that TSST-1 is the major etiologic agent in TSS comes from rabbit protection studies using antibodies against TSST-1. Rabbits passively immunized with anti-TSST-1 polyclonal antibodies prior to infection with a TSS S. aureus strain did not succumb to the disease (Scott et al., 1983). A monoclonal antibody against TSST-1, designated mAb 8-5-7, has also been found to provide protection in rabbits inoculated with either TSST-1-producing S. aureus strains or with purified TSST-1 (Best et al., 1988; Bonventre et al., 1988; Scott et al., 1989). Rabbits given mAb 8-5-7 developed an attenuated illness and were protected from the hypocalcemia, lipemia, and hepatic and renal insufficiency seen in control rabbits (Bonventre et al., 1988). These studies add strong support to the pathological role of TSST-1.

In humans, TSST-1 has been detected in the serum, urine, and vaginal washings (Melish et al., 1984) as well as in breast milk (Vergeront et al., 1982) of TSS patients. Using a noncompetitive ELISA for TSST-1 (sensitivity = 0.5 ng/ml), we previously demonstrated that TSST-1 could be detected in vaginal

washings of women with acute menstrual TSS but not in healthy controls. The timing of specimen collection was critical since TSST-1 could only be detected within 3 days after the onset of symptoms. Antistaphylococcal antibiotic therapy to eradicate S. aureus made detection of TSST-1 difficult after this period (Rosten et al., 1987).

As mentioned earlier, most TSS patients initially lack TSST-1 antibodies in their sera (Bonventre et al., 1984; Notermans et al., 1983), but soon develop detectable antibody titres as early as seven days after the onset of illness (Notermans et al., 1983; Stoltz et al., 1985). In our laboratory, we have analysed serum antibody responses to TSST-1 and the staphylococcal enterotoxins by immunoblot analysis of acute- and convalescent-phase sera from TSS- and non-TSS-associated S. aureus infections (Whiting et al., 1989). Compared with non-TSS cases, seroconversion to TSST-1 was significantly more frequent among both menstrual and nonmenstrual TSS patients. However, seroconversion to staphylococcal enterotoxin A (SEA) was also more frequent among both menstrual and nonmenstrual TSS patients, implicating that staphylococcal enterotoxins might also be involved in TSS as well (Whiting et al., 1989).

The possible role of staphylococcal enterotoxins (SE) A, B, C, D, and E has also been postulated by other investigators, particularly in nonmenstrual TSS cases. Garbe et al. (1985) found that whereas 93% of menstrual TSS S. aureus isolates produce TSST-1, only 62% of nonmenstrual isolates produce the toxin. More importantly, the TSST-1-negative TSS-associated S. aureus isolates were able to induce TSS-like symptoms in a rabbit model, suggesting that toxins other than TSST-1 may be involved. Schlievert (1986) also confirmed that significantly fewer nonmenstrual S. aureus isolates produced TSST-1; however, in his study, he showed that SEB production was more prevalent in nonmenstrual TSS isolates than in nonmenstrual non-TSS isolates. Therefore, he concluded that SEB is significantly associated with TSS cases where TSST-1 is not involved. His hypothesis is consistent with our analysis of acute- and convalescent-phase paired sera from TSS patients. In general, patients with TSS associated with TSST-1-positive S. aureus were more likely to seroconvert exclusively to TSST-1 whereas those

associated with TSST-1-negative S. aureus were more likely to seroconvert exclusively to the enterotoxins (Whiting et al., 1989). Furthermore, concurrent seroconversions to multiple exoproteins were more frequent among both menstrual and nonmenstrual TSS patients compared with control subjects. Of interest is a report by Scott et al. (1986) that a TSST-1-negative nonmenstrual TSS S. aureus strain (D4508) produces a 30 kd exoprotein that cross-reacts with antiserum to TSST-1. Because this strain causes a TSS-like illness in rabbits, Scott and co-workers proposed to designate this protein as TSST-2. However, the authors did not address the possibility that the TSST-1 preparation used in raising anti-sera may have been originally contaminated by the 30 kd protein. Using immunoblot analysis, subsequent work by McCollister et al. (1990) demonstrated that the 30 kd exoprotein was not TSST-2, but SEA. The addition of anti-SEA serum to culture supernatant of D4508 prior to its administration to rabbits in miniosmotic pumps protected the animals from the fever, diarrhea, weight loss and death normally observed in animals receiving culture supernatant or purified SEA alone. Thus, the authors concluded that SEA was definitively the causative agent of TSS-like illness in this S. aureus strain. Moreover, Bergdoll et al. (1982) screened 130 TSST-1-positive isolates of S. aureus and found that 41.5% produced SEA, 14% produced SEC, and none produce SEB. We have also shown that SEA is significantly co-secreted with TSST-1 (66% of TSST-1-positive isolates) in urogenital TSS S. aureus isolates compared with either nonurogenital TSS or non-TSS isolates (Chang et al., 1991). These results, coupled with the finding that staphylococcal enterotoxins share many of the same biological properties as TSST-1, not only implicate a role for these toxins in TSS, but also suggest the possibility that they may interact additively or synergistically with TSST-1 in producing the clinical manifestations of TSS (Whiting et al., 1989).

1.6 Characterization Of TSST-1 And Its Gene

TSST-1 consists of a single polypeptide chain with an isoelectric point of 7.2 and a molecular weight of 22,049 (Schlievert et al., 1981). The mature

protein is 194 amino acids in length with no disulfide bonds and has a serine as the N-terminal residue (Blomster-Hautamaa et al., 1986b). The lack of cysteine residues in TSST-1 distinguishes it from the staphylococcal enterotoxins, which characteristically contain a disulfide loop (Bergdoll et al., 1981). TSST-1 shares no homology with SEB and SEC and although minimal homology was observed between TSST-1 and streptococcal pyrogenic exotoxin A, it was not found to be significant (Blomster-Hautamaa et al., 1986). No cross-reactivity between TSST-1 and SEB or SEC was observed by Western blot analysis using TSST-1 antiserum or antisera against the other toxins (Blomster-Hautamaa et al., 1986b). TSST-1 is resistant to trypsin digestion but susceptible to pepsin proteolysis at pH 4.5 (Bergdoll et al., 1981). The toxin has been purified to homogeneity from S. aureus culture supernatants by a variety of procedures (Bergdoll et al., 1981; Igarashi et al., 1984; Reeves et al., 1986; Rosten et al., 1989; Schlievert et al., 1981).

TSST-1 is produced by S. aureus during late exponential and early stationary phase. Production of toxin is optimal under aerobic conditions at 37°C and at pH 7 (Schlievert and Blomster, 1983). TSS-associated S. aureus isolates also produce more TSST-1 in the presence of CO_2 (Lee-Wong and Bergdoll, 1990), tryptophan (Chu et al., 1985), and low levels of both glucose (Schlievert and Blomster, 1983) and magnesium (Mills et al., 1985). As mentioned earlier, the production of TSST-1 as well as several other exoproteins is under the regulation of the *agr*, which acts at a transcription level (Rescei et al., 1986).

The TSST-1 gene (*tst*) has been cloned into E. coli using shotgun cloning of a partial *MboI* digest of total S. aureus DNA into pBR322 (Kreiswirth et al., 1983; Poindexter and Schlievert, 1985a). The *tst* gene is located on the bacterial chromosome of TSST-1-producing S. aureus isolates and encodes a 234 amino acid protein of which 40 amino acids are part of the signal peptide sequence. The *tst* gene is also flanked by unique sequences of DNA since they are present in TSST-1-positive isolates but not in the chromosomal sequences of TSST-1-negative isolates. Thus, the *tst* gene and its flanking sequences make up a larger TSST-1 element (Kreiswirth et al., 1989). Preliminary sequence data and Southern

hybridization results have revealed that the TSST-1 element is 4 to 7 kb in size (Kreiswirth et al., 1989). Hybridization analysis of whole-cell DNA from two genetically mapped TSST-1-positive isolates indicate that the TSST-1 element has at least two chromosomal locations. These findings have led to the suggestion that the TSST-1 element is a staphylococcal transposon (Kreiswirth et al., 1989).

1.7 Staphylococcal Enterotoxins-Structure And Immunological Relatedness

As discussed above, there is now strong evidence that the staphylococcal enterotoxins are involved in the pathogenesis of TSS, particularly in cases where TSS-associated S. aureus strains do not produce TSST-1. The staphylococcal enterotoxins were originally named because of their ability to produce an emetic reaction when given intragastrically to monkeys (Bergdoll, 1983). In humans, these toxins are noted for the causation of staphylococcal food poisoning (Bergdoll, 1983). The enterotoxins consist of low molecular weight proteins (26 kd to 30 kd) with similar biological properties. The enterotoxins are classified into 5 major serological groups (SEA, SEB, SEC, SED and SEE); in the case of SEC, they are further subdivided into 3 groups (SEC_1, SEC_2, and SEC_3) based on specific minor determinants that can be detected with serogroup-specific antibodies (Bergdoll et al., 1983). A summary of the biochemical characteristics of each of the enterotoxins as well as TSST-1 is given in Table IV. The enterotoxins contain large amounts of lysine, aspartic and glutamic acid and tyrosine. Each enterotoxin contains two half-cystine residues which act to form a cystine loop. Previous studies had indicated that the disulfide loop was not essential for the biological activity of the enterotoxins (Fraser, 1989; Noskova et al., 1984). However, in these studies the reduction and alkylation processes were not performed efficiently to completely destroy the disulfide loop. Using optimized conditions for full reduction and alkylation of the enterotoxin disulfide loop, Grossman et al. (1990) subsequently showed that the cystine loop was important for T cell mitogenic activity.

Table IV

Biochemical Properties Of TSST-1, Staphylococcal Enterotoxins And Streptococcal Pyrogenic Exotoxins

Toxin	Isoelectric Point	Number of Amino Acids (mature protein)	Molecular Weight
TSST-1	7.2	194	22,049
SEA	7.3	233	27,100
SEB	8.5	239	28,336
SEC_1	8.5	239	27,531
SEC_2	7.0	239	27,589
SEC_3	8.0	239	27,563
SED	7.4	228	26,360
SEE	7.0	230	26,425
SPE A	5.0-5.5	221	25,787
SPE C	6.7-7.0	208	24,354

Modified from Bergdoll (1983) and Schlievert et al., (1990)

The nucleotide sequence for each of the enterotoxins has been determined. (Bayles and Iandolo, 1989; Betley et al., 1988; Bohach and Schlievert, 1987; Couch et al., 1988; Jones and Khan, 1986). The genes for SEA (*sea*) and SEE (*see*) are the most closely related (84% nucleotide sequence homology) followed by *seb* and *sec* gene pair (75% nucleotide sequence identity) (Bohach and Schlievert, 1987; Couch et al., 1988). In terms of amino acid sequence, there is 82% homology between SEA and SEE (Couch et al., 1988), 53% homology between SEA and SED (Bayles and Iandolo, 1989), 55% homology between SED and SEE (Bayles and Iandolo, 1989), and 69% homology between SEB and SEC_1 (Bohach and Schlievert, 1987). Over 96% amino acid sequence identity exists between the SECs, with SEC_2 and SEC_3 being the most similar (98.3%) (Hovde et al., 1990). Interestingly, significant amino acid homology (50%) has also been found between SEB and streptococcal pyrogenic exotoxin A (SPEA) made by Streptococcus pyogenes (Johnson et al., 1986). TSST-1 does not share significant amino acid homology with any of the staphylococcal enterotoxins although it does belong to the same general family based on biological properties (Blomster-Hautamaa et al., 1986b). Thus, the staphylococcal toxins can be divided into three major groups on the basis of amino acid residue homology: 1) SEA, SED and SEE; 2) SEB, SEC_1, and SPEA (from S. pyrogenes); and 3) TSST-1 (Bayles and Iandolo, 1989; Marrack and Kappler, 1990). The evolutionary and genetic basis for the relatedness of these toxins have not been determined since sequencing studies have not revealed a well-defined pattern among the various toxins (Hovde et al., 1990). Possibly, the toxin family may have arisen as a result of divergence from an ancestral staphylococcal or streptococcal toxin protein and subsequent recombination between similar toxin genes (Hovde et al., 1990). Evidence to support the role of recombination is that the signal peptide of SEC_3 is identical to that of SEB (with the exception of one amino acid) but only 77.7% homologous to that of the other SECs. Furthermore, SEC_3 is clearly more related to other SECs than to SEB (Hovde et al., 1990).

1.8 Streptococcal Pyrogenic Exotoxins

The streptococcal pyrogenic exotoxins (SPEs) from group A streptococci are most noted for their ability to induce the symptoms of scarlet fever (Hooker and Follensby, 1934). There are three antigenically distinct forms of SPEs (A, B, and C) (Hooker and Follensby, 1934; Stock and Lynn, 1961). SPEA, the first SPE to be identified, has a charge microheterogeneity with an isoelectric point between 4.5 to 5.5 (Cunningham et al., 1976). Although initial estimates of the molecular weight of SPEA were conflicting, sequence studies have recently shown this protein to be 221 amino acids long with a molecular weight of 25,787 (Bohach et al., 1990). Studies with SPEB also demonstrate that this toxin has charged microheterogeneity (pI forms of 8.0, 8.3, and 9.0), but only the pI 8.3 species was biologically active when tested in rabbits (Barsumian et al., 1978a). Various molecular weights have been reported for SPEB (range 17,500 to 21,900) because of its sensitivity to proteolysis during the purification process (Barsumian et al., 1978a; Cunningham et al., 1976). However, studies with the cloned toxin show that it has a molecular weight of 27,600 and is more stable in E. coli (Hauser and Schlievert, 1990). Moreover, studies with SPEC show this toxin to have a molecular weight of 24,300 and a single isoelectric point (Bohach et al., 1990). Of the SPEs, SPEA and SPEC are the two most related in terms of amino acid homology (Barsumian et al., 1978a; Cunningham et al., 1976).

SPEs from group A streptococci have been implicated in the causation of a TSS-like illness (Cone et al., 1987). Patients with streptococcal TSS present with hypotension, renal dysfunction, hypoalbuminemia, hypocalcemia, and respiratory failure, features similar to those seen in staphylococcal TSS cases (Stevens et al., 1989). The main difference between the two diseases is the presence of extensive soft-tissue infection (eg. necrotizing fasciitis) and bacteremia in patients with streptococcal TSS (Stevens et al., 1989). A higher mortality rate was observed among patients with streptococcal TSS (Cone et al., 1987; Stevens et al., 1989).

The reasons for the recent increase in streptococcal TSS cases are not clear although some suggestions indicate that it may be due to the reemergence of SPEA-producing S. pyogenes isolates. Stevens et al. (1989) showed that of 80 S. pyogenes isolates obtained from a variety of clinical sources in the United States between 1976 and 1986, none produce SPEA, whereas many isolates recovered earlier in the century were able to express this toxin. In contrast, analysis of 26 isolates of group A streptococci from streptococcal TSS cases between 1987 and 1988 showed that 88% produced SPEA, whereas SPEB and SPEC were produced by 38% and 30% of the isolates, respectively (Lee and Schlievert, 1989). Further studies showed that these isolates produced significantly more SPEA than the other two SPEs. In addition, SPEA administered in miniosmotic pumps implanted subcutaneously in rabbits resulted in a higher lethality rate than rabbits challenged with SPEC. Thus, SPEA was not only produced in larger amounts by SPEA-positive S. pyogenes isolates, but also showed more toxicity relative to the other SPEs (Lee and Schlievert, 1989).

A recent study by multilocus enzyme electrophoresis of 108 isolates of S. pyogenes from patients with streptococcal TSS and other invasive diseases in the United States revealed the presence of 33 multilocus enzyme genotypes (Musser et al., 1991). However, nearly half the disease episodes and more than two-thirds of streptococcal TSS cases were caused by isolates of two related multilocus genotypes, ET 1 and ET 2. Since evolutionary convergence to the same multilocus genotype is highly unlikely, the authors postulated that these isolates descended from a common ancestral cell or clone (Musser et al., 1991). Furthermore, analysis of these isolates with a *speA* gene probe revealed that the majority of SPEA-positive S. pyogenes belong to either genotypes ET 1 or ET 2. The study also reported a high frequency of SPEA production (alone or in combination with SPEB and SPEC) among S. pyogenes isolates from streptococcal TSS cases, providing further support for an etiologic role for this toxin in streptococcal TSS (Musser et al., 1991).

SPEA, which is bacteriophage-encoded, shares nearly 50% amino acid homology with Staphylococcus aureus enterotoxins B and C_1 (Bohach and Schlievert, 1987;

Johnson et al., 1986). Immunologic cross-reactivity between SPEA, SEB, and SEC_1 has been demonstrated by Ouchterlony double diffusion, Western immunoblot, and immunodot analyses (Hynes et al., 1987). Studies show that the carboxyl terminal sequences and the sequences flanking the enterotoxin cystine loop are highly conserved among all three proteins (Bohach and Schlievert, 1987). As will be discussed below, although the three toxins share several biological properties, there are distinct differences. For example, only the enterotoxins cause nausea and vomiting after ingestion. SPEA, on the other hand, can predispose the host to myocardial damage (Bohach and Schlievert, 1987). Thus, one can speculate that the common biological properties between the toxins may be attributed to homologous amino acid sequences whereas the dissimilar regions may be responsible for the toxin's unique toxicities.

1.9 Biological Properties Of TSST-1, Staphylococcal Enterotoxins, And Streptococcal Pyrogenic Exotoxins

TSST-1, the staphylococcal enterotoxins and the streptococcal pyrogenic exotoxins are grouped together as a family based on their shared biochemical, biological, and serological activities, sequence homology, and involvement in related illness (Bohach et al., 1990). Collectively, these toxins have been called pyrogenic toxins because of their distinctive capacity to induce fever in the host (Bohach et al., 1990). In this section, although the properties of TSST-1 will be mainly discussed, the concepts also apply to other staphylococcal and streptococcal toxins since the modes of action of these toxins are very similar.

A. Fever Induction

One of the most prominent features of TSS is the rapid onset of fever (>38.9°C). Biologically, TSST-1 and the other pyrogenic toxins are potent inducers of fever when injected intravenously into rabbits (Bergdoll, 1983; Kim and Watson, 1970; Schlievert et al., 1981). Typically, there is a fairly linear rise in temperature with maximal responses at approximately 4 hours after

intravenous injection (Schlievert and Kelly, 1982b). In contrast, rabbits given endotoxin intravenously show a biphasic response, peaking at 1 and 3 hours post-injection (Schlievert and Kelly, 1982b). Fever has also been reported in other systems where rabbits have been infected with a TSST-1-producing S. aureus strain or have been implanted subcutaneously with a miniosomotic pump to allow controlled release of purified TSST-1 (Arko et al., 1984; Parsonnet et al., 1987; Rasheed et al., 1985).

One reason for the pyrogenic response in animals after TSST-1 administration is the effect of the toxin on the preoptic area of the anterior hypothalamus (Poindexter and Schlievert, 1985a). TSST-1 injected intravenously, subcutaneously, intradermally, and intracisternally into the hypothalamic area all result in fever responses (Schlievert, 1984). The induction of fever by TSST-1 appears to be mediated through the release of cytokines which then stimulate the fever center of the hypothalamus (Poindexter and Schlievert, 1985a). As will be discussed later in this chapter, TSST-1 is a strong inducer of interleukin-1 (IL-1) and tumor necrosis factor (TNF) from human peripheral blood mononuclear cells (Parsonnet et al., 1985; Parsonnet and Gillis, 1988a). These cytokines are able to induce fever by stimulating the production of prostaglandin E_2 in the preoptic area of the anterior hypothalamus via the oxidation of arachidonic acid by the cyclooxygenase pathway (Dinarello and Wolff, 1982). The role of prostaglandins in the causation of fever is suggested by studies that inhibitors of the cyclooxygenase pathway (eg. indomethacin) are able to reduce the pyrogenic response caused by purified IL-1, TNF or TSST-1 (Dinarello and Wolff, 1982; Igarashi et al., 1989). Thus, the pyrogenic response to TSS-associated exotoxins is thought to result from the effect of cytokines (induced by TSST-1 or other staphylococcal or streptococcal toxins) on the hypothalamus (Bohach et al., 1990).

B. Mitogenicity For B and T Lymphocytes.

TSST-1, the staphylococcal enterotoxins, and the streptococcal pyrogenic exotoxins are all potent T cell mitogens (Barsumian et al., 1978b; Bergdoll,

1983; Carlsson et al., 1988; Poindexter and Schlievert, 1985b). In the case of SEA, significant activation of human T cells occurs at concentrations as low as 1 fg/ml, making this toxin one of the most potent mitogens known today (Carlsson et al., 1988). Both $CD4^+$ and $CD8^+$ subpopulations of T lymphocytes are stimulated to proliferate (Calvano et al., 1984).

TSST-1-induced stimulation of T lymphocyte proliferation occurs only when accessory cells are present. Poindexter and Schlievert (1985b) showed that purified human and murine T lymphocytes did not respond to TSST-1 stimulation unless macrophages were added to T cells. Subsequent studies with staphylococcal enterotoxins also indicate that antigen-presenting cells are required for stimulation of T cell proliferation (Carlsson et al., 1988). Chatila et al. (1988) reported that in the absence of monocytes, TSST-1 does not induce IL-2 receptor expression or IL-2 synthesis in T lymphocytes. Upon the addition of monocytes to T cells, IL-2 receptor expression, IL-2 synthesis, and T cell mitogenic activity were all enhanced greatly (Chatila et al., 1988).

The accessory cell requirement for toxin stimulation of T lymphocyte proliferation does not appear to involve mediators such as IL-1. Fixed macrophages were found to support SEA-induced T lymphocyte proliferation as well as viable cells (Carlsson et al., 1988). Moreover, the addition of purified IL-1 or IL-2 did not replace the accessory cell requirement for stimulation of T cell proliferation by SEA or TSST-1, suggesting that the role of antigen-presenting cells was not solely to provide these cytokines (Carlsson et al., 1988; Chatila et al., 1988). However, the use of the phorbol ester, PMA, in combination with TSST-1 or SEA could bypass the requirement for accessory cells in inducing T lymphocyte proliferation (Carlsson et al., 1988; Chatila et al., 1988). The authors postulated that T cell proliferation was driven as a result of TSST-1-induced increases in intracellular calcium levels and PMA activation of protein kinase C (Chatila et al., 1988).

In contrast to the mitogenic effect of TSST-1 on T cells, B lymphocytes do not respond to the toxin, even in the presence of monocytes or macrophages (Poindexter and Schlievert, 1985b). Moreover, Poindexter and Schlievert (1985b)

reported that the addition of mitomycin-treated T cells to untreated B lymphocytes in a 1:1 ratio did not increase the mitogenic activity of the latter cell type after the addition of TSST-1. The authors did find that B cells alone proliferated in response to bacterial lipopolysaccharide (LPS). Contrary to this work, Mourad et al. (1989) were able to show that TSST-1-stimulated tonsillar B lymphocytes required T cells for proliferative activity. In the presence of 5% irradiated T cells, TSST-1 successfully triggered the proliferation of tonsillar B lymphocytes, with maximal responses noted at a 1:1 T/B ratio (Mourad et al., 1989). Direct contact between B and T lymphocytes was important since the separation of the two cell types by a 0.4 μm membrane insert abolished TSST-1-stimulated B cell mitogenic activity. The addition of various cytokines, including IL-1, IL-2, IL-4, IL-5, IL-6, IFN-τ, and TNF-ß, alone or in combination, or of supernatants from TSST-1-activated T cells, did not replace the T cell requirement (Mourad et al., 1989).

The structural domains within TSST-1 responsible for T cell mitogenic activity have been investigated by a number of laboratories (Blomster-Hautamaa et al., 1986a; Edwin et al., 1988; Kokan-Moore and Bergdoll, 1989). Blomster-Hautamaa et al. (1986a) showed indirectly that the T cell mitogenic activity of TSST-1 resides within an internal 14 kd cyanogen bromide-generated fragment since an anti-TSST-1 mAb (designated B-14) which blocks this function also binds to this fragment, as demonstrated by Western blot analysis. Murphy et al. (1988) further localized the binding epitope of B-14 to a region of the TSST-1 molecule encompassing residues 34 to 43. Edwin et al. (1988) used papain to digest TSST-1 into three fragments of 10 kd, 12 kd and 16 kd in size. The 12 kd fragment (amino acids 88 to 194), which forms 75% of the 16 kd fragment, was found to induce significantly higher T cell mitogenic activity than the other fragments. Amino acid sequencing studies revealed that the 12 kd fragment was located towards the carboxyl end of the TSST-1 molecule (Edwin et al., 1988). Kokan-Moore and Bergdoll (1989) chemically modified histidine or tyrosine residues to determine whether these amino acids were important for biological activity of TSST-1. Their results showed that both amino acids were important for the mitogenic activity

of TSST-1. Blanco et al. (1990) extended these findings by generating mutant TSST-1 gene constructs in E. coli in which histidine and tyrosine residues were replaced by alanines at various positions. Their data indicate that histidine 141 and tyrosine 144 near the carboxyl end of the TSST-1 molecule were essential for the mitogenic activity of the toxin. More recently, a synthetic peptide to TSST-1 [TSST-1 (58-68)] has been found to be directly mitogenic for T lymphocytes in the presence of accessory cells (Edwin et al., 1991). Since the residues in this peptide are not included in the 12 kd papain digestion fragment which is also mitogenic for T cells, it has been hypothesized that there may be more than one mitogenic epitope on the TSST-1 molecule (Edwin et al., 1991).

Unlike the staphylococcal enterotoxins, TSST-1 does not contain a disulfide loop. Previous attempts to demonstrate the importance of the disulfide loop in the mitogenic activity of entertoxins have been unsuccessful (Fraser, 1989; Noskova et al., 1984). However, Grossman et al. (1990), using more efficient methods of alkylation and reduction of the enterotoxin disulfide loop, recently demonstrated that the loop is required for mitogenic activity of SEA and SEB. The authors hypothesized that the disulfide loop either interacts with the T cell antigen receptor (TCR) itself or helps maintain the conformation of some other portion of the enterotoxin molecule that is necessary for TCR interaction. As indicated earlier, TSST-1 is structurally distinct from the staphylococcal enterotoxins although both possess similar biological properties. It is possible that TSST-1 lacks a disulfide loop because it already possesses the proper structural features required for interaction with the TCR. On the other hand, the structural features necessary for interaction with T cells may not be present in the staphylococcal enterotoxins unless the disulfide loop is intact (Grossman et al., 1990).

C. Induction of Cytokines From Human Peripheral Blood Mononuclear Cells.

The ability of TSST-1 to stimulate the release of a variety of cytokines from human immune blood cells suggested that these secondary mediators may play

an important role in the pathogenesis of TSS (Parsonnet, 1989). IL-1 was first implicated in TSS when culture filtrates from TSS- but not non-TSS-associated S. aureus isolates were able to stimulate the release of this mediator from human monocytes (Ikejima et al., 1984). A subsequent study showed that the bacterial product responsible for IL-1 induction in culture filtrates was TSST-1 (Parsonnet et al, 1985).

Since these initial studies, there is now strong evidence that the macrophage-derived cytokines, IL-1 and TNF, play an important role in mediating the actions of TSST-1 in the host (Parsonnet, 1989). TSST-1 is a potent inducer of both IL-1 and TNF release from human blood monocytes in vitro (Fast et al., 1989; Jupin et al., 1988; Parsonnet et al., 1985; Parsonnet and Gillis, 1988a). A mAb (designated as mAb 8-5-7) against TSST-1, known to block TSST-1-induced IL-1 release from human monocytes, also protected rabbits from the toxic effects of TSST-1 (Bonventre et al., 1988). Ikejima et al. (1988) noted that within 3.5 h of injection of purified TSST-1 into rabbits, a TNF-like activity could be detected in their sera. More importantly, the authors noted that the appearance of the TNF-like activity coincided with the onset of shock-related hemodynamic changes. TNF has additionally been demonstrated in the sera of TSS patients but not in healthy controls (Ikejima et al., 1989a). The direct involvement of TNF in TSS pathogenesis has been shown by Parsonnet et al. (1988). In their study, rabbits were immunized with goat anti-rabbit TNF prior to the subcutaneous administration of TSST-1. Significant mortality was found in animals given TSST-1 alone or TSST-1 in combination with normal goat serum antibodies. In contrast, rabbits passively immunized with goat anti-rabbit TNF IgG were protected from the lethal effects of the toxin. Although rabbits receiving goat anti-rabbit TNF developed less lipemia and cachexia than did normal goat serum antibody-treated animals, there were no apparent differences in fever or levels of blood urea nitrogen, calcium, and serum glutamic-pyruvic transaminase levels between the two groups. This suggests that although TNF is responsible for mediating shock, other cytokines such as IL-1 may be responsible for other aspects of the disease (Parsonnet and Gillis, 1988b).

As indicated in Table V, TNF and IL-1 have many biological properties in common. Both cytokines are potent pyrogens and both are involved in the acute phase response (Dinarello, 1988). TNF is also known as a key mediator of septic shock. The injection of purified TNF into rats results in a shock state similar to that seen with endotoxin (Beutler and Cerami, 1988). Hypotension, tachypnea, diarrhea, hematuria, hemoconcentration, metabolic acidosis, pulmonary leukostasis, necrosis of gastrointestinal tract, and acute renal tubular necrosis were some of the effects observed (Tracey et al., 1986; Tracey et al., 1987a). Protection studies using monoclonal antibodies strongly support the role of TNF as a major mediator of the effects of bacterial LPS (Tracey et al., 1987b). Moreover, a second cytokine structurally and biologically related to TNF called lymphotoxin or TNFß is now known to be produced by T lymphocytes and B lymphoblastoid cell lines in response to activating stimuli other than LPS (Beutler and Cerami, 1988). Lymphotoxin is secreted in smaller amounts than TNF and appears with later kinetics than TNF (Beutler and Cerami, 1988).

Because of their similarities in biological properties, it is not surprising that TNF and IL-1 may act synergistically to induce a shock-like state in animals. Okusawa et al. (1988) reported that injecting IL-1 and TNF together into rabbits was more potent in inducing shock-related hemodynamic and hematological changes than injecting either cytokine alone. Thus, although IL-1 and TNF are unrelated at the amino acid, secondary or even tertiary structural level, their common mechanism of action seems to be due to the stimulation of similar intracellular messages as indicated by recent protein phosphorylation studies (Guy et al., 1991).

Stimulation of IL-1 and TNF release from monocytes by staphylococcal products does not appear to be restricted to TSST-1. Parsonnet et al. (1986) observed that the culture filtrates of TSST-1-negative TSS-associated S. aureus isolates were potent inducers of IL-1 from human monocytes. Upon examination of toxin production, all but one produce one or more of the staphylococcal enterotoxins, implicating a role for these toxins in TSS. Furthermore, subsequent studies have shown that staphylococcal enterotoxins are capable of stimulating

Table V

Comparison Of The Biological Properties Of IL-1 And TNF

Biological Property	IL-1	TNF
Endogenous pyrogen fever	+	+
Slow-wave sleep	+	+
Hemodynamic shock	+	+
Increased hepatic acute-phase protein synthesis	+	+
Decreased albumin synthesis	+	+
Activation of endothelium	+	+
Decreased lipoprotein lipase	+	+
Decreased cytochrome P-450	+	+
Decreased plasma Fe/Zn	+	+
Increased fibroblast proliferation	+	+
Increased synovial cell collagenase and PGE_2	+	+
Induction of IL-1	+	+
T/B cell activation	+	-

From Dinarello, 1988.

TNF and IL-1 release from human monocytes (Fast et al., 1989; Gjorloff et al., 1991). As a result, a common property of both menstrual- and nonmenstrual TSS S. aureus isolates is their ability to generate exotoxins capable of inducing IL-1 and TNF release from monocytes (Parsonnet et al., 1986).

Aside from TNF and IL-1, TSST-1 and the staphylococcal enterotoxins are strong inducers of many other cytokine products from human peripheral blood mononuclear cells. TSST-1 has been shown to stimulate the release of interleukin-2 (IL-2) from T cells, but only in the presence of accessory cells (Uchiyama et al., 1986). TSST-1 also triggers the release of interferon-τ (IFN-τ) from human peripheral blood cells (Jupin et al., 1988; Micusan et al., 1989). IFN-τ is a well-known activator of macrophages (Beutler and Cerami, 1986). Although it does not stimulate IL-1 or TNF release itself, it has been shown to amplify the endotoxin-induced release of these cytokines by macrophages and peripheral blood monocytes by stimulating transcription (Collart et al., 1986; Nedwin et al., 1985). Therefore, the possibility exists that priming of monocytes or macrophages with IFN-τ may enhance TSST-1-stimulated TNF or IL-1 release from these cells.

Structure-function studies of TSST-1 and staphylococcal enterotoxins have focussed mainly on their mitogenic rather than their cytokine-inducing properties. Edwin et al. (1991) showed that the synthetic peptide, TSST-1 (58-78), was capable of stimulating both TNF release from human monocytes and monocyte-dependent T cell proliferation. Grossman et al. (1990) observed that although the disulfide loop was critical for enterotoxin-induced mitogenicity, its cleavage did not affect monocyte production of TNFα. Hence, the T cell stimulatory activities of staphylococcal enterotoxins can be dissociated from that of monocyte stimulatory activities (Grossman et al., 1990).

D. Immunosuppression

TSST-1, the staphylococcal enterotoxins, and the streptococcal pyrogenic toxins have the capacity to nonspecifically suppress immunoglobulin production by B cells (Bergdoll, 1983; Cunningham et al., 1978; Poindexter and Schlievert, 1986). In vitro, Poindexter and Schlievert (1986) have shown that TSST-1

suppresses the response of immunoglobulin-secreting cells to pokeweed mitogen. The immunosuppressive effect appeared to be mediated through soluble factors released by TSST-1-activated T lymphocytes (Poindexter and Schlievert, 1986). These findings appeared to contradict those of Mourad et al. (1989) who showed that TSST-1 stimulated B cell proliferation and differentiation into immunoglobulin-secreting cells in the presence of T lymphocytes. However, a recent report by Moseley and Huston (1991) indicates that a dichotomy exists regarding exotoxin effects on B cell proliferation and immunoglobulin production. The authors showed that TSST-1- or SEA-stimulated B cells proliferated and produced immunoglobulins in the presence of irradiated or mitomycin C-treated T lymphocytes. However, the ability of SEA or TSST-1 to inhibit B cell proliferation and immunoglobulin synthesis in response to Staphylococcus aureus Cowan I stimulus strain (SAC) depended on the nature of the T cells. SEA-induced inhibition of immunoglobulin synthesis was observed in the presence of proliferating T cells whereas pretreatment of the T cells with irradiation or mitomycin C abolished the inhibition. This suggests that either the T cell proliferation process itself or the production of a T cell-derived inhibitory factor is important for the induction of the inhibitory effect by these exotoxins (Moseley and Huston, 1991). This hypothesis is supported by the finding that mAbs which block TSST-1-induced T cell mitogenicity also block TSST-1-mediated suppression of immunoglobulin synthesis (Blomster-Hautamaa et al., 1986a). One potential inhibitor from T cells is IFN-τ, whose secretion is induced by exposure of peripheral blood mononuclear cells to TSST-1 or staphylococcal enterotoxins (Jupin et al., 1988; Micusan et al., 1989). This cytokine has several biological properties, including the ability to inhibit polyclonal immunoglobulin production (Finkelman et al., 1988).

The inhibition of immunoglobulin production by TSST-1 and the other exotoxins may explain the absence of TSST-1 antibodies in TSS patients or why some individuals do not seroconvert to TSST-1 after a disease episode (Notermans et al., 1983; Stolz et al., 1985). In one study, total IgG levels in acute sera from TSS patients were significantly lower than those of healthy controls, which

is indicative of polyclonal antibody suppression of immunoglobulin production (Christensson et al., 1986). Of interest, IgG1 and IgG4 against TSST-1 were elevated in TSS patients that seroconverted to the toxin, suggesting that these subclasses may be important in neutralizing the effects of this toxin (Christensson et al., 1986).

E. Cytotoxic Effect Of TSST-1 On Porcine Aortic Endothelial Cells

TSST-1 has been shown to cause a direct cytotoxic effect on porcine aortic endothelial cells (PAEC) as measured by a ^{51}Cr release assay (Lee et al., 1991b). The cytotoxic effect did not depend on LPS since polymyxin B sulfate (which inactivates lipid A of LPS) and the addition of various concentrations of LPS did not affect TSST-1-induced PAEC cytotoxicity. The cytotoxic activity was dependent on binding and internalization of TSST-1 to PAEC since inhibitors of this process were able to abolish the effect. In addition, TSST-1-stimulated damage to PAEC was blocked by an antioxidant and iron chelator, deferoxamine, suggesting that oxidants were involved in mediating the cytopathic effect (Lee et al., 1991b). The significance of the TSST-1-induced cytotoxic damage on PAEC was examined in the same study by monitoring the migration of ^{125}I-BSA across the transendothelial membrane in the presence of TSST-1. The authors showed that an increase in the permeability of PAEC-monolayers was observed in the presence of TSST-1, as demonstrated by migration of ^{125}I-BSA through the cells. Thus, aside from the effects of TNF and IL-1 on the vascular endothelium, direct cytotoxic effects by TSST-1 may contribute to the vascular leak and shock observed in TSS (Lee et al., 1991b). Recent data show that fluid replacement was highly successful in preventing the lethal effects of TSST-1 and SPEA in rabbits, suggesting that toxin interactions causing vascular leakage is of major importance in the development of shock in TSS (Lee et al., 1991a).

F. Enhancement of Host Susceptibility to Lethal Endotoxic Shock and the Role of Endotoxin in TSS

TSST-1 has been shown to enhance the susceptibility of rabbits to lethal endotoxin shock by up to 50,000 fold (Schlievert, 1982c). Rabbits administered with TSST-1 followed by doses of endotoxin 1/500 of the LD_{50} developed fever, hypothermia, labored breathing, diarrhea, evidence of vascular collapse and finally death (Poindexter and Schlievert, 1985; Sclievert, 1982c). Control rabbits given TSST-1 or endotoxin intravenously developed fever only. The animals were protected from the enhanced susceptibility to endotoxin by prior immunization with TSST-1 (Schlievert, 1982c).

Another example of synergistic interactions between TSST-1 and endotoxin has also been demonstrated in renal tubular cells. Endotoxin alone induces a dose-related necrosis of these cells which involves a calcium-dependent generation of reactive oxygen species like superoxide and hydrogen peroxide (Keane et al., 1986). In contrast, exposure of these cells to TSST-1 results in minimal cytotoxic effects. Pretreatment of renal tubular cells with TSST-1 prior to the addition of endotoxin results in a significant enhancement of cytotoxicity compared with that observed with endotoxin alone. The mechanism by which TSST-1 sensitizes renal tubular cells for endotoxin-triggered necrosis appears to involve receptor-mediated endocytosis of the staphylococcal exotoxin and an increase in intracellular calcium (Keane et al., 1986).

The synergistic effects observed with the combination of TSST-1 and endotoxin initially created some controversy as to whether TSST-1 was a pathological agent by itself or whether its role was to sensitize the host to small amounts of endogenous endotoxin, as had been demonstrated previously by Schlievert (1982c). Moreover, variable results obtained in animals given lethal doses of TSST-1 by intravenous bolus injection did not help to clarify matters any further. A number of investigators have noted that lethality due to bolus injection of TSST-1 depended on the age and breed of the animals as well as sex-related differences (de Azavedo and Arbuthnott, 1984a; Reeves et al., 1986; Melish et al., 1989b). The role of endotoxin in TSS pathogenesis was directly

addressed by Melish et al. (1989) using a live staphylococcal infection model or a subcutaneous TSST-1 depot model which was found to reproduce the major physiologic abnormalities of human TSS more reliably than the intravenous bolus injection of TSST-1. The authors showed that neutralization of the effects of endotoxin with antiserum to core lipopolysaccharide or with the antibiotic polymyxin B sulfate did not change the clinical course or mortality among animals infected with live TSS-associated staphylococci or among animals challenged with a subcutaneous depot of TSST-1. In contrast, TSST-1 antibodies were effective in preventing disease and death in these models (Melish et al., 1989). These results clearly demonstrate that endotoxin is not required for TSS pathogenesis and that TSST-1 by itself is capable of inducing the full spectrum of TSS.

G. Lethality in Rabbits

As mentioned in Section 1.5, studies in rabbits using purified TSST-1, live TSS-associated S. aureus isolates or recombinant strains bearing the *tst* gene reproduce many of the clinical features of the disease in humans, with the exception of the rash and desquamation (de Azavedo, 1989). Interestingly, mice, rats, hamsters, guinea pigs, and monkeys are relatively resistant to the lethal effects of the toxin unless high doses are used (de Azavedo, 1989; Quimby and Nguyen, 1985). However, some mouse strains do show symptoms of rapid weight loss, thymus atrophy and immunosuppression when exposed to mild doses of toxins like SEB (Marrack et al., 1990). The resistance of these animals may be related to their inability to produce cytokines at the translational level in response to the staphylococcal exotoxins. Ikejima et al. (1989b) noted that a 10-fold higher dose of TSST-1 was required to induce fever in endotoxin-resistant C3H/HeJ mice compared with endotoxin-sensitive A/HeJ mice. The amount of recombinant IL-1 required to induce fever in both mouse strains was similar, indicating that murine macrophages from the two strains were responding differently to TSST-1. Furthermore, both endotoxin and TSST-1 were able to induce the transcription of murine IL-1α and IL-1ß genes. Surprisingly, the synthesis of IL-1α and IL-1ß mRNA was more significantly induced in the resistant C3H/HeJ strain than in the

sensitive A/HeJ strain. These results suggest that the C3H/HeJ resistance to endotoxin or TSST-1 is not at the transcriptional level but may be due to a defect in the translational or processing of the IL-1 precursor (Ikejima et al., 1989b).

Various models have been used to examine the lethal effects of TSST-1-positive S. aureus isolates in rabbits. Most studies utilize either subcutaneous polyethylene whiffle balls (Arko et al., 1984) or micropore diffusion chambers consisting of a silicone tubing with a 0.22 μm membrane at either end (Scott et al., 1983). Both models produce fever, conjunctival hyperemia, respiratory distress and shock similar to the human syndrome (de Azavedo, 1989). A more analogous model of human TSS is the vaginal tampon model described by Melish et al., (1989). Finally, the administration of purified TSST-1 via a subcutaneous infusion pump has also helped to define the role of this toxin in TSS (Parsonnet et al., 1987).

Interestingly, there are age- and species-related differences in the susceptibility of rabbits to TSST-1. Older rabbits (> 1 to 2 years) were more susceptible to TSST-1 than younger animals (de Azavedo et al., 1984b). At present, there is no adequate explanation for this difference (de Azavedo, 1989). Different strains of rabbits have also been found to differ in their susceptibility to TSST-1; both conventional and specific pathogen-free New Zealand white rabbits were more susceptible to TSST-1 than the corresponding Dutch-belted strain (de Azavedo and Arbuthnott, 1984a; de Azavedo et al., 1985b). Male rabbits are also more susceptible to the lethal effects induced by TSST-1-positive S. aureus isolates than female rabbits (Best et al., 1984). Hormonal differences may play a role in the latter case since either castration or the administration of estrogen protects male rabbits and prolongs survival (Best et al., 1984; Best et al., 1986). It has been hypothesized that sex hormones act by inhibiting in vivo TSST-1 production by S. aureus strains since there are no gender differences in susceptibility when purified TSST-1 is used (de Azavedo, 1989).

1.10 Pathogenesis Of TSS: An Overview

Having provided an overview of TSS and the role of TSST-1 in the pathogenesis of the disease, an important question remains as to how this toxin causes the manifestations seen among TSS patients. As shown in Figure 1, host factors such as the ability of TSST-1-producing *S. aureus* isolates to colonize the human genital tract or the lack of serum antibodies to TSST-1 play just as an important role in the causation of TSS as do the biological effects of TSST-1 itself. Consequently, TSS can be considered a multifactorial disease with many contributors to the pathogenic process.

There is now strong evidence that staphylococcal exotoxins are an important bacterial mediator of TSS. These agents act by directly exerting toxic effects on host tissues or by inducing the release of endogenous mediators such as monokines or lymphokines. Molecules such as IL-1 and TNF, when released in small quantities are of considerable benefit to the host in protection against diseases. For example, TNF is known to enhance many neutrophil and macrophage functions, to induce HLA antigen expression, to stimulate IL-1 secretion by many cell types, and to activate macrophages (van der Merwe, 1988). In contrast, when released in massive amounts, these cytokines can produce harmful effects in the host such as shock or cachexia. Thus, the host may suffer from harmful physiological effects as a consequence of its own response to environmental insult.

The accumulated evidence to date suggests that many of the effects of TSST-1 is mediated by its interaction with human peripheral blood mononuclear cells and the subsequent massive release of inflammatory cytokines such as IL-1, IL-2 and TNF. The release of these particular cytokines as well as other mediators may then lead to the production of fever, the acute phase response, hypotension, and other manifestations seen among TSS patients as shown if Figure 2.

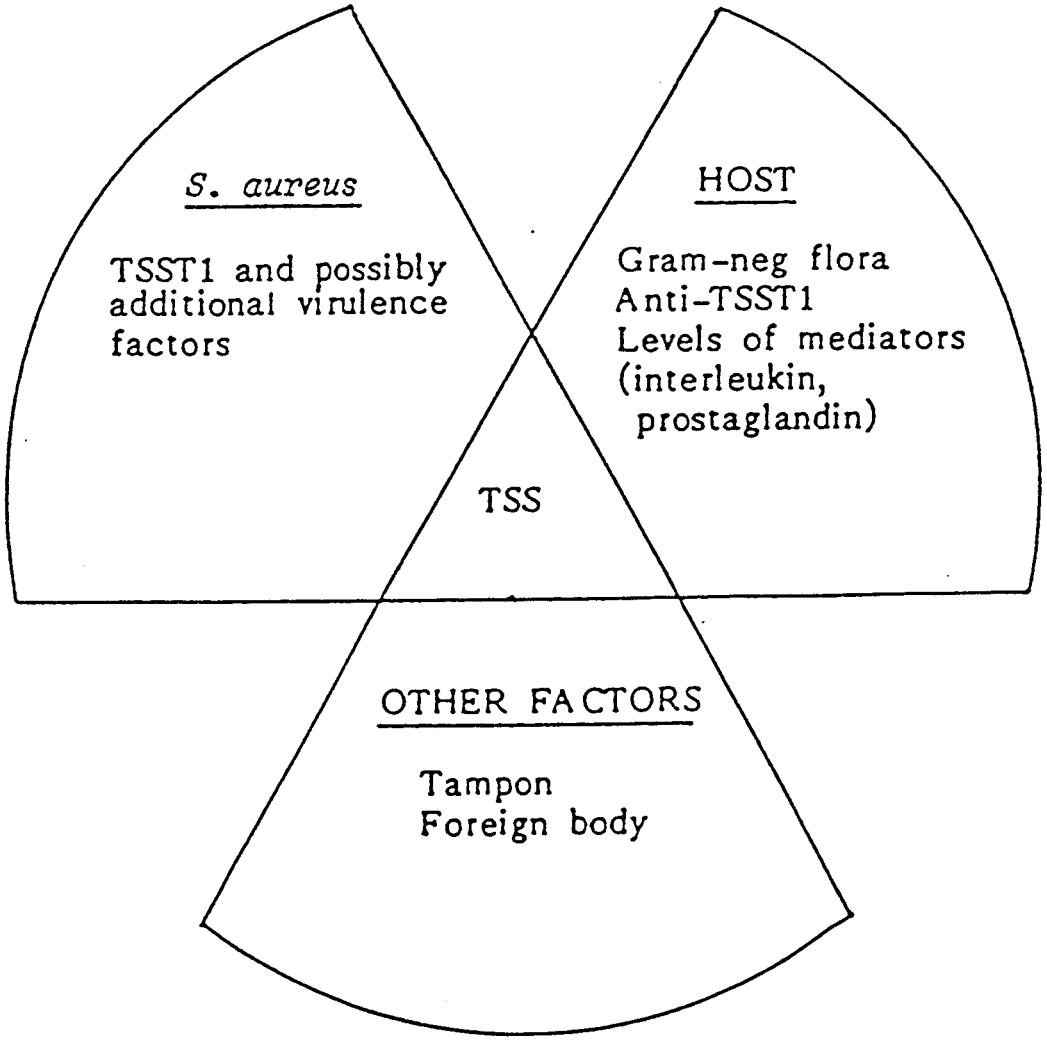

Figure 1. Factors in TSS pathogenesis.

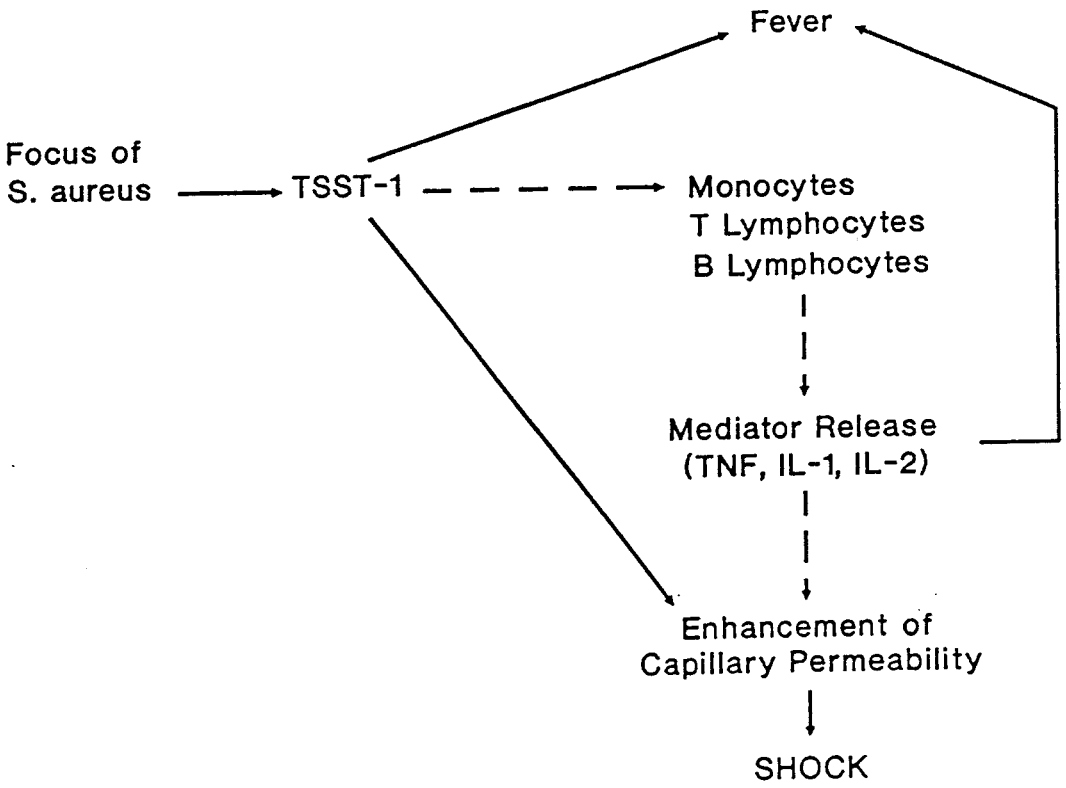

Figure 2. Hypothesis of TSS pathogenesis.

1.11 Thesis Objectives And Rationale

Although the biologic properties of TSST-1 have been well-studied, little is understood about the mechanisms by which this toxin exerts its physiological effects. For instance, few studies have addressed the question of how TSST-1 stimulates the release of cytokines from human peripheral blood monocytes or T cells. The interaction of TSST-1 with specific receptors may be crucial for initiating the sequence of events leading to the processes of cytokine induction or T cell proliferation. Although receptors have been demonstrated for TSST-1 on conjunctival epithelial cells (Kushnaryov et al., 1984) and on a few selected peripheral blood mononuclear cells (Poindexter and Schlievert, 1985), little is known about the identity of the receptor itself or how it interacts with TSST-1. Characterization of the receptor for TSST-1 and other staphylococcal enterotoxins on human blood cells may help elucidate some of the molecular mechanisms by which these toxins act. Perhaps the similarities in biological properties between TSST-1 and staphylococcal enterotoxins are due to binding of a common receptor on these cells.

Although there are many similarities between gram-negative septic shock and TSS, there are also obvious differences (eg. rash and desquamation). A comparison of the biological properties between TSST-1 and LPS indicates that only the former has a potent effect on T lymphocytes. This suggests that the mechanism of shock induction by TSST-1 and endotoxin is different. Further studies need to be performed to address this question.

The major objective of this thesis is to further our understanding of how TSST-1 interacts with human peripheral blood mononuclear cells and the manner by which this interaction leads to cytokine induction. I have elected to focus on human monocytes for several reasons. Monocytes are known target cells for TSST-1 since they respond to this toxin by releasing IL-1 and TNF, two cytokines thought to play a key role in TSS. Furthermore, it is now well-known that activation of T cell proliferation and lymphokine release by TSST-1 is monocyte-dependent. Thus, the monocyte appears to be a focal point in TSS pathogenesis. Finally,

since monocytes are also target cells for LPS, comparisons can be made regarding the manner by which TSST-1 and LPS exert their toxic effects.

The study of TSST-1-monocyte interaction is divided into several phases. The first phase (described in Chapter 3) examines whether TSST-1 receptors exist on human blood monocytes and if so, what is the identity of these receptors. Characterization of TSST-1 receptors will be carried out by cross-linking studies with radiolabeled toxins or by the use of antibodies directed against monocyte surface antigens. In addition, the ability of staphylococcal enterotoxins to compete for TSST-1 receptor sites on human monocytes will be determined. These studies will provide some insight as to why the biological properties of TSST-1 and enterotoxins are so similar.

In the second phase of the thesis (described in Chapter 4), the signal transduction pathways by which TSST-1 activates human monocytes will be explored. Specifically, protein phosphorylation of monocyte cellular proteins in response to TSST-1 stimulation will be examined by two-dimensional gel electrophoresis. The phosphorylation patterns induced by TSST-1 will then be compared with those of other monocyte agonists such as staphylococcal enterotoxins, LPS, and the phorbol ester, PMA. The results from this chapter will help determine whether these agents share similar signal transduction pathways of monocyte activation.

The third phase of the thesis (Chapter 5) will examine whether T lymphocytes are involved in cytokine production by TSST-1-stimulated monocytes. Previous cytokine studies have used only peripheral blood mononuclear cells or adherence-purified human monocytes. It is possible that contaminating T lymphocytes in these preparations may be a contributing factor for cytokine secretion by human monocytes in response to TSST-1 stimulation. To resolve this issue, highly purified human monocytes will be stimulated with TSST-1 in the presence and absence of T lymphocytes and the resulting culture supernatants examined for TNF and IL-1 production. Moreover, the mechanism by which T cells participate in cytokine production by TSST-1-triggered monocytes will be explored. The role of lymphokines, direct cell contact and adhesion molecules will also be addressed in this section.

Finally, in the last phase of this thesis (Chapter 6), protein kinase inhibitors will be used to determine the types of kinases involved in cytokine production by human peripheral blood cells. Inhibitors of protein kinase C, tyrosine kinases and cAMP- and cGMP-dependent kinases will provide valuable clues as to the second messengers used during secretion of IL-1 and TNF by TSST-1-induced monocytes and T cells.

In summary, the goal of this thesis is to explore the mechanisms by which staphylococcal toxins activate human monocytes. The findings will not only increase our understanding of the pathogenesis of TSS but will also add to our knowledge of other staphylococcal and streptococcal infections.

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Chapter 2

Materials and Methods

2.1 Purification Of TSST-1

2.1.1 Glassware

Reagents and glassware used in the purification of TSST-1 were maintained pyrogen-free to prevent endotoxin contamination. All glassware were baked at 180°C overnight and autoclaved prior to use. Reagents were prepared using pyrogen-free water at all times.

2.1.2 Bacterial Strains

Staphylococcus aureus MN8, isolated from the vagina of a woman with typical menstrual TSS, was provided by P. Schlievert (University of Minnesota). This strain has been previously shown to be a high producer of TSST-1 and was maintained on brain heart infusion (BHI) agar (Difco) containing 5% sheep blood at 4°C. It was regularly transferred into fresh medium to maintain purity and viability.

2.1.3 TSST-1 Production

A single colony of strain MN8 was picked from a BHI-blood agar plate and inoculated into 20 ml of dialyzable brain-heart infusion broth (dBHI). 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, 1 ml amounts were inoculated into 6x 1000 ml flasks containing 250 ml dBHI. After shaking for 24 h, culture medium was centrifuged at 15,000 x g for 30 min at 4°C (J2-21 Superspeed centrifuge; Beckman instruments, Palo Alto, CA) to remove bacterial cells. Supernatants were then concentrated 10-fold with an Amicon Spiral Cartridge concentrator (Model CH2PRS; Acrylic Reservoir RA2000; Spiral Cartridge

S1 Y10) with a molecular weight cutoff of 10 kd. The concentrated supernatants were filter-sterilized and stored at 4°C until purification.

2.1.4 Preparative Isoelectric Focusing And Chromatofocusing

TSST-1 was purified from culture supernatants by a series of preparative isoelectric focusing (PIEF) and chromatofocusing as previously described (WWS Kum, J Wong, RH See, and AW Chow, manuscript submitted). Briefly, 45 ml of concentrated culture supernatant were mixed with Pharmalyte, pH 6.7-7.7 (Sigma Chemical Co., St. Louis, Mo), to give a final concentration of 2% (w/v) of the latter. The mixture was focused at 12 W on a Rotofor Cell (Bio-Rad Laboratories, Mississauga, Ont.) for 4 h at 15°C. 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 a non-competitive ELISA previously developed in our laboratory (Rosten et al., 1987). Fractions containing >50 μg/ml TSST-1 were pooled, dialyzed extensively at 4°C against deionized water 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, Dorval, Quebec) 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, Dorval, Quebec) at a flow rate of 36 ml/h. Fractions positive for TSST-1 as determined by ELISA were dialyzed against several changes of deionized water at 4°C and lyophilized. The overall recovery of TSST-1 by the PIEF/chromatofocusing method was 43% of that in the crude supernatants with a yield of 2 mg/l culture supernatant. The final TSST-1 preparations were endotoxin-free as measured by the Limulus amoebocyte lysate test (sensitivity limit, 10 pg/ml) (Levin and Bang, 1984).

2.1.5 Preparation Of Antisera Against S. Aureus MN8 Culture Supernatant

Antisera to S. aureus MN8 exoproteins were prepared by injecting 100 μg of crude culture supernatant in complete Freund's adjuvant into female New Zealand White rabbits (20 weeks old, R and R Rabbitry, Stanwood, WA)

intramuscularly and subcutaneously. The crude supernatants were injected 5 more times (100 μg per rabbit) at 2 week intervals in incomplete adjuvant. Rabbits were bled and tested for the presence of antibodies one week after injection.

2.1.6 SDS-PAGE Analysis

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as described previously (Laemmli, 1970), using a Protean XII apparatus (16 cm x 18 cm x 1.5 mm slab gel, Bio-Rad Laboratories, Mississauga, Ont.). Toxin preparations at different stages of purification were analysed as follows. Samples were adjusted to 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol (SDS-sample buffer) and boiled for 3 min prior to electrophoresis in a 4% stacking gel and a 14% resolving gel. Rainbow-colored molecular weight protein markers (range = 14.3 kd to 200 kd, Amersham, Arlington Heights, Ont.) were used to estimate molecular size of proteins in the gel lanes. Protein bands were visualized in gels using a Bio-Rad silver staining kit. (Bio-Rad Laboratories, Mississauga, Ont.).

2.1.7 Western Blot Analysis Of Toxin Preparations Using Antisera Against S. Aureus MN8 Culture Supernatants

Immunoblot analysis was performed as previously described (Burnette, 1981). Following SDS-PAGE, proteins were transferred to 0.45 μm nitrocellulose at 150 mA for 1 h using the semi-dry Electroblotter A (Ancos, Denmark). The nitrocellulose was incubated in 5% skim milk for 1 h at 20°C to block non-specific sites followed by incubation for 18 h at 20°C with 1% polyclonal rabbit antiserum raised against crude culture supernatant from the MN8 strain. After washing 3 times with 20 mM Tris-buffered saline, pH 7.5, with 0.1% Tween 20 (TBS-T), the nitrocellulose was rocked with biotinylated goat anti-rabbit IgG (1/1000 dilution in TBS with 1% BSA, Burlington, Ont.) for 2 h at 20°C. Following another 3 washes with TBS-T, streptavidin horse-radish peroxidase (1/1000 dilution in TBS with 1% BSA, Gibco/BRL, Burlington, Ont.) was added and the nitrocellulose incubated for another 30 min at 20°C. After extensive washing with TBS-T and once

with TBS, immunoreactive bands were visualized with 4-chloronapthol substrate (BRL, Burlington, Ont.).

2.2 Characterization Of Receptors For TSST-1 On Human Peripheral Blood Mononuclear Cells (PBMC).

2.2.1 Purification Of Monoclonal Antibodies (mAb) To Monocyte Surface Antigens

Monoclonal antibodies (L243, L203, and 2.06) against monomorphic determinants of the class II Major Histocompatibility Antigen (MHC), HLA-DR, as well as the monocyte-specific CD11b antigen (OKM1) were produced by hybridomas obtained from the American Type Culture Collection (Bethesda, MD). The hybridoma cell lines were expanded in culture and then injected intraperitoneally into pristane-primed BALB/c mice to generate ascites. The mAbs were then purified from ascitic fluid by ammonium sulphate precipitation followed by protein G chromatography using the MAb Trap kit (Pharmacia Fine Chemicals, Dorval, Quebec).

2.2.2 Purification Of Human PBMC

Fresh human peripheral blood mononuclear cells (PBMC) were obtained from normal healthy donors and prepared by centrifugation of plateletpheresis buffy coats over Histopaque 1.077 (Sigma Chemical Co., St. Louis, Mo.). Cells at the interface were washed five times with Hank's balanced salt solution and additionally separated into T and non-T cell populations by rosetting with sheep red blood cells as described (Madsen and Johnson, 1979). Monocytes were then separated from B lymphocytes by density centrifugation using Percoll (Pharmacia Fine Chemicals, Dorval, Quebec, Canada) as outlined by de Boer et al. (1981). Briefly, the non-T cell fraction was suspended in RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, Utah). The cells were then mixed with Percoll to give a final specific gravity of 1.062 g per ml. One ml of RPMI 1640 with 10% FBS was gently layered on top of each suspension. The gradient was then centrifuged at 850 x g for 15 min. The monocyte-containing interface was removed, washed three times and suspended in RPMI 1640 containing 10% FBS. Purity

of the monocytes was >90% as assessed by non-specific esterase staining of cytospin preparations (Yam et al., 1971). For isolation of purified T lymphocytes, E-rosetted cells were treated with ammonium chloride to remove sheep red blood cells, washed three times and subjected to antibody-directed complement-mediated lysis using an antibody directed against HLA-DR antigen, L243, and to the monocyte antigen OKM1 and pooled rabbit complement as described (Chatila et al., 1987). T lymphocytes were suspended in the above medium. Purity of T cells, confirmed by flow cytometric analysis, was >98% $CD2^+$ and <2% $HLA\text{-}DR^+$.

2.2.3 Iodination Of TSST-1 And SEA

TSST-1 and SEA were iodinated by the chloramine T procedure (Palaszynski et al.,1984) with the following modifications (See et al., 1990). Briefly, 0.1 M sodium phosphate buffer (pH 7.4) containing 100 μg/ml polyethylene glycol and 10% dimethyl sulfoxide was added to 100 μg of TSST-1 or highly purified SEA (>95%, Toxin Technology, Madison, WI) in a total volume of 135 μl. A quantity (10 μl) of freshly prepared chloramine T solution (1 mg/ml) was added, and this was followed by 20 μl (2 mCi) of ^{125}I (carrier-free NaI; 100 mCi/ml; ICN Radiochemicals, Irvine, CA). After 20 min incubation at 20°C, the reaction was stopped by the addition of 10 μl of sodium metabisulfite (3 mg/ml) and the mixture put on ice for 5 min. Iodinated toxins were separated from free iodine by gel filtration through Sephadex G-25 (Pharmacia Fine Chemical, Dorval, Quebec) columns equilibrated with phosphate-buffered saline (PBS) containing 0.25% gelatin. Pooled fractions corresponding to the protein peak were adjusted to 0.1% with BSA and stored in 200-μl aliquots at -70°C. More than 95% of the radioactivity in the pooled fractions was precipitable with 10% trichloroacetic acid. Typically, the specific activity of radioiodinated TSST-1 or SEA ranged from 30 to 55 $\mu C/\mu g$ of protein, with loss of <10% of biologic activity, as determined by their mitogenic effect on human PBMC and their ability to induce TNFα secretion in these cells. Preparations of ^{125}I-TSST-1 or ^{125}I-SEA were stable for at least 2 months when stored at -70°C and exhibited no changes in binding

characteristics over this time period. Protein recovery was approximately 50% to 70%, as determined by standard ELISAs for the toxins.

2.2.4 Binding Studies With ^{125}I-TSST-1 And ^{125}I-SEA

Binding assays were performed as described (See et al., 1990; See et al., 1992) by suspending human PBMC in binding medium (RPMI 1640 containing 1% BSA) at a concentration of 1-3 x 10^8 cells/ml and transferring 100 μl aliquots to 1.5 ml Eppendorf microfuge tubes. The binding reaction was initiated by incubating PBMC at 4°C with various concentrations of ^{125}I-TSST-1 or ^{125}I-SEA in final volumes of 300 μl. Nonspecific binding was determined by adding a 100-fold or greater molar excess of unlabeled toxin. The assay mixtures were gently rotated for various periods, and unbound radiolabeled toxin was then separated from the cells by transferring triplicate 90-μl portions of assay mixture to pre-cooled microcentrifuge tubes containing 280 μl of 1.1:1 mixture of dibutyl phthalate and dioctyl phthalate oils (BDH, Vancouver, BC) and centrifuging the tubes at 16,000 x g for 1 min. The tubes were then immediately frozen at -70°C, and the tube tips containing the cell pellets were excised. Cell-associated radioactivity was determined with a Searle 1185 gamma counter. All data are reported in terms of specific binding (total binding minus nonspecific binding). Receptor numbers and dissociation constants were determined from the binding studies with the aid of the LIGAND binding program (Munson and Rodbard, 1980). For competitive binding experiments, PBMC were incubated for 1 h at 4°C with radiolabeled TSST-1 plus increasing concentrations of unlabeled TSST-1 or staphylococcal enterotoxins (A,B,C,D, and E, Toxin Technology, Madison, WI).

Binding studies with purified human monocytes were performed by suspending the cells at a concentration of 5 x 10^7 to 1 x 10^8 cells/ml. Approximately 3 x 10^7 monocytes were incubated with radiolabeled TSST-1 or SEA at 4°C and processed as described above. For binding inhibition assays with HLA-DR mAbs, monocytes were incubated for a minimum of 1 h at 4°C with anti-HLA-DR mAbs (L243, L203, or 2.06) or isotype control mAb against the monocyte surface antigen, OKM1, before the addition of radiolabeled toxin.

2.2.5 Receptor Cross-Linking

Cross-linking studies were performed using the method previously described (Sorenson et al., 1986). Specifically, 1 x 10^7 human PBMC or monocytes were incubated with 60 nM ^{125}I-TSST-1 or ^{125}I-SEA for at least 1 h at 4°C. The cells were washed twice and resuspended in 200 μl of ice-cold PBS, pH 7.4. Disuccinimidyl suberate (DSS) (Pierce, Rockford, IL), freshly dissolved in dimethyl sulfoxide at 40 nM, was added to give a final concentration of 1 mM. After rotating at 4°C for 30 min, the reaction was stopped by the addition of 200 μl of 50 mM Tris-HCL, pH 7.4, and the cells pelleted at 250 x g for 5 min. Monocytes were then resuspended in 1 ml ice-cold 10 mM Tris-HCL, pH 7.4, containing 10 μg/ml leupeptin, 100 kallikrien inhibitor units/ml aprotonin, and 2 mM freshly added phenylmethylsulfonyl fluoride (PMSF). After swelling for 10 min at 4°C, cells were lysed by passing through a 26-gauge needle 6 times. Released nuclei and unbroken cells were removed by centrifugation at 800 x g for 5 min at 4°C. Membranes were then pelleted at 16,000 x g for 30 min in a microfuge. The resulting pellet was dissolved in 40 μl of SDS-sample buffer containing 2% SDS and 10% glycerol, boiled for 3 min and analysed by SDS-PAGE using a 12% separating gel. After electrophoresis, the gel was dried and exposed to Kodak X-OMAT-AR film at -70°C in the presence of Cronex lighting plus intensifying screens. For monoclonal antibody inhibition experiments, monocytes were pretreated with monoclonal antibodies for 1 h at 4°C, washed and cross-linked to radiolabeled toxin as described above.

2.3 Stimulation Of Human PBMC Proliferation By TSST-1

2.3.1 Mitogenicity Assay

Mitogenicity assays were performed as previously described (Poindexter and Schlievert, 1985). Human PBMC were suspended at a concentration of 3 x 10^6 cells/ml in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, Utah), 25 mM HEPES, 2 mM L-glutamine, and 10 μg/ml polymyxin B sulphate. 3 x 10^5 cells were cultured in 200 μl volumes with various

concentrations of TSST-1 or SEA in round-bottom 96-well tissue culture plates (Falcon Plastics) for 3 days at 37°C in 5% CO_2. For mAb inhibition studies, PBMC were incubated with the indicated concentrations of anti-HLA-DR mAbs or control mAb OKM1 for 1 h at 37°C before toxin stimulation. At 48 h, cells were pulsed with 1 μCi of [^{3}H]-thymidine (5 Ci/mmole; Amersham, Arlington Heights, Ont.) and harvested 18 h later onto glass-filter paper using an automatic harvester (Skatron, Norway). Samples were counted in a liquid scintillation counter (Beckman LS 1800). All assays were carried out in quadruplicates and controls included PBMC alone, PBMC plus mAb, and PBMC plus toxin.

2.4 Phosphorylation Changes In Monocyte Cellular Proteins Induced By TSST-1 And Staphylococcal Enterotoxins

2.4.1 Phosphate-Labeling Of Human Monocytes

Human monocytes prepared as described earlier were washed 3 times with phosphate-free RPMI 1640 and suspended at a concentration of 5 x 10^7 cells/ml in the same medium containing 2% dialyzed FBS (Hyclone, Logan, Utah). Monocytes were then incubated with 0.5 mCi/ml carrier-free ^{32}P-orthophosphate for 2 h at 37°C. The cells were then washed 3 times with phosphate-free RPMI 1640 and resuspended in the same buffer at 5 x 10^6 cells/ml.

2.4.2 Stimulation With Factors And Preparation Of Cell Lysates

^{32}P-labeled monocytes were stimulated with TSST-1, SEA, and SEB at 37°C for various time intervals. Studies were also done with two other monocyte agonists, *E. coli* lipopolysaccharide (LPS) 026:B6 and phorbol-12-myristate-13-acetate (PMA), both from Sigma Chemical Co. (St. Louis, MO). The reaction was stopped after 15 min by the addition of 10 volumes of ice-cold PBS containing phosphatase inhibitors (100 mM sodium fluoride, 10 mM tetrasodium pyrophosphate, 4 mM EDTA, 2 mM sodium orthovanadate, and 1 mM sodium molybdate, pH 7.4) and the cells were centrifuged for 5 min at 225 x g. The supernatant was aspirated and the pelleted cells lysed in 60 μl lysis buffer (50 mM HEPES, 2 mM sodium

orthovanadate, 4 mM EDTA, 10 mM tetrasodium pyrophosphate, and 100 mM sodium fluoride) containing 1% NP-40, 10 μg/ml leupeptin, 100 KIU/ml aprotonin, 10 μg/ml soybean trypsin inhibitor, 1 μM pepstatin, and 2 mM PMSF). After 20 min on ice, the lysates were cleared of insoluble material by microfuging at 16,000 x g for 15 min at 4°C and the supernatant gently layered onto Sephadex G-25 beads (Pharmacia Fine Chemicals, Dorval, Quebec,) packed in a 1 ml syringe. ^{32}P-labeled proteins were separated from free ^{32}P-orthophosphate by centrifuging the columns at 600 x g for 4 min at 4°C. The eluents were then denatured in 1% SDS (final concentration) and boiled for 3 min prior to isoelectric focusing.

2.4.3 Two-dimensional Gel Electrophoresis Of ^{32}P-Labeled Proteins

Proteins were resolved by two-dimensional gel electrophoresis as previously described (O'Farrell, 1975) with the following modifications. Samples were loaded onto 125 x 3.0 mm tube gels containing 2% ampholytes (9:1 mixture of ampholines with pI values of 5-7 and 3-10, respectively, Bio-Rad Laboratories, Mississauga, Ont.). Isoelectric focusing was performed for 18 h at 400 V followed by 2 h at 1000 V. Second dimension SDS-PAGE was carried out using 12.5% polyacrylamide gels. Gels were fixed in 40% methanol/10% glacial acetic acid (v/v) for 1 h, washed in distilled water, dried and exposed to Kodak X-OMAT-AR film using double intensifying screens. The pH gradient in the first dimension was determined by cutting gels into 1 cm slices and incubating each slice in 1 ml degassed deionized water for 2 h before measuring the pH.

2.4.4 Western Blot Analysis Of Monocyte Cytosolic And Membrane Proteins With Anti-phosphotyrosine Antibodies

Analysis of phosphotyrosine-containing proteins in both cytosolic and membrane fractions from stimulated monocytes was performed by immunoblotting using an anti-phosphotyrosine mAb, 4G10 (Upstate Biotechnology, Lake Placid, NY) as previously described (Sorensen et al., 1989). Approximately 2 x 10^7 unlabeled monocytes were stimulated with TSST-1, SEA, SEB, or PMA at 37°C for 5 min and 10 volumes of ice-cold PBS containing phosphatase inhibitors were then added, and

the cells pelleted as described above. The cells were resuspended in 1 ml ice-cold hypotonic buffer (10 mM Tris-HCL, pH 7.4, 2 mM sodium orthovanadate, 1 mM sodium molybdate, 4 mM EDTA, 5 mM sodium fluoride, 10 mM tetrasodium pyrophosphate, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 2 mM freshly added PMSF) and allowed to swell for 15 min on ice. Monocytes were then homogenized at 4°C using 30 strokes of a Wheaton homogenizer. The homogenates were then passed 6 times through a 26-gauge needle and centrifuged in a tabletop centrifuge (800 x g, 5 min at 4°C) to pellet nuclei and unbroken cells. The supernatants were then microfuged at 16,000 x g for 30 min at 4°C to pellet the membranes. The resulting pellets were then suspended in the above lysis buffer with phosphatase and protease inhibitors. Cytosolic proteins were precipitated overnight at -20°C with 9 volumes of HPLC-grade acetone, pelleted at 16,000 x g for 15 min and suspended in lysis buffer. Both membrane and cytosolic proteins in lysis buffer were adjusted to 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and boiled for 3 min prior to SDS-PAGE on one-dimensional 14% gels. Electrophoretic transfer of proteins to nitrocellulose was performed as described earlier with the following modifications. Unreacted sites on nitrocellulose were blocked by incubating with 5% BSA in TBS (20 mM Tris-HCL, 150 mM NaCl, pH 7.5) for 4 h at 20°C. After 3 washes with TBS-T, transferred proteins were probed with mAb 4G10 (1 μg/ml dilution in TBS with 1% BSA) overnight at 20°C. Blots were washed and incubated with biotinylated goat anti-mouse IgG (Cappel) for 1 h at 20°C. Following washes with TBS-T, blots were incubated with 0.25 μCi/ml of ^{125}I-streptavidin (Amersham, Arlington, Ont.) in TBS with 1% BSA for 30 min at 20°C. After extensive washing, phosphotyrosine proteins were visualized by exposure of blots to Kodak X-OMAT-AR film at -70°C.

2.4.5 Quantititative Determination Of Protein Phosphorylation

The amount of protein phosphorylation or dephosphorylation on autoradiographs after two-dimensional gel electrophoresis was analysed by computerized densitometry using a Visage 110 Image analysis system (BioImage Products, Ann Arbor, MI). The data are expressed as a ratio of the densitometric

intensity of the spot of interest divided by the intensity of a reference internal standard for that particular autoradiograph.

2.5 Induction Of Cytokine Secretion In Human Blood Monocytes By TSST-1

2.5.1 Paraformaldehyde Fixation Of Cells

Purified human monocytes or T lymphocytes were fixed by incubating in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 10 min at 20°C as previously described (Harlow and Lane, 1988). Fixed cells were then washed 3 times with Hank's balanced salt solution and suspended in RPMI 1640 with 10% FBS.

2.5.2 Culture Of Human Monocytes And T Lymphocytes With TSST-1

Approximately 1 x 10^6 purified human monocytes, alone or with 1 x 10^6 purified T lymphocytes, were cultured with TSST-1 in a total volume of 1 ml of RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 25 mM HEPES, and 10 μg/ml polymyxin B sulphate. Cells were stimulated with toxin for 24 h at 37°C in 24-well plates. At the end of the incubation period, culture supernatants were centrifuged (800 x g, 5 min) to remove cells and frozen at -70°C until cytokine analysis.

2.5.3 Cell Culture In Two-Chamber Plates

In some experiments, the effects of physical separation of monocytes and T lymphocytes were studied by using 24-well plates with 12-mm Transwell units (0.45 μm pore size, Costar Transwell Cell Culture Chambers). Monocytes were cultured in the upper chambers or lower chambers while an equivalent number of T lymphocytes were added to the lower chambers or upper chambers, respectively. Cells were stimulated with TSST-1 and the culture supernatants harvested as described above.

2.5.4 Effects Of Antibodies On Cytokine Induction By TSST-1

Mixed cultures of human monocytes and T lymphocytes were preincubated with various concentrations of antibodies for at least 2 h at 37°C before the addition of TSST-1. Antibodies used in these studies were as follows: 1) mAb L243 (anti-HLA-DR); 2) mAb OKM1; 3) rabbit anti-human interferon-gamma polyclonal antibody and pre-immune rabbit IgG (Endogen, Boston, MA); 4) mAb IOT16 (anti-CD11a or anti-LFA-1α chain, AMAC Inc., Westbrook, ME); 5) mAb IOT18 (anti-CD18 or anti-LFA-1ß chain, AMAC Inc., Westbrook, ME); and 6) mAb IOM2 as a negative control (anti-CD14, AMAC Inc., Westbrook, ME). Additional negative controls for these cytokine studies consisted of the above antibodies alone without TSST-1.

2.5.5 Effect Of Protein Kinase Inhibitors On Induction Of Cytokine Secretion By TSST-1

The second messenger pathways involved in cytokine induction by TSST-1 were investigated using various protein kinase inhibitors. Human monocytes, co-cultured 1:1 with T lymphocytes, were incubated with TSST-1 for 24 h at 37°C in the presence or absence of the following protein kinase inhibitors: H7 [1-(5-isoquinolinylinylsulfonyl)-2-methylpiperazine, inhibitor of protein kinase C], HA1004 [N-(2-guanidinoethyl)-5-isoquinolinesulfonamide, inhibitor of cAMP-and cGMP-dependent protein kinases], both purchased from Sigma Chemical Co. (St Louis, MO), and genistein (inhibitor of tyrosine kinases, ICN, Mississauga, Ont.). Cells were pretreated with the protein kinase inhibitors for 15 min prior to stimulation with TSST-1. Culture supernatants were harvested for cytokine analysis as described earlier.

2.5.6 Detection Of TNF Activity By Cytotoxicity Assay

A standard L929 cytotoxicity assay was used to detect TNF activity in supernatants of TSST-1-stimulated cell cultures (Flick and Gifford, 1984). Specifically, the murine fibroblast L929 cell line (American Type Culture Collection, Rockville, MD) was cultured in 96-well microtitre plates at a concentration of 5 x 10^4 cells per well in RPMI 1640 containing 10% FBS and 4

μg/ml actinomycin D (Sigma Chemical Co., St Louis, MO). Serial dilutions of recombinant human TNFα standards (R & D Systems, Minneapolis, MN) or test samples, preincubated for 2 h at 37°C with or without goat anti-human TNFα or anti-TNFß (R & D Systems, Minneapolis, MN) were placed in 50 μl volumes into appropriate wells. After incubation for 20 h at 37°C, culture medium was removed and 0.5 mg/ml MTT dye (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide, Sigma Chemical Co., St. Louis, MO) in phenol red-free RPMI 1640 was added and the culture plate incubated for a further 3 h at 37°C. The dye was removed and 100 μl dimethyl sulfoxide was added to dissolve the dye precipitate. The purple color was quantitated at an optimal density of 540 nm with a Titertek multiscan spectrophotometer (Flow Laboratories, Inc., McLean, VA). The cytotoxicity was calculated as follows: percent cytotoxicity = [1-(O.D.$_{540}$ of sample/O.D.$_{540}$ of control)] x 100.

2.5.7 Biotinylation Of Antibodies For Cytokine ELISAs

Goat anti-human TNFα or goat anti-human IL-1ß antibodies (R & D Systems, Minneapolis, MN) were biotinylated using freshly prepared biotinyl-Σ-amino caproic acid N-hydroxysuccinimide ester (biotin-X-NHS, Calbiochem, La Jolla, CA) at a biotin-X-NHS to antibody ratio of 100:1 as previously described (Goding, 1986). The reaction was stopped after 4 h at 20°C by the addition of 10% (v/v) 1 M Tris-HCL, pH 8.0. Biotinylated antibodies were separated from free biotin by gel filtration using a PD-10 gel filtration column (Pharmacia Fine Chemicals, Dorval, Quebec) and stored at 4°C in the presence of 1% BSA and 0.02% sodium azide.

2.5.8 ELISAs For TNFα And IL-1ß

Levels of TNFα and IL-1ß in culture supernatants were determined by ELISAs. Goat anti-human TNFα or goat anti-human IL-1ß (R & D Systems, Minneapolis, MN), at 2 μg/ml in 0.05 M bicarbonate-carbonate buffer, pH 9.6, was coated in flat-bottomed 96-well microtiter plates (Immulon I, Dynatech Laboratories, Inc., Alexandria, Va) for 20 h at 20°C (100 μl per well). Unbound

antibodies were removed by three 2-min washes with PBS containing 0.1% Tween 20 (PBS-T), followed by one wash with PBS alone. Human recombinant TNFα or IL-1ß standards (R & D Systems, Minneapolis, MN), serially diluted from 8,000 to 62.5 pg/ml in PBS with 3% BSA, were added in triplicate 100 μl volumes to respective wells and incubated at 37°C for 1.5 h. After washing with PBS-T, 100 μl of biotinylated goat anti-human TNFα or biotinylated goat anti-human IL-1ß (diluted 1/4000 in PBS with 3% BSA) were added to respective wells and the plates incubated for 1.5 h. Plates were washed with PBS-T and incubated with 100 μl streptavidin alkaline-phosphatase (diluted 1/2000 in PBS with 3% BSA) for 20 min at 37°C. Wells were then washed 5 times with 200 μl 50 mM Tris-buffered saline, pH 7.5. An ELISA amplification system (Gibco/BRL, Burlington, Ont.) was then used to increase the sensitivity of the assay. Briefly, 50 μl of BRL substrate was pipetted into each well and incubated at 20°C for 15 min in the dark. Without removing the substrate solution, 50 μl of BRL amplifier reagent was added and the plate incubated a further 15 min at 20°C in the dark. The reaction was stopped by the addition of 50 μl of 0.3 M H_2SO_4 and the $O.D._{495}$ measured in a Titertek multiscan spectrophotometer. For the TNFα ELISA, no cross-reactivity was observed with IL-1α, IL-1ß, or IFN-τ. The IL-1ß ELISA showed no cross-reactivity with IL-1α, TNFα, or IFN-τ. The coefficients of variation for both assays were approximately 10%.

2.5.9 Statistical Analyses

The student t test was used to determine the statistical significance of differences in mitogenicity assay results where applicable. Phosphorylation studies were analysed by the student t test and the sign test. Statistical analyses of cytokine levels in culture supernatants were performed by the Wilcoxon's signed-rank test (one-tailed). The Spearman rank correlation test was used to compare the TNF cytotoxicity bioassay with the TNFα ELISA.

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Chapter 3

Characterization Of The Receptor For TSST-1 On Human Peripheral Blood Mononuclear Cells

3.1 Introduction

Staphylococcal TSST-1 has been shown to have potent biological effects on human PBMC such as the induction of IL-1 and TNF production in human monocytes as well as the activation of T lymphocyte proliferation. These properties are also shared with another group of exoproteins, the staphylococcal enterotoxins (SE). The molecular mechanisms by which TSST-1 and staphylococcal enterotoxins exert these various effects are still unclear. The first event may be binding to a specific cell surface receptor. Binding sites for TSST-1 have been demonstrated on a number of cell types including human conjunctival epithelial cells (Kushnaryov et al., 1984), human T lymphocytes (Poindexter and Schlievert, 1987), and endothelial cells from human umbilical cord veins and arteries (Kushnaryov et al., 1989). Although receptor numbers and dissociation constants were reported in these studies, the identity of the TSST-1 receptor was not determined.

In the present study, purified TSST-1 was labeled to a high specific activity with ^{125}I to study the characteristics and distribution of TSST-1 receptors on human PBMC and monocytes. Characterization of TSST-1 receptor numbers and binding constants in blood cells of normal donors may provide us with normal values with which we can subsequently compare with those of TSS patients. The analysis of receptor numbers and affinities in TSS patients may reveal anomalies that may explain why these individuals are more susceptible to the effects of TSST-1.

Further characterization of the TSST-1 receptors on human peripheral blood mononuclear cells was performed by determining whether any of the staphylococcal enterotoxins could compete for TSST-1 binding sites. Staphylococcal enterotoxins have been implicated in a number of TSS cases (Schlievert, 1986; Whiting et al.,

1989). Furthermore, our laboratory has previously demonstrated a significantly higher seroconversion rate to SEA as well as to TSST-1 using paired acute and convalescent sera from TSS patients compared with non-TSS-associated control patients with S. aureus infection (Whiting et al., 1989). We recently observed that a single clone of S. aureus which produces both TSST-1 and SEA is associated with the majority of urogenital TSS cases (71%) in contrast with non-urogenital TSS (24%) or non-TSS-associated control patients (12%) (Chang et al., 1991). These findings, along with the fact that the biological properties of TSST-1 and the staphylococcal enterotoxins are very similar led us to investigate whether these toxins were binding to a common receptor on human blood cells.

Specific Aims

1) To determine the number and dissociation constant of receptors for TSST-1 on human PBMC.
2) To determine whether the biologically-related staphylococcal enterotoxins can compete for TSST-1 binding sites on human PBMC.
3) To examine the nature of the TSST-1 receptor on purified human monocytes and to determine the molecular size of these receptors by cross-linking experiments.
4) To examine the importance of the TSST-1 receptor on human monocytes in human T cell stimulation.

3.2 Results

3.2.1 Assessment Of The Purity of TSST-1

The purity of TSST-1 was evaluated by SDS-PAGE. Results show that the toxin migrated as a homogeneous band with a relative molecular weight of 22 kd as determined by silver staining of gels (Figure 3) and by immunoblotting the toxin preparation with antisera directed against the culture filtrates of S. aureus MN8 (data not shown). Furthermore, the ^{125}I-TSST-1 preparation used in the

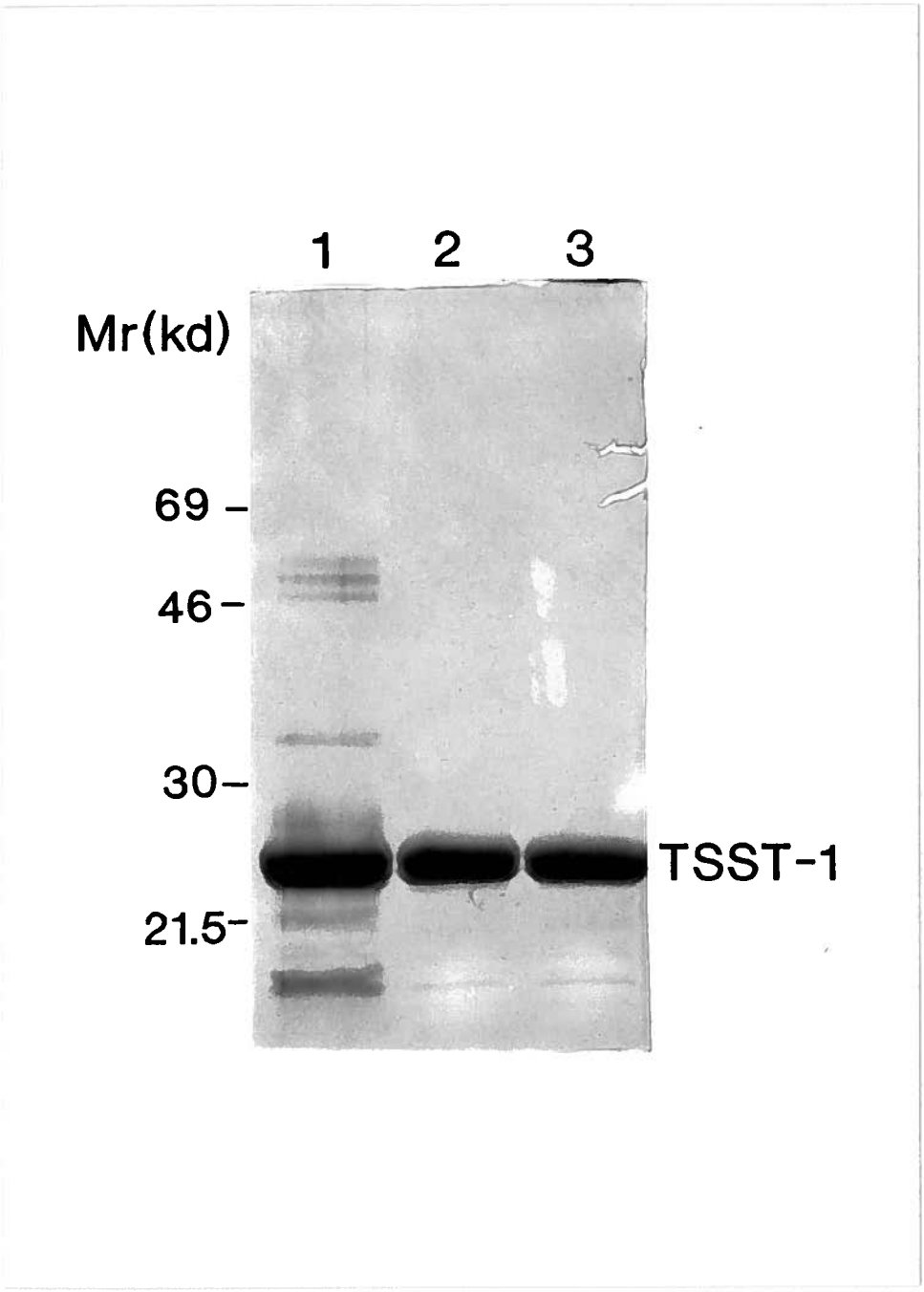


Figure 3. Purity of TSST-1 as demonstrated by silver staining of SDS-PAGE gels (14%). Lane 1, 10 μg commercial TSST-1 (Toxin Technology Inc.,); lanes 2 and 3, 10 μg of in-house preparation of TSST-1.

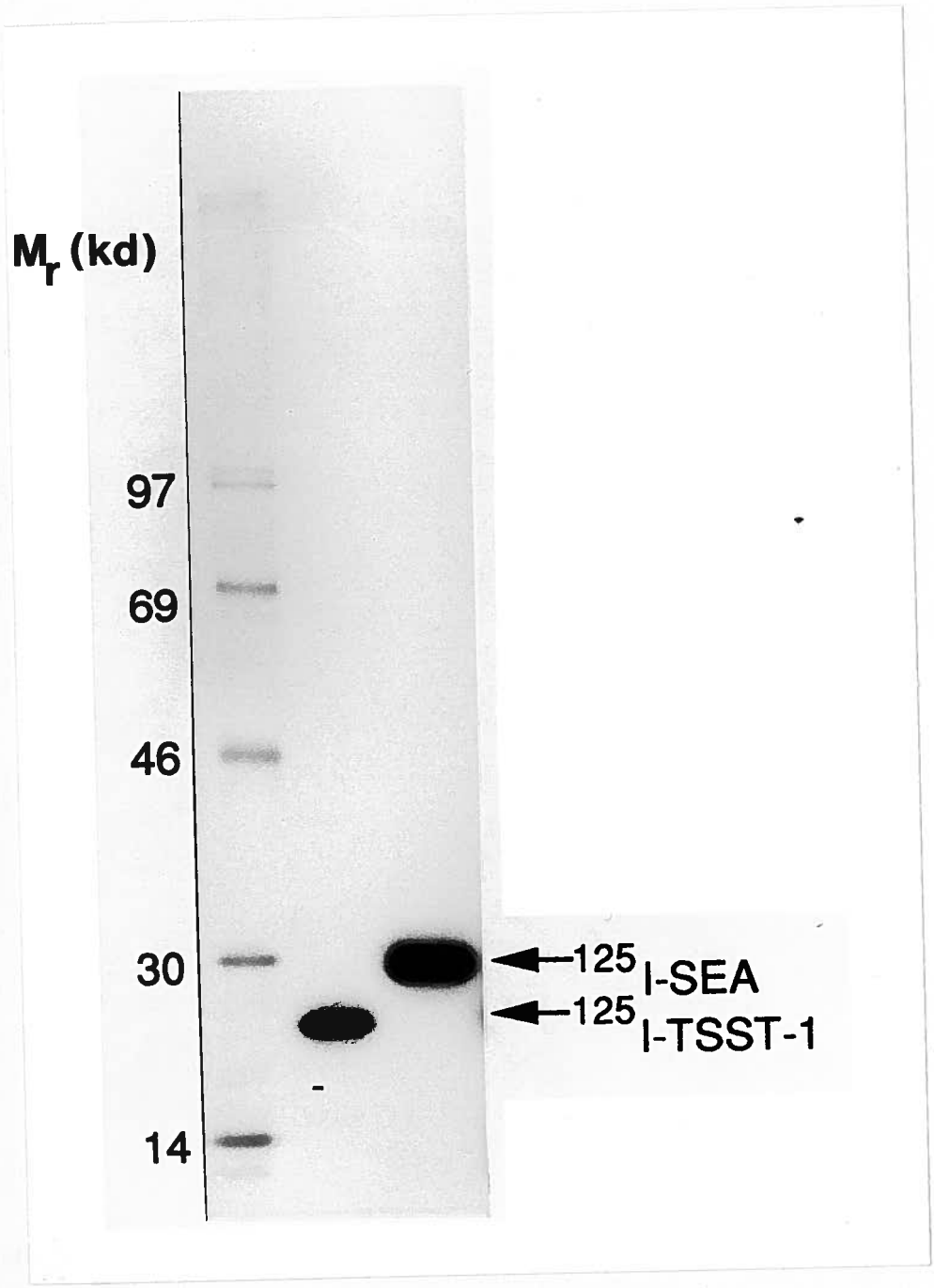


Figure 4. Purity of ^{125}I-labeled TSST-1 (in-house preparation) and SEA (Toxin Technology, Inc.) preparations used in binding assays as determined by SDS-PAGE and autoradiography.

binding studies were judged to be relatively pure as indicated in Figure 4. Analysis of ^{125}I-SEA showed that this toxin was >95% pure with no TSST-1 contamination (Figure 4).

3.2.2 Binding Of ^{125}I-TSST-1 To Specific Receptors On Human Peripheral Blood Mononuclear Cells

Human PBMC, isolated as described in Materials and Methods were incubated with saturating amounts of radioiodinated TSST-1 in the presence or absence of unlabeled TSST-1. Studies were performed at 4°C to prevent internalization of radiolabeled toxin. Figure 5 shows that binding of ^{125}I-TSST-1 to 3 x10^6 PBMC was both dose-dependent and saturable. In general, specific binding represented 70% or more of the total binding. Time course studies, as indicated in Figure 6, showed that saturation of TSST-1 receptors was achieved within 10 min (Figure 6). For subsequent binding studies, cells were incubated with radiolabeled toxins for 60 min. Binding data from 8 donors were statistically analysed by the computer-associated LIGAND program developed by Munson and Rodbard (1980) in order to determine the number of receptors and dissociation constants. A Scatchard plot for a representative donor is shown in Figure 7. A single class of high affinity receptors for TSST-1 on PBMC was determined by the LIGAND program, as evidenced by the straight line of the graph. A composite analysis of data from 8 different normal donors by the LIGAND program revealed an average of 5430 ± 923 TSST-1 receptors per PBMC (mean ± SEM) with a dissociation constant of 18.0 ± 5.9 nM (mean ± SEM).

3.2.3 Specificity Of ^{125}I-TSST-1 Binding And Competition Binding Experiments With Staphylococcal Enterotoxins

The specificity of ^{125}I-TSST-1 binding to human PBMC was determined in the presence of unlabeled TSST-1. The results show that binding of ^{125}I-TSST-1 to PBMC was inhibited in a dose-dependent manner by increasing concentrations of unlabeled toxin (Figure 8).

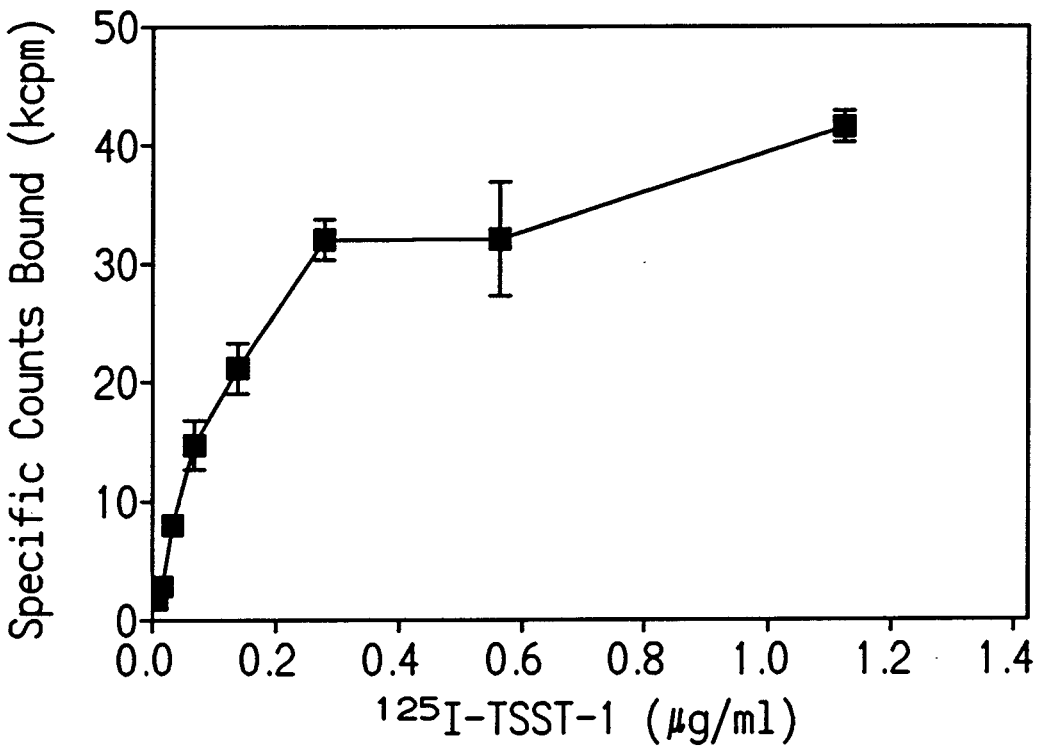


Figure 5. Dose-dependent binding of ^{125}I-TSST-1 to human PBMC. 3 x 10^6 cells were exposed to increasing concentrations of TSST-1 for 1 h at 4°C. Aliquots of sample were then separated through phthalate oils and the radioactivity measured as described in Materials and Methods. Nonspecific binding determined in the presence of 100-fold molar excess of unlabeled TSST-1 has been subtracted. Each point represents the mean ± SD for triplicate determinations.

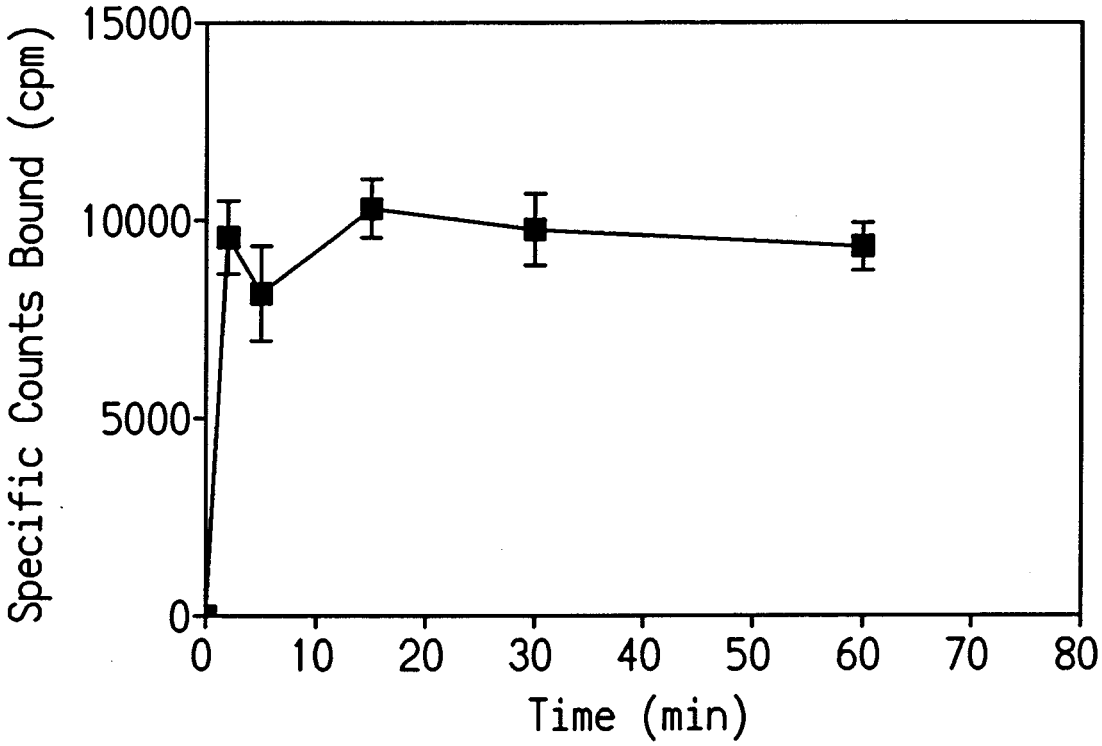


Figure 6. Time course of ^{125}I-TSST-1 binding to human PBMC. 3 x 10^6 cells were incubated with 20 nM labeled toxin in the presence or absence of 100-fold molar excess of unlabeled TSST-1 for the indicated times at 4°C. Non-specific binding has been subtracted. Results represent the mean ± SD for triplicate determinations.

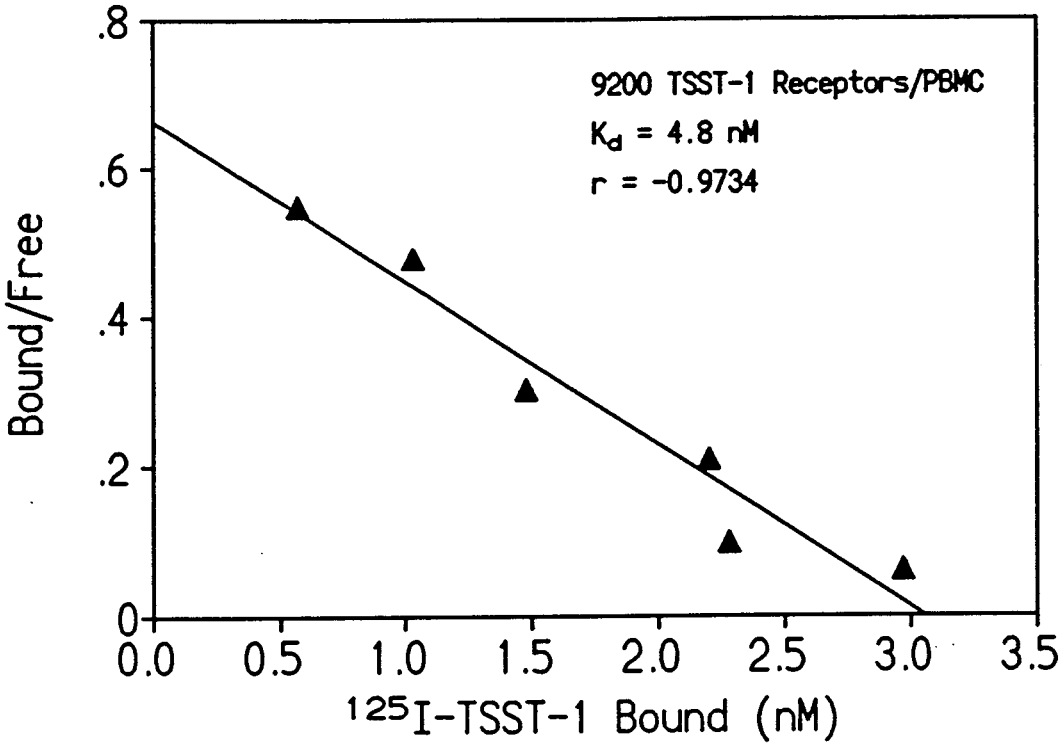


Figure 7. Determination of receptor number and dissociation constant (K_d) for TSST-1 on human PBMC by the LIGAND program. Approximately 1.2 x 10^7 PBMC were incubated with increasing concentrations of ^{125}I-TSST-1 (0.4 nM to 50 nM) for 1 h at 4°C. Nonspecific binding, determined in the presence of 100-fold molar excess of unlabeled toxin, has been subtracted. Data presented are representative of one of eight donors.

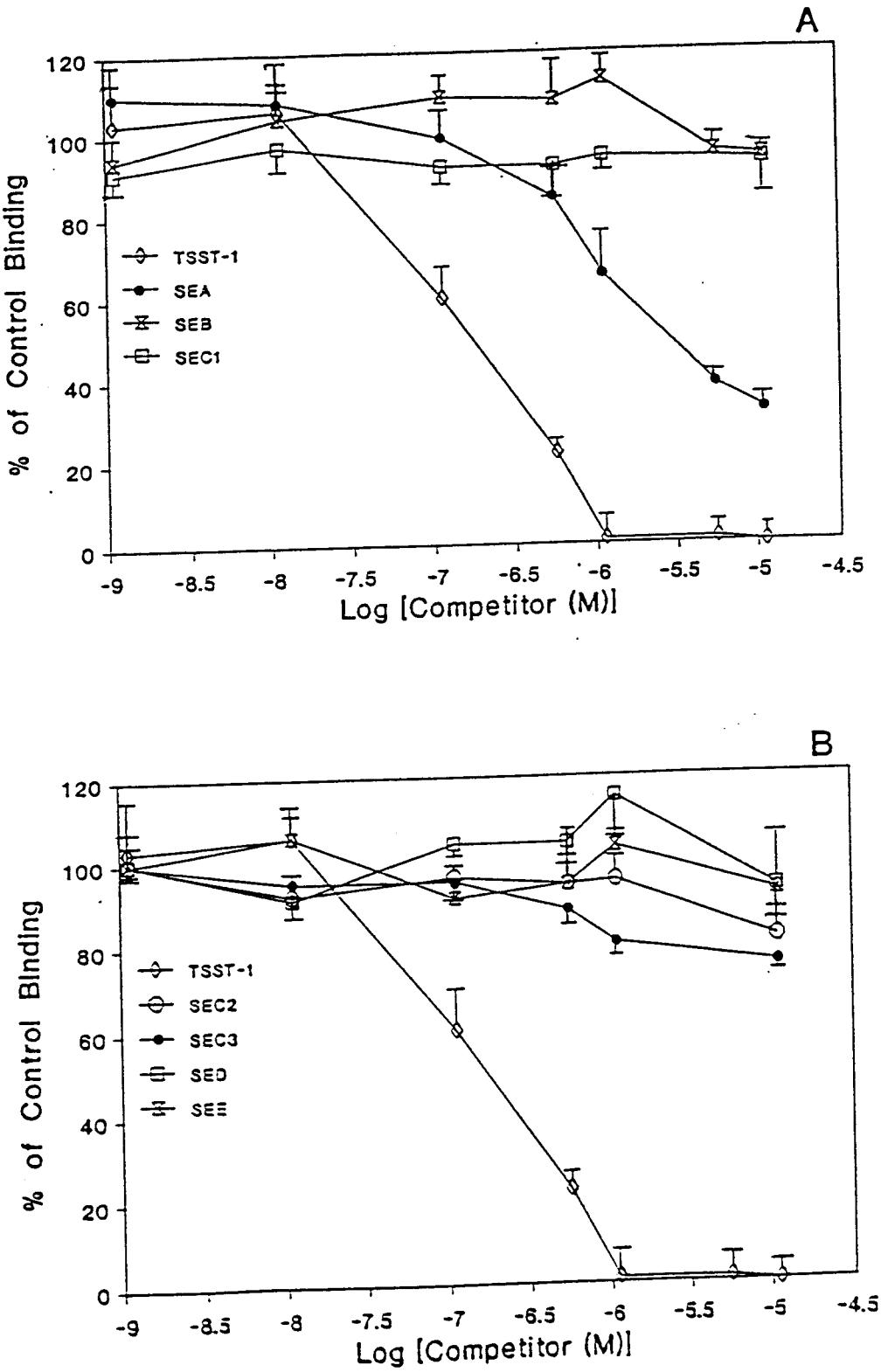


Figure 8. Competition for ^{125}I-TSST-1 binding to human PBMC by unlabeled homologous toxin and staphylococcal enterotoxins. 1.3 x 10^7 PBMC were incubated with 10 nM ^{125}I-TSST-1 for 1 h at 4°C in the presence or absence of increasing concentrations of unlabeled TSST-1, SEA, SEB and SEC_1 (A) and TSST-1, SEC_2, SEC_3, SED and SEE (B). Binding of ^{125}I-TSST-1 in the absence of competitors was normalized to 100%. A 100-fold molar excess of unlabeled TSST-1 was included in all experiments as a positive control. Each datum point represents the mean ± SD for triplicate determinations. All data have been corrected for nonspecific binding.

Further studies were then performed to determine whether any of the biologically-related staphylococcal enterotoxins (A, B, C1, C2, C3, D and E) could compete for TSST-1 binding sites. Of the staphylococcal enterotoxins tested, only SEA could compete with radiolabeled TSST-1 for binding sites. None of the other staphylococcal enterotoxins could inhibit ^{125}I-TSST-1 binding even when tested up to a 1000-fold molar excess (Figure 8). Unlabeled SEA inhibited ^{125}I-TSST-1 binding strongly (maximum inhibition = 80%) at 1000-fold molar excess.

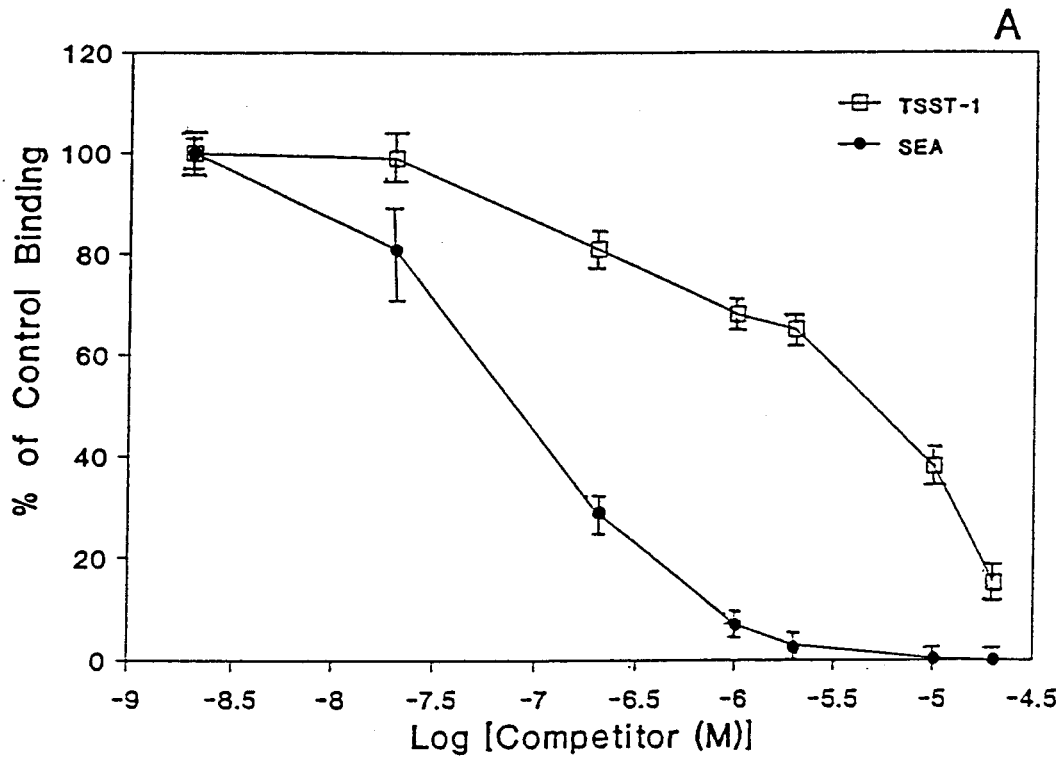
Since the binding of ^{125}I-TSST-1 was blocked by SEA, it was important to establish whether this inhibition was unidirectional or whether the two toxins actually cross-competed for binding sites. To address this question, SEA was iodinated and cross-competition binding experiments were performed using the same source of donor PBMC. Figure 9 shows a representative experiment in which a fixed concentration of radioligand (20 nM) was incubated with increasing concentrations of homologous or heterologous competitor. Although unlabeled TSST-1 significantly inhibited ^{125}I-SEA to human PBMC, unlabeled SEA was by far a better competitor on a molar basis (Figure 9A). Conversely, in the same experiment, TSST-1 showed greater effiency in competing for ^{125}I-TSST-1 binding sites than did SEA (Figure 9B). To confirm that the two toxins were binding to a common receptor, receptor numbers and dissociation constants were determined for each toxin separately using the same donor cells. Using the LIGAND program, 6080 receptors per PBMC (K_d = 20 nM) were found for TSST-1 (Figure 10A) and 6680 receptors per PBMC (K_d = 21 nM) were determined for SEA (Figure 10B). The LIGAND program also indicated one class of receptors for TSST-1 and SEA.

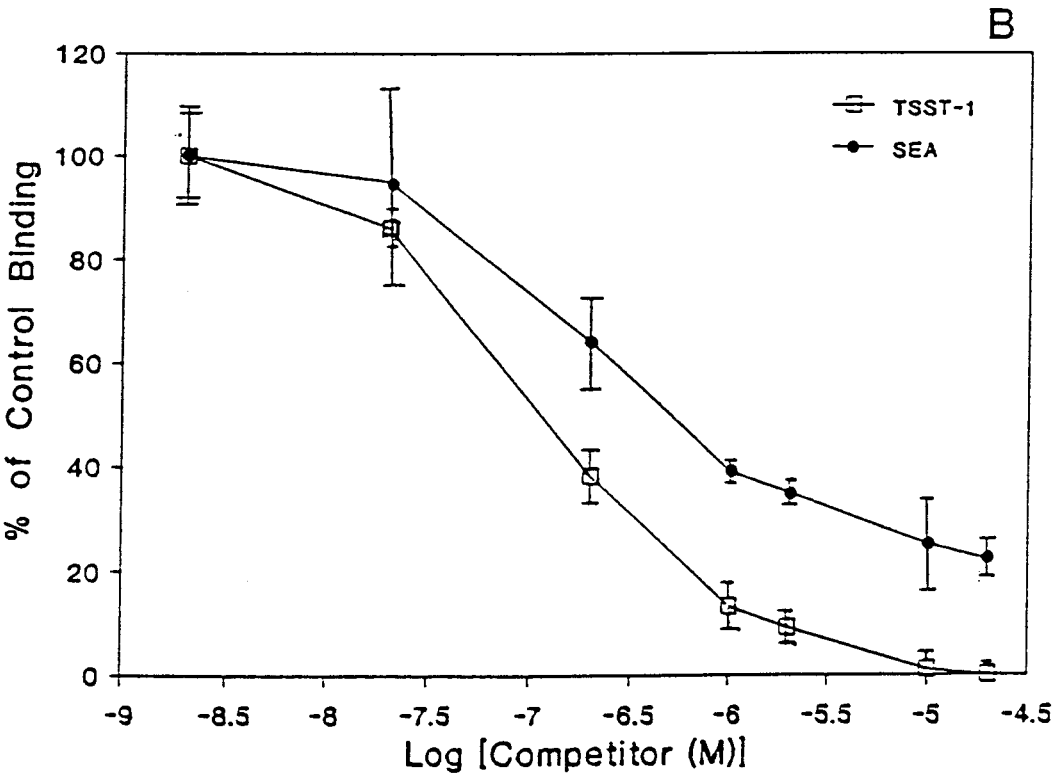
3.2.4 Demonstration Of TSST-1 And SEA Receptors On Human Monocytes

The binding studies above were performed on human PBMC which consist of a mixed population of cells (monocytes, T and B lymphocytes). To make subsequent studies less complicated, only one cell type was examined in the characterization of the receptors for TSST-1 and SEA. I decided to examine human monocytes for the following reasons. Firstly, these cells have been documented to respond to TSST-1 and SEA by secreting the cytokines IL-1 and TNF, two mediators thought to play

Figure 9 (next page).

Cross-competition between TSST-1 and SEA for receptors on a single source of human PBMC. PBMC (1 x 10^7 cells) were incubated for 1 h at 4°C with 20 nM samples of either ^{125}I-SEA (A) or ^{125}I-TSST-1 (B) in the presence of unlabeled SEA or TSST-1. Maximum binding was measured with ^{125}I-toxins alone, while background binding was determined with a 100-fold molar excess of unlabeled homologous ligand. Datum points represent the means ± SD of triplicate determinations. Nonspecific binding has been subtracted.


A
TSST-1
SEA
% of Control Binding
120
100
80
60
40
20
0
-9
-8.5
-8
-7.5
-7
-6.5
-6
-5.5
-5
-4.5
Log [Competitor (M)]


B
TSST-1
SEA
% of Control Binding
120
100
80
60
40
20
0
-9
-8.5
-8
-7.5
-7
-6.5
-6
-5.5
-5
-4.5
Log [Competitor (M)]

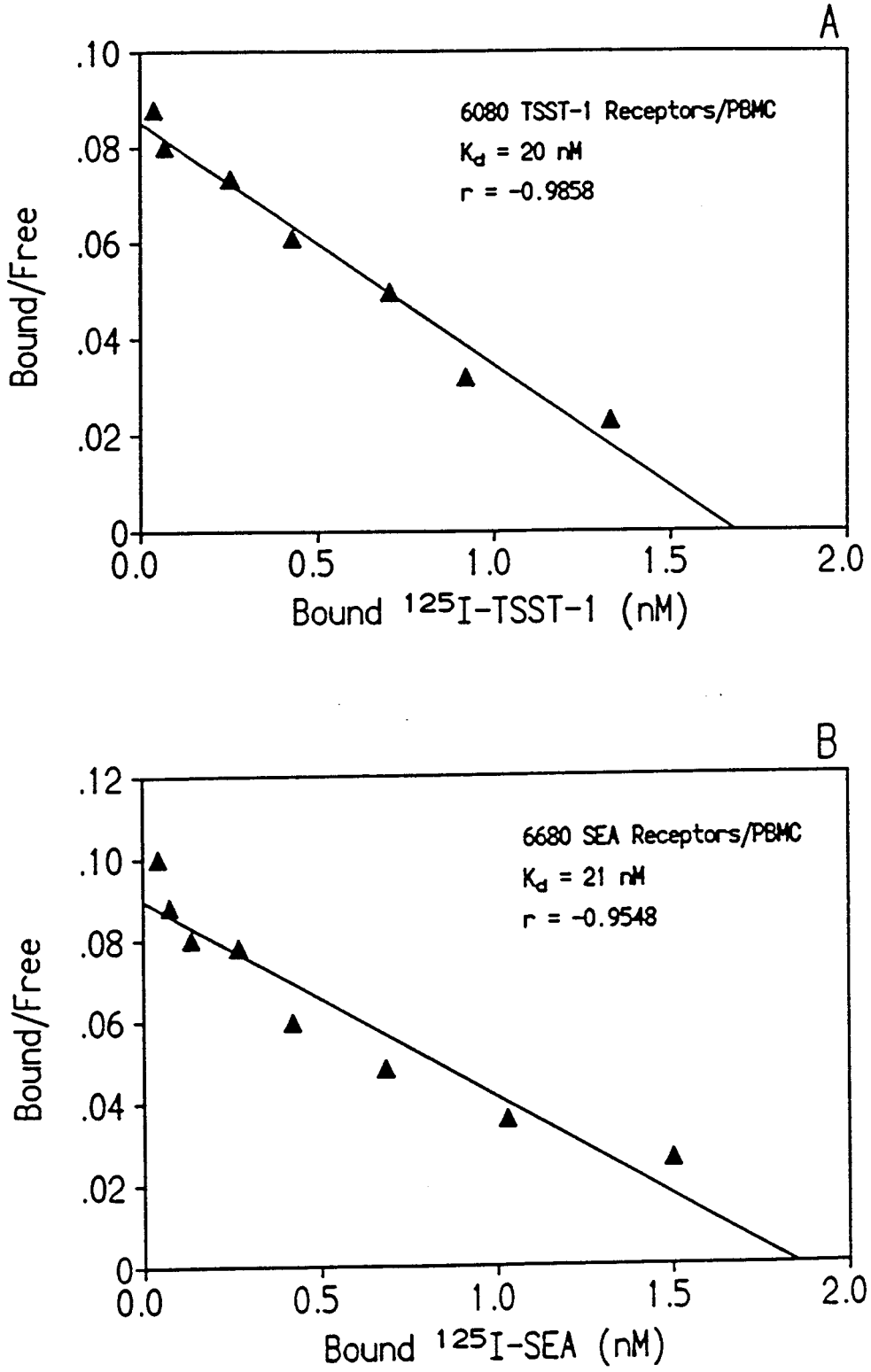


Figure 10 Parallel analysis of ^{125}I-TSST-1 (A) and ^{125}I-SEA (B) binding to human PBMC (1 x 10^7 cells) from the same donor. Cells were incubated with 0.5 nM to 60 nM radiolabeled toxins for 1 h at 4°C and processed as described in Materials and Methods, Chapter 2. Receptor numbers and dissociation constants (K_d) were determined by the LIGAND program.

a major role in TSS. Secondly, superantigen activation of T lymphocyte proliferation appears to be dependent on the presence of monocytes. Thus, monocytes appear to be a major target cell and are likely to bear receptors for these toxins. Using a combination of sheep red blood cell rosetting to remove T lymphocytes and Percoll density centrifugation to eliminate B cells, highly purified monocyte fractions (>90%, determined by non-specific esterase staining) were obtained. The purified monocytes were then used in binding experiments to ensure that they contained receptors for TSST-1 and SEA. As indicated in Table VI, monocytes possessed an average of 34,010 ± 8658 (K_d = 40 ± 15 nM) receptors for TSST-1. Similar receptor numbers were found for SEA (Table VI).

3.2.5 Characterization Of The TSST-1 And SEA Receptors On Human Monocytes

Having shown that TSST-1 receptors exist on human monocytes, the next objective was to identify them. At about this time, three papers were simultaneously published indicating that the receptor for SEA and SEB was the class II major histocompatibility (MHC) antigen, HLA-DR (Fischer et al., 1989, Fraser, 1989, Mollick et al., 1989). Based on our cross-competition binding experiments with TSST-1 and SEA, it was decided that the HLA-DR molecule may also be a potential receptor for TSST-1. To investigate this hypothesis, three mAbs directed against monomorphic regions of HLA-DR were used in an attempt to block ^{125}I-TSST-1 binding to its receptor on human monocytes. Anti-HLA-DR mAbs were preincubated with human monocytes for at least 1 h prior to the addition of labeled toxin. Results show that the anti-HLA-DR mAb, L243, strongly inhibited both ^{125}I-TSST-1 and ^{125}I-SEA binding to monocytes in a dose-dependent manner (Figures 11A and 11B). In 3 separate experiments, L243 was consistently more potent in blocking ^{125}I-TSST-1 binding to HLA-DR than ^{125}I-SEA to the same receptor (data not shown). A monocyte-specific isotype control mAb, OKM1,had no effect on binding of TSST-1 or SEA (Figures 11A and 11B). Two other anti-HLA-DR mAbs, L203 and 2.06, did not block binding of either toxin.

Table VI

Receptor Numbers And Dissociation Constants For TSST-1 And SEA On Normal Human Blood Monocytes

Donor No.	TSST-1		SEA	
	R[a]	K_d	R	K_d
1	39,200	37	31,800	12
2	26,000	35	ND[c]	ND
3	30,250	19	ND	ND
4	28,000	57	ND	ND
5	46,600	53	ND	ND
6	ND	ND	13,500	23
Mean ± SD[d]	34,010 ± 8658	40 ± 15.2	22,650 ± 12,940	18 ± 7.8

[a] R = receptor number per monocyte as determined by the LIGAND program.

[b] K_d = dissociation constant (nM) as determined by the LIGAND program.

[c] ND = not determined.

[d] mean ± standard deviation.

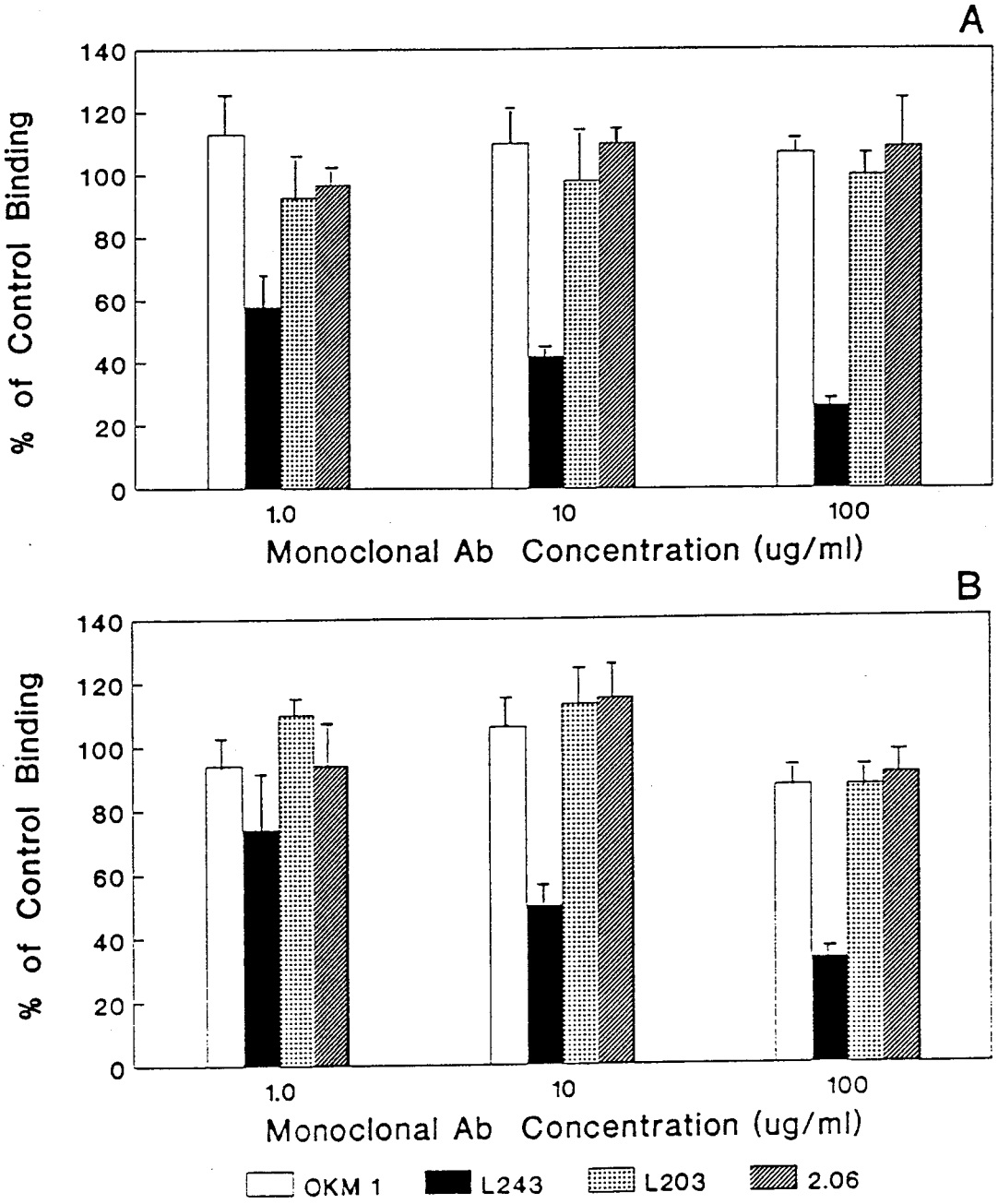


Figure 11. Inhibition of ^{125}I-TSST-1 (A) or ^{125}I-SEA (B) binding to purified human monocytes by anti-HLA-DR mAbs. 1 x 10^6 cells were preincubated with either anti-HLA-DR mAbs (L243, L203, 2.06) or a monocyte-specific isotype control mAb (OKM1) for 1 h at 4°C. 30 nM ^{125}I-TSST-1 or ^{125}I-SEA was added and the cells rocked for 1 h at 4°C. The percentage of radiolabeled toxin bound in the presence of antibodies was normalized to 100% (ordinate). Values represent the mean of triplicate determinations ± standard deviation. Nonspecific counts have been subtracted.

3.2.6 Determination Of The Molecular Weight Of TSST-1 And SEA Receptor On Human Monocytes By Chemical Cross-Linking

Further characterization of the monocyte receptors for TSST-1 and SEA was performed by cross-linking studies using the bifunctional cross-linker, dissuccinimidyl suberate (DSS). Radiolabeled TSST-1 or SEA was chemically cross-linked to their plasma membrane receptors on monocytes by DSS, and the cross-linked toxin-receptor complex resolved by SDS-PAGE. After subtracting the molecular weight of TSST-1 and SEA (22 kd and 28 kd, respectively), autoradiograms revealed 2 major receptor species at 35 kd and 28 kd (Figures 12, arrows A and B, respectively). The specificity of binding to these 2 proteins was demonstrated by their absence when a 100-fold molar excess of unlabeled TSST-1 or unlabeled SEA was included (Figure 12, lanes 2 and 6, respectively). Furthermore, no evidence of ^{125}I-TSST-1 or ^{125}I-SEA cross-linking was observed in the absence of cells (data not shown). To determine whether the receptor species were related to HLA-DR, monocytes were preincubated with L243 for at least 1 h before cross-linking with ^{125}I-TSST-1 or ^{125}I-SEA. Results show that L243 inhibited the affinity labeling of the 35 kd and 28 kd bands by both radiolabeled TSST-1 and SEA (Figure 12, lanes 3 and 7, respectively). Inhibition of the receptor species was not evident when monocytes were pretreated with the isotype control mAb, OKM1 (Figure 12, lanes 4 and 8). These results provide suggestive evidence that the 35 kd and 28 kd bands represent the alpha and beta chains of the human class II HLA-DR antigen.

3.2.7 The Role Of HLA-DR In The Activation Of Monocyte-Dependent T Cell Proliferation By TSST-1

It is well-documented that TSST-1 and SEA are two of the most potent T cell mitogens known today. As illustrated in Figure 13, significant activation of PBMC proliferation was achieved at <0.01 pM levels of both toxins, with SEA being the more potent of the two. However, highly purified T cells cannot be activated by TSST-1 or SEA unless accessory cells such as monocytes are also present. Work in our laboratory as well as in others (Carlsson et al., 1988; Fleischer et al.,

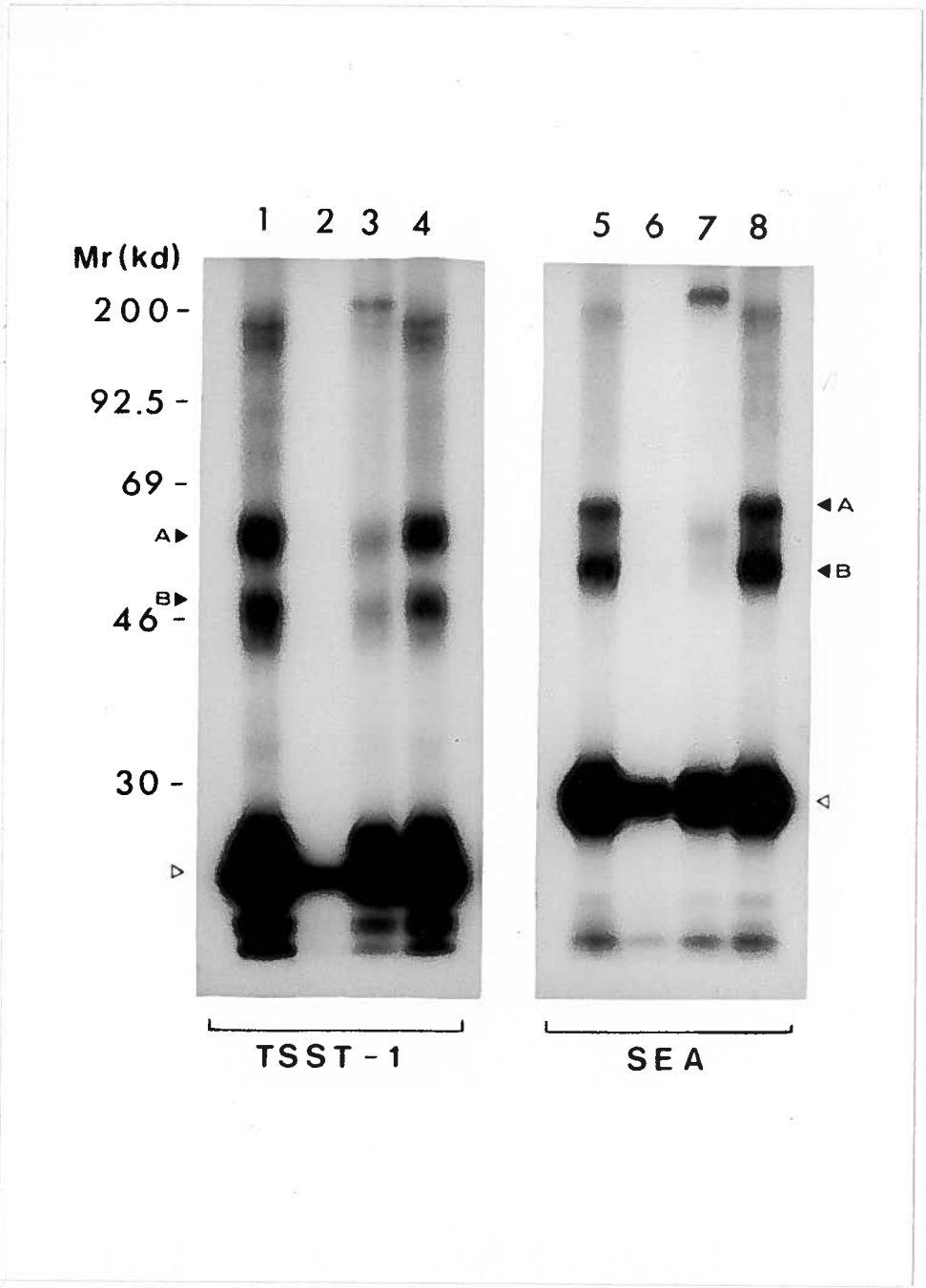


Figure 12. Characterization of TSST-1 and SEA receptors on human monocytes by affinity cross-linking. Monocytes were incubated with 60 nM of ^{125}I-TSST-1 for 1 h at 4°C, treated with DSS and analysed by SDS-PAGE under nonreducing conditions. Cells were exposed to ^{125}I-TSST-1 alone (lane 1), or 100-fold molar excess of unlabeled TSST-1 plus ^{125}I-TSST-1 (lane 2). Similarly, ^{125}I-SEA was cross-linked to monocytes in the absence (lane 5) or presence (lane 6) of unlabeled SEA. Monocytes were preincubated with 100 μg/ml L243 (lanes 3 and 7) or OKM1 (lanes 4 and 8) before cross-linking with ^{125}I-TSST-1 or ^{125}I-SEA. Arrows A and B represent the 35 and 28 kd receptor species, respectively, after subtracting the apparent molecular weight of uncross-linked ^{125}I-TSST-1 or ^{125}I-SEA (indicated by unshaded arrows).

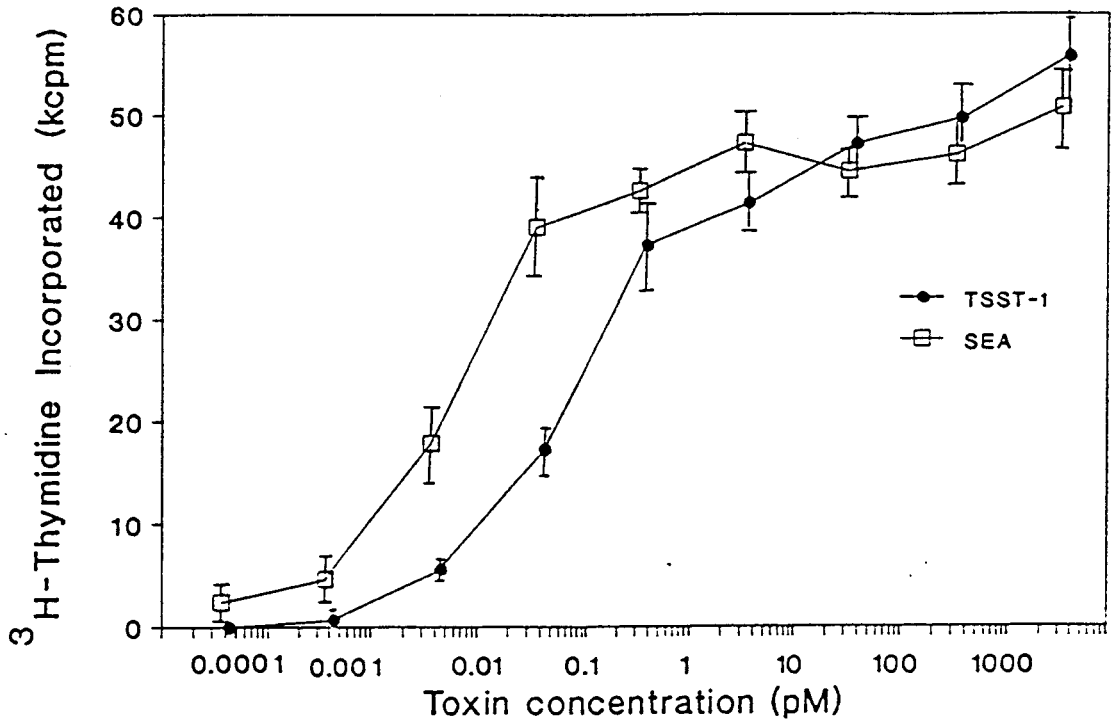


Figure 13. Proliferative response of human PBMC to TSST-1 and SEA as measured by tritiated-thymidine incorporation assay. 3 x 10^5 PBMC were cultured with the indicated concentrations of TSST-1 or SEA for 72 h at 37°C. Results represent the mean ± SD for quadruplicate cultures.

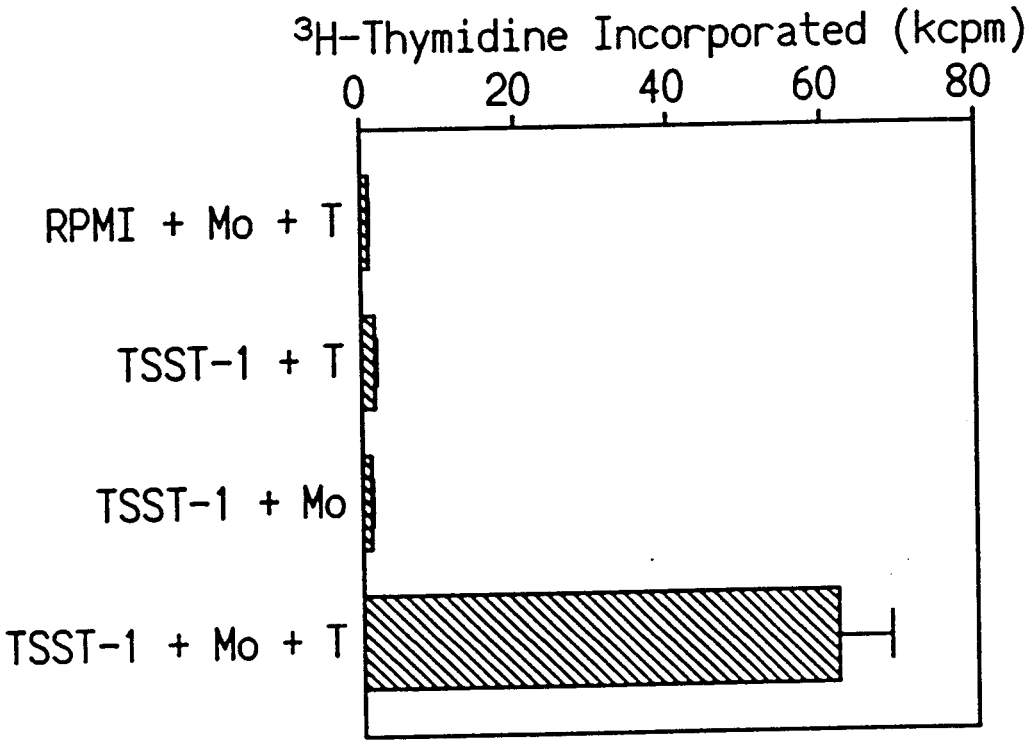


Figure 14. Proliferative response of purified T lymphocytes or monocytes to TSST-1 stimulation. 3 x 10^5 T cells or monocytes were exposed to 10 μg/ml TSST-1 for 72 h at 37°C. Where monocytes and T lymphocytes were co-cultured together, 10% monocytes were added to 3 x 10^5 T lymphocytes and exposed to the same concentration of TSST-1. No proliferation was observed in the presence of RPMI 1640 medium control. Results represent the mean ± SD for triplicate determinations. Data from M.Sc. thesis of Alex Chang.

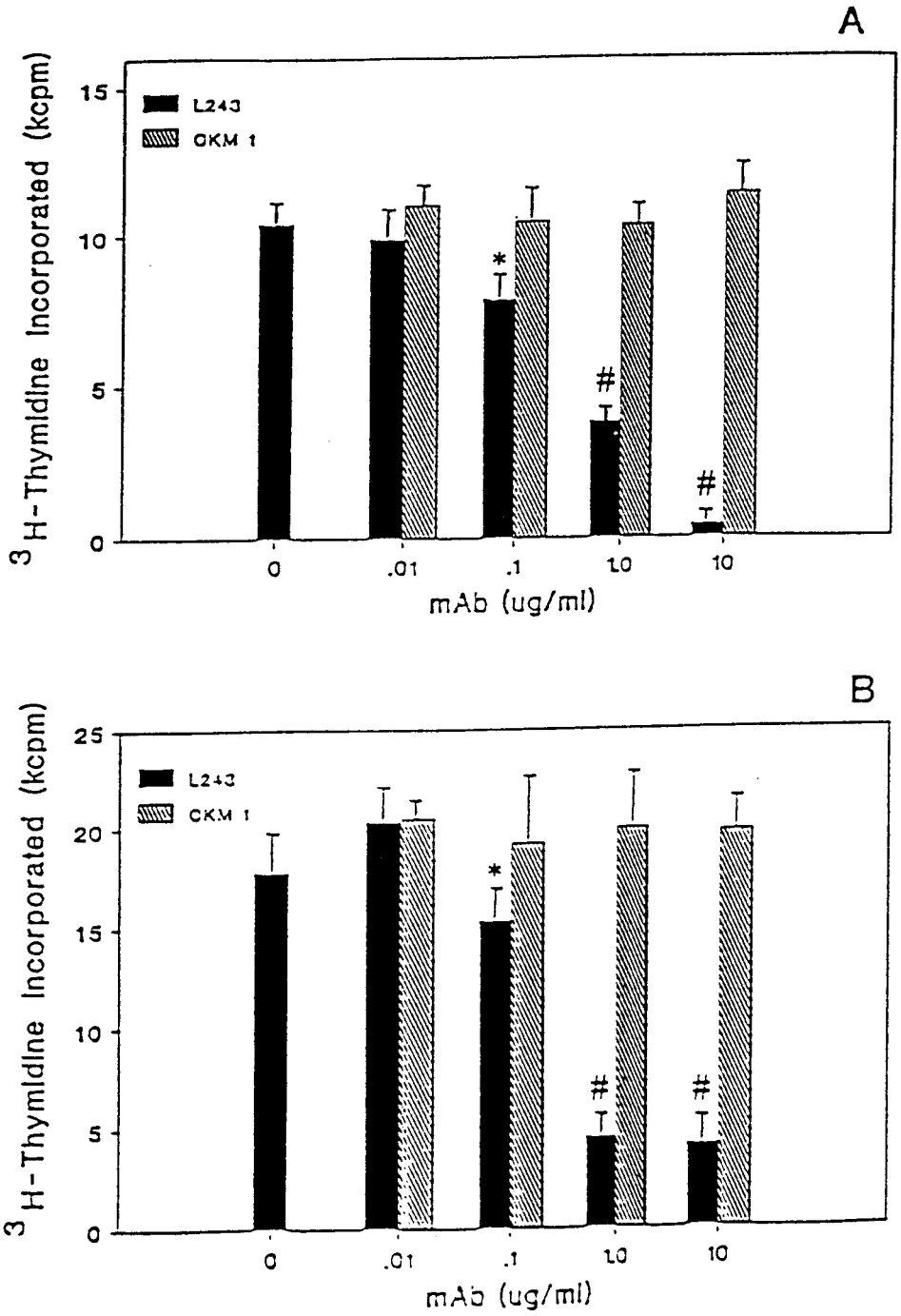


Figure 15. Inhibition of TSST-1- or SEA-induced proliferation of human PBMC by anti-HLA-DR mAb, L243. 3 x 10^5 PBMC were preincubated with the indicated concentrations of L243 or control mAb OKM1 for 1 h at 37°C. Cells were then stimulated with 0.1 μg/ml TSST-1 (A) or SEA (B) for 72 h at 37°C. Each value represents the mean ± SD for quadruplicate determinations. Background stimulation has been subtracted. (*) denotes significant difference ($P < 0.05$, one-tailed student t test) compared to toxin control without antibodies. (#) denotes significant difference ($P < 0.001$, one-tailed student t test) compared to toxin control without antibodies. No significant inhibition of TSST-1- or SEA-induced mitogenicity was found in the presence of OKM1 mAb.

1989) have shown that the addition of monocytes restores the proliferative response of human T cells to TSST-1 or SEA. This is exemplified in Figure 14 where highly purified T cells alone did not proliferate when stimulated with different concentrations of TSST-1 unless 10% monocytes were added. Monocytes in the absence of T lymphocytes also did not proliferate (Figure 14).

Since monocytes were required for T cell activation and our earlier work showed that TSST-1 and SEA bound to HLA-DR, we then set out to determine the role of HLA-DR in T cell stimulation by these toxins. The importance of HLA-DR antigens in the T cell mitogenic activity of TSST-1 or SEA was investigated by using the anti-HLA-DR mAb, L243, to block the biologic response. L243 or control mAb OKM1 were preincubated with PBMC for at least 1 h before the addition of the toxins. Figure 15 shows that L243 blocked the mitogenic response of PBMC to either TSST-1 (A) or SEA (B) in a concentration-dependent manner. L243 did not affect the viability of the cells as judged by trypan blue exclusion (data not shown). In contrast to L243, no inhibition of toxin-stimulated T cell proliferation was observed with the isotype control OKM1 mAb at the concentrations tested (Figure 15, A and B).

3.3 Discussion

Our present study demonstrates that TSST-1 and SEA bind to specific receptors on normal human peripheral blood monocytes. That the receptor on these cells represents the class II HLA-DR heterodimer is supported by the following: 1) binding of either ^{125}I-TSST-1 or ^{125}I-SEA to monocytes was blocked by preincubating cells with an anti-HLA-DR mAb, L243, and 2) cross-linking studies revealed 2 receptor species with molecular weights consistent with those of the α and ß subunits of HLA-DR. Our results corroborate with a number of earlier reports that TSST-1 and the staphylococcal enterotoxins bind to the MHC class II antigen. Specifically, others have shown that TSST-1, SEA and SEB bind to HLA-DR by using B cell lines as well as murine fibroblasts transfected with HLA-DR

molecules (Fischer et al., 1989; Fraser, 1989; Mollick et al., 1989; Scholl et al., 1989a). The demonstration of TSST-1 and SEA binding sites on normal human monocytes in our study has several implications. Human monocytes have been identified as the key producers of the cytokines IL-1 and TNF in response to TSST-1 and other staphylococcal exoproteins (Ikejima et al., 1984; Jupin et al., 1988; Parsonnet et al., 1985). Both cytokines may be involved in mediating some of the characteristic features observed in TSS such as fever and shock. Presumably, binding to MHC class II molecules on human monocytes may represent the initial step by which TSST-1 or SEA stimulates the release of these monokines. The determination of receptor numbers and dissociation constants for TSST-1 on normal monocytes may also allow one to draw comparisons with those of monocytes from TSS patients. Although no monocytes from TSS patients were examined in this thesis, future studies may reveal abnormalities either in receptor numbers or affinities that may predispose these individuals to the effects of TSST-1. Finally, the demonstration of TSST-1-specific receptors on normal monocytes is important from the point of view of T cell proliferation (See et al., 1992a). Several groups have demonstrated that TSST-1- or staphylococcal enterotoxin-triggered T cell proliferation is dependent on the presence of accessory cells such as monocytes (Carlsson et al., 1988; Fischer et al., 1989; Fleischer et al., 1989). Only HLA class II-positive accessory cells can support T cell proliferation since class II-negative mutants neither bind TSST-1 or enterotoxins nor affect T cell responses (Fleischer et al., 1989). Our own study also demonstrates that binding of TSST-1 to HLA-DR in human monocytes is important for T cell proliferation as indicated by the inhibition of this activity with the anti-HLA-DR mAb, L243 (See et al., 1992a). The importance of presentation of staphylococcal toxins by MHC class II molecules is further discussed below.

TSST-1 was found to bind to HLA-DR molecules on monocytes with a K_d of 40 nM (average of 34,000 sites/cell), in close agreement with that found for TSST-1 receptors on human B lymphocytes (K_d = 31 nM, 76,000 sites/cell) (Mourad et al., 1989). These dissociation constant values are similar to those reported for

binding of TSST-1 to murine spleen cells (Scholl et al., 1990b) or L cells transfected with the mouse class II molecules, I-A^b or I-A^k (Uchiyama et al., 1990).

The effects of MHC class II isotype and allotype on toxin binding and T cell proliferation have been examined in a number of other studies. In addition to HLA-DR, TSST-1 has also been reported to bind strongly to HLA-DQ (Scholl et al., 1989a). No binding to HLA-DP was found in the same study although in a subsequent report, Scholl et al. (1990a) found that a HLA-DP-transfected cell line could support TSST-1-induced T cell proliferation. This suggests that the affinity of TSST-1 for HLA-DP may be below the detection limits of the radioligand binding assay but not the T cell proliferation assay (nM vs. pM sensitivity, respectively). In similar studies, Mollick et al. (1991) also showed that SEA, SEB, TSST-1 and SEC_1 bind to HLA-DR and HLA-DQ transfectants efficiently. Binding to HLA-DP transfectants was observed only with SEA and SEB (Mollick et al., 1991). Overall though, there is a general hierarchy by which these toxins bind to human class II antigens (HLA-DR > HLA-DQ > HLA-DP) (Herman et al., 1991) although exceptions have been found with SEC_2 (Herrmann et al., 1989). Interestingly, in the murine system, TSST-1 binds strongly to I-A class II molecules (the mouse DQ analog) and only weakly to I-E molecules (the murine equivalent of DR) (Mollick et al., 1991; Scholl et al., 1990b; Uchiyama et al., 1990). This is in contrast to SEA and SEB, both of which bind I-A and I-E readily (Mollick et al., 1991). Thus, staphylococcal toxins appear to bind preferentially to particular MHC class II isotypes (Herrmann et al., 1989; Mollick et al., 1991; Uchiyama et al., 1990).

Quantitative differences in the binding of toxins by allelic forms of class II molecules have been noted. The HLA-DR7 transfectant, for example, has been found to bind 10 times more TSST-1 than the HLA-DRw53 transfectant (Scholl et al., 1990a). These differences were attributed to differences in the surface expression of MHC class II molecules (Scholl et al., 1990a). In mice, I-A^b and I-A^d have been shown to bind TSST-1 with a much higher affinity than I-A^k (Scholl et al., 1990b; Uchiyama et al., 1990).

For the most part, the affinity of staphylococcal toxins for class II molecules have correlated well with their ability to stimulate T cell proliferation. HLA-DR and HLA-DQ molecules, which bind strongly to SEA, SEB and TSST-1, also supported proliferation of T lymphocytes in response to these toxins (Mollick et al., 1991). The only exception was the TSST-1/HLA-DP combination which was found to stimulate T cell proliferation despite the lack of detectable binding of the toxin to this class II isotype. The latter result indicates that even modest binding of toxins to class II molecules may lead to amplified responses in T cell proliferation. Mollick et al. (1991) have observed that for each unit increase in the affinity of the toxin for class II molecules, a 10-fold amplification in T cell responsiveness was found. It has been estimated for SEA that only 0.1% of class II molecules need to be occupied for the activation of T cell proliferation by this toxin (Mollick et al., 1991). This may explain why SEA is one of the most potent mitogens known today. The potency of SEA and other staphylococcal toxins suggests that each toxin/class II complex may be activating multiple T cells (Mollick et al., 1991). In our studies, the half-maximal stimulatory concentration for T cell stimulation (~0.1 pM) by TSST-1 was much lower than the dissociation constant (average = 40 nM) found for binding of TSST-1 to HLA-DR on human monocytes. The difference between these values may be due to the temperature (ie. 4°C for binding vs. 37°C for proliferation) at which these assays were performed. Alternatively, there may be an unidentified receptor molecule on human monocytes that associates with HLA-DR only at physiological temperatures to increase the affinity of the HLA-DR antigen for TSST-1. It is of note that the binding affinity of SEA for DR1-transfected fibroblasts is a few fold higher at 37°C than at 4°C (Mollick et al., 1991).

Recently, TSST-1 and the staphylococcal enterotoxins have been called "superantigens" because of their ability to stimulate a broader range of T cells compared with conventional antigens (Choi et al., 1989; Kappler et al., 1989; Marrack and Kappler, 1990). The explanation for the activation of a larger number of T cells lies in the finding that superantigens, in association with MHC class II proteins interact with only one chain, the ß chain of the T cell antigen

receptor (TCR). Normally, the 5 other variable elements of the TCR (V_α, J_α, V_β, D_β, and J_β) are used by T cells to recognize conventional peptide antigens presented in the context of class II MHC molecules (Kronenberg et al., 1986). The millions of possible combinations of these TCR variable elements mean that the frequency of T cells responsive to a conventional antigens is very low (Herman et al., 1991). Since these elements contribute little to the recognition of superantigens, the frequency of responding T cells is much higher (Marrack and Kappler, 1990; White et al., 1989). In mouse, for example, whereas an ordinary antigen stimulates 1% of all T cells, the bacterial superantigens can activate as many as 10% of the mouse's T cell repertoire (Herman et al., 1991; Marrack and Kappler, 1990). That the TCR α chain was not required for TCR interaction with superantigen/class II MHC complex was further demonstrated by the binding of a soluble TCR ß chain with SEA complexed to cell-surface class II molecules (Gascoigne and Ames, 1991). However, the bacterial superantigens also differ from conventional antigens in a number of other ways as indicated in Table VII. Unlike normal antigens, superantigens do not require processing prior to binding to HLA-DR nor is stimulation of T cells MHC-restricted (Fischer et al., 1989; Fleischer et al., 1989). Fixed macrophages or monocytes bind bacterial antigens and support T cell proliferation as efficiently as viable cells (Fischer et al., 1989). In fact, treatment of SEA with a variety of proteases hinders its ability to bind to class II molecules and stimulate T cell proliferation (Fraser, 1989; Marrack and Kappler, 1990). Moreover, allogeneic and even xenogeneic MHC class II molecules can bind and present bacterial superantigens to T cells, suggesting that there is no restriction by polymorphic determinants of HLA molecules (Fleischer et al., 1989). Neither CD4 nor CD8 is required for recognition of superantigens since T cell clones lacking these molecules are generally able to respond to superantigen stimulation (Fleischer and Schrezenmeier, 1988; Fleischer et al., 1989). Possibly, the affinity of the TCR for class II/superantigen complex may override the necessity of CD4 and CD8 molecules normally required for conventional antigen/MHC complexes (Herman et al., 1991). The superantigen activation of T cells through the V_β-sequences of their TCR may account for the

Table VII

Comparison Of Properties Between Superantigens And Conventional Antigens

Property	Superantigens	Conventional Antigens
Accessory cell requirement for T cell proliferation	yes	yes
Direct binding to MHC class II	yes	no
Antigen processing	no	yes
MHC class II restriction	no	yes
Allogeneic or xenogeneic restriction	no	yes
TCR recognition	V_β	$V_\alpha, J_\alpha, V_\beta, D_\beta, J_\beta$
Frequency of responding T cells	1/4-1/20	$1/10^4$-$1/10^6$

Modified from Herman et al., 1991, and Janeway, 1990.

reason why both $CD4^+$ and $CD8^+$ T cells are activated (Marrack and Kappler, 1990). Superantigens are also able to induce nonresponsiveness in murine T cells either by clonal deletion (White et al., 1989) or functional inactivation (Rammensee et al., 1989). In humans, however, the deletion of certain TCR V_β families following superantigen stimulation has not been demonstrated (Goronzy et al., 1992). Finally, unlike conventional antigens which trigger $CD8^+$ T cell-mediated cytotoxicity in the context of class I molecules, very low concentrations of superantigens can direct cytotoxic T lymphocytes to mediate strong cytotoxicity against MHC class II-bearing target cells (Hedlund et al., 1990).

The availability of a limited number of V_β-specific mAbs allowed the initial demonstration that staphylococcal exotoxins selectively stimulated T cells bearing particular V_β sequences (Kappler et al., 1989). Using a quantitative polymerase chain reaction method, Choi et al. (1989) further showed that the patterns of V_β-specificity was determined by the toxin itself. A summary of the V_β-specificities for murine and human T cells is shown in Table VIII. TSST-1, for example, interacts with human T cells bearing $V_{\beta 2}$ whereas SEB shows a broader V_β-specificity in reacting with 6 different subsets (Table VIII). Therefore, it appears that each toxin has different specificities for $V_{\beta s}$. The reasons why different toxins use unique patterns of V_β-specificities is not immediately known. Callahan et al. (1990) have hypothesized that after binding to MHC class II molecules, different toxins have different affinities for particular V_β elements. They believe that the affinity of the toxin for either the TCR or class II molecule may be related to the amino acid sequence of the toxins themselves. Based on amino acid homology, the staphylococcal toxins can be divided into 3 major groups. Staphylococcal enterotoxins A, D and E belong to one group, staphylococcal enterotoxins B and C to another, and TSST-1 and the staphylococcal exfoliative toxins (ExT) to the third (Abe et al., 1991; Marrack and Kappler, 1990). In support, Callahan et al. (1990) found that the closely related toxins, SEC_1, SEC_2, and SEC_3, have similar although not identical patterns of V_β-usage in mice. In contrast, the two toxins most distantly related to the

Table VIII

V_β Specificities Of Staphylococcal Exotoxins

Toxin	V_β Specificity	
	Human	Mouse
SEA	?	1,3,11,12[a]
SEB	3,12,14,15,17,20	3,7,8.1,8.2,8.3,17
SEC_1	12,?	3,8.2,8.3,11,17
SEC_2	12,13.1,13.2,14,15,17,20	3,8.2,10,17
SEC_3	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	3,15,17
ExFT[b]	2	3,10,11,15,17
MAM[c]	?	6,8.1,8.2,8.3

Modified from Marrack and Kappler, 1990 and Herman et al., 1991.

[a] Derived from Takimoto et al., 1990

[b] ExFT = exfoliative toxin

[c] MAM = M. arthriditis mitogen

enterotoxins, TSST-1 and ExT, stimulated $V_{\beta 15}$ T cells, which none of the enterotoxins could affect. $V_{\beta 8}$ T cells, a common target for staphylococcal enterotoxins, were not activated by TSST-1 or ExT (Callahan et al.,1990). One other interesting feature is that V_β-usage by superantigens appears to be conserved in mice and humans. TSST-1, for example, stimulates human $V_{\beta 2}$ T cells; interestingly, the human homolog in mice, $V_{\beta 15}$, is also a major target for TSST-1. Thus, despite only 45% amino acid homology between human $V_{\beta 2}$ and mouse $V_{\beta 15}$, the capacity of these $V_{\beta s}$ to interact with TSST-1 and class II molecules have been retained (Callahan et al., 1990; Marrack and Kappler, 1990).

The importance of V_β-specific stimulation of T cells in TSS has been demonstrated by examining the expansion of $V_{\beta 2+}$ T cells in TSS patients. Choi et al. (1990b) showed that five of eight TSS patients showed markedly increased levels of $V_{\beta 2+}$ T cells compared to controls a few days after the onset of symptoms. In one patient $V_{\beta 2}$ rose from 10% in normal individuals to about 70% of total peripheral T cell population (Choi et al., 1990b). The magnitude of this T cell response is not observed in response to conventional antigens. The $V_{\beta 2+}$ T cell percentage returned to normal values 45 to 60 days after the acute episode (Choi et al., 1990b).

Besides the staphylococcal toxins, there is now a growing number of bacterial proteins that can also function like superantigens. Streptococcal pyrogenic exotoxins (SPEs), known to cause TSS-like syndromes, as well as the streptococcal M protein have also been shown to stimulate T cells in a V_β-specific manner (Abe et al., 1991; Kotb et al., 1990; Leonard et al., 1991). All of these toxins activate $CD4^+$ and $CD8^+$ T cells and require MHC class II expression on accessory cells (Fleischer et al., 1991; Leonard et al., 1991). Streptococcal pyrogenic exotoxin A (SPEA) shares structural homology with the first group (SEA, SED, and SEE) and the second group (SEB and SECs) of enterotoxins. The fact that SEE stimulates human $V_{\beta 8}$, SEC_2 stimulates $V_{\beta 12}$ and $V_{\beta 14}$ and SPEA stimulates $V_{\beta 8}$, $V_{\beta 12}$, and $V_{\beta 14}$ suggests that these toxins share common determinants for T cell recognition (Abe et al., 1991). Thus, group A

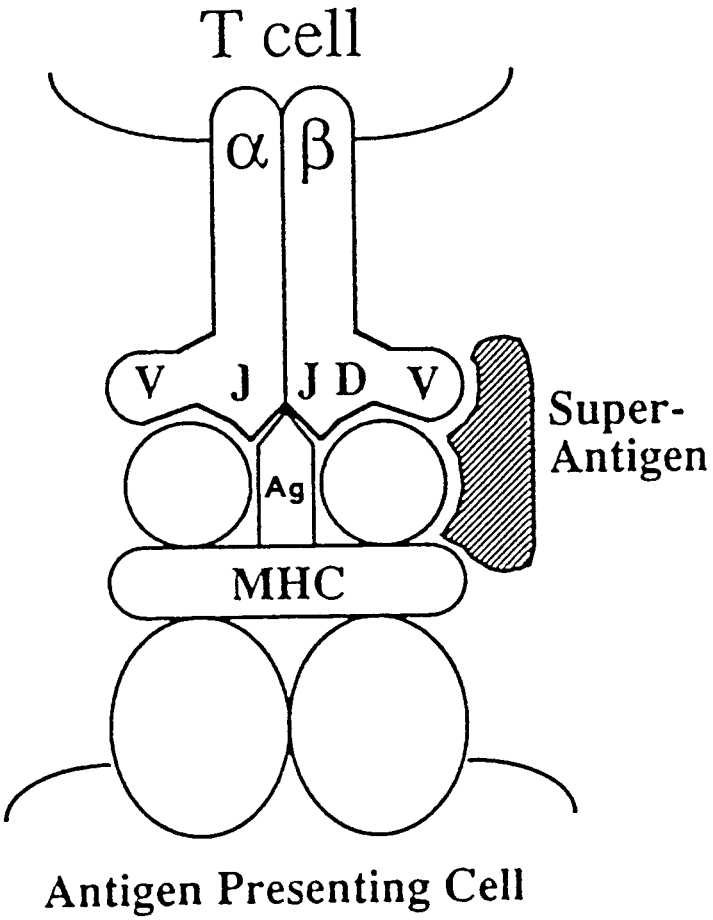


Figure 16. Hypothetical structure for the complex of class II MHC, TCR, and superantigens as proposed by Marrack and Kappler, 1990. Note that the superantigen binds to the sides of class II MHC and V_β away from the normal peptide groove of conventional antigens. Figure taken from Herman et al., 1991.

streptococci may cause TSS-like diseases in a manner similar to staphylococcal toxins. The ability of SPE to stimulate TNF release from macrophages (Fast et al., 1989) coupled with the activation of a number of V_{β}-specificities by SPEA, SPEB, and streptococcal M protein may account for their wide spectrum of human diseases (Abe et al., 1991).

Based on the above studies, Marrack and Kappler (1990) have proposed a hypothetical model for binding of superantigens by MHC class II molecules and TCR. As diagrammed in Figure 16, they believe that superantigens interact with the ß-pleated sheets of the V_{β} domain of the TCR and with the MHC class II molecule. Unlike conventional antigens, superantigens bind outside the normal peptide groove to clamp both the class II molecules and the TCR (Marrack and Kappler, 1990). There is supporting evidence that superantigens are presented to T cells in a different manner compared with conventional antigens. Since superantigens are not processed by accessory cells, they are unable to bind to the peptide groove because of their large molecular size. Moreover, Dellabonna et al. (1990), using a panel of mutant-presenting cell lines, showed that mutatations in the α chain of $I\text{-}A^{k}$ inhibited presentation of conventional antigens but not superantigens to T cells. In addition, peptides made towards the high affinity binding groove inhibited presentation of conventional antigens to a responsive T cell line while having no effect on SEB (Dellabonna et al., 1990). Finally, some of the residues in the TCR V_{β} chain thought to be important for binding of the MHC class II/superantigen complex have been found to lie on the side of the TCR molecule, outside the conventional antigen/MHC binding site (Choi et al., 1990a).

Within the last two years, researchers have also focused on defining the binding site of bacterial superantigens on MHC class II molecule. MHC class II molecules, which consist of the HLA-DR, -DQ, and - DP antigens in humans, and I-A and I-E antigens in mice, are composed of a monomorphic α chain (35 kd) and a polymorphic ß chain (28 kd) (Trowsdale et al., 1985). Since mAbs directed against the monomorphic regions of HLA-DR have been shown in our study and those of others (Moseley et al., 1991; Mourad et al., 1989; Scholl et al., 1990a) to

inhibit TSST-1 binding to HLA-DR and subsequent activation of T cell proliferation, it is reasonable to suggest that the α chain might be the superantigen binding site in class II molecules. Karp et al. (1990) showed that the α-1 region (ie. the amino-terminal portion) of the HLA-DR α chain was important for binding of TSST-1. L cells expressing the DR or DP α-chain were transfected with cDNAs for chimaeric ß-chains and tested for their ability to bind TSST-1. These chimaeric ß-chains consisted of DR ß and DP ß chain amino acid sequences. The authors found that cells consisting of chimaeric ß-chains paired with DR α were able to bind TSST-1. In contrast, TSST-1 did not bind to DP α-chains paired with the same chimaeric ß-chains. These results show that the α-chain of HLA-DR is important for binding of TSST-1 (Karp et al., 1990). However, there also appears to be a contribution from the ß-chain for binding of TSST-1 and the staphylococcal enterotoxins. It has previously been shown that SEA and SEE bind well to most HLA-DR alleles with the exception of HLA-DRw53 (Herman et al., 1990). Since the α-chain was common to both HLA-DR alleles, evidence pointed to a region of the ß-chain for toxin binding (Herman et al., 1990). Karp and Long (1992) recently showed that mutation of histidine 81 in an α helical region of the DR1 ß-chain to tyrosine reduced SEA/SEE binding. In contrast, introduction of histidine 81 in the DRw53 ß chain restored binding of the two toxins. Thus, the binding site of SEA/SEE may lie on the outer face of the ß-chain α helix (Karp and Long, 1992). Using a different approach, Russell et al. (1990, 1991) made synthetic peptides to the α- and ß-chains of murine MHC class II antigen in order to determine the binding site of SEA. They found that peptide I-$A_ß^b$-(65-85) directly bound SEA and strongly inhibited this toxin from binding to the mouse B-cell lymphoma line, A20 (H-2^d) or the HLA-DR-bearing Raji cell line. This peptide also strongly blocked SEA-induced proliferation of human PBMC. However, it also appears that the α-chain is involved since the peptide I-$A_α^b$-(51-80) bound SEA in solid phase ELISAs and blocked stimulation of human PBMC by SEA (Russell et al., 1991). Interestingly, this peptide was not able to block SEA binding to A20 or Raji cells. The authors postulated that the α-chain is required for SEA function but not for binding to MHC class II molecules. In contrast, the

ß-chain is necessary not only for SEA function, but also for SEA binding (Russell et al., 1991). Therefore, these studies indicate that the ß-chain is important for binding of TSST-1 and the staphylococcal toxins to class II molecules; the exact role of the α-chain is still under investigation (Karp et al., 1990).

At present, the mechanism by which TSST-1 and the staphylococcal enterotoxins stimulate V_{β}-specific T cells is unknown. There is some controversy as to whether receptors directly exist for these toxins on human T lymphocytes. Poindexter and Schlievert (1987) demonstrated the presence of TSST-1 receptors on human T lymphocytes. However, a number of subsequent studies have failed to demonstrate toxin binding to human T lymphocytes (Carlsson et al., 1988; Fischer et al., 1989; Fraser, 1989; Mollick et al., 1989; Uchiyama et al., 1989). We have recently reported that highly purified human T lymphocytes do not bind radiolabeled TSST-1 or SEA unless HLA-DR-bearing human monocyte membrane fragments are present (Chang et al., 1992). Fleischer et al. (1989) have shown that SEA and SEB can interact with resting human T cells or Jurkat T cell lines since they increase cytosolic Ca^{2+} in the absence of MHC class II antigen. Presumably, this toxin-lymphocyte interaction is receptor-mediated. As mentioned earlier, it is possible that low number of receptors or low affinity interactions between staphylococcal toxins and T cells may not be detectable by the radioligand binding assay, but may be detected by the more sensitive functional assay.

Nevertheless, MHC class II molecules are important for toxins to stimulate T cell proliferation as demonstrated by the inhibition of the mitogenic activity by mAbs to HLA-DR in this study and in those of others (Fischer et al., 1989; Fleischer and Schrezenmeier, 1988). Several hypotheses have been put forth to explain the role of the MHC class II molecules in presenting bacterial superantigens to the TCR (Fischer et al., 1989). One possibility is that the toxins alone are unable to interact with the TCR and that their polyvalent presentation by class II molecules might increase their affinity for the TCR. However, Fischer et al. (1990) have found that the polyvalent presentation of SEA on Sepharose beads did not stimulate T cell proliferation, even in the presence

of exogenous IL-1. In similar experiments, Fleischer et al. (1991) showed that the superantigens, SEB and streptococcal erythrogenic toxins A (ETA), when covalently bound to the surface of silica beads, did not induce proliferation of resting T cells. The authors noticed that T cell proliferation did occur if ETA or SEB were co-cross-linked on beads together with anti-CD8 or anti-CD2 antibodies in the presence of IL-2. These results suggest that although the toxins may bind to the TCR complex in the absence of class II molecules, full activation of T cells does not occur unless the TCR is co-cross-linked with accessory molecules such as CD2 or CD8 (Fleischer et al., 1991). Based on these studies, the authors proposed that the toxins are functionally bivalent proteins crosslinking MHC class II molecules with the variable regions of the TCR beta chain (Fleischer et al., 1991). Alternatively, there are some suggestions that upon binding to MHC class II molecules, the superantigens become conformationally altered to interact with the V_{β}-sequence in the TCR although there has not been direct experimental proof (Fischer et al., 1989; Marrack and Kappler, 1990; Yagi et al., 1991).

Although TSST-1 and the staphylococcal enterotoxins bind to class II HLA-DR molecules on human monocytes, there is suggestive evidence that the binding epitopes for these toxins may be overlapping or even distinct. This was supported in our experiments where unlabeled TSST-1 significantly inhibited radiolabeled SEA binding to human blood monocytes; however on a molar basis, unlabeled SEA was by far a better competitor. Conversely, unlabeled TSST-1 was more effective per mole than unlabeled SEA in blocking radiolabeled TSST-1 binding to the same source of cells (See et al., 1990). The most likely interpretation of these results is that although TSST-1 and SEA are binding to the same receptor, the epitopes on HLA-DR are not entirely identical. Furthermore, the anti-HLA-DR mAb, L243, was consistently more potent in blocking radiolabeled TSST-1 binding than SEA binding to human monocytes from the same donor. Simultaneous with our study, Scholl et al. (1989b) showed that both TSST-1 and SEB bind to HLA-DR and HLA-DQ molecules on transfectant cell lines. However, no cross-inhibition of TSST-1 and SEB was observed in competitive binding assays, indicating that the epitopes for

these toxins on HLA-DR and HLA-DQ were distinct (Scholl et al., 1989b). Their results are consistent with our finding that an excess of unlabeled SEB was unable to inhibit ^{125}I-TSST-1 binding to human monocytes. Previous work by others has shown that SEA and SEB also share overlapping binding sites on HLA-DR (Fraser, 1989). We propose from the above experiments that at least two binding sites for staphylococcal toxins may exist on the HLA-DR molecule, one which is shared by TSST-1 and SEA, and the other which is shared by SEA and SEB. Thus, SEA occupies a binding site within HLA-DR that overlaps both TSST-1 and SEB, while the binding sites for TSST-1 and SEB appear either topographically or sterically distinct.

Although the above hypothesis may be correct, it still does not explain the shapes of the cross-competition curves shown in Figures 8A and 8B. The ability of SEA to cross-inhibit radiolabeled TSST-1 binding to human monocytes was far more effective than the ability of unlabeled TSST-1 to block radiolabeled SEA binding. Additionally, in receptor cross-linking experiments, the intensity of the 35 kd and the 28 kd HLA-DR subunit bands were generally more intense with SEA. One possible explanation to account for the difference is that TSST-1 and SEA may be binding to other MHC class II molecules such as HLA-DQ and HLA-DP in addition to HLA-DR. Previous studies have shown that TSST-1 binds to HLA-DR and HLA-DQ, but not to HLA-DP (Scholl et al., 1989b). SEA was found to bind to all three MHC class II isotypes in one study (Mollick et al., 1991) and to only HLA-DR in two others (Fischer et al., 1989; Fraser, 1989). LIGAND analysis of the binding data indicated only one class of receptors for TSST-1 and SEA in our study, implying that the HLA-DR may be the main receptor in normal human monocytes. However, since the expression of HLA-DQ and HLA-DP are much lower than that of HLA-DR, one cannot totally exclude the possibility that TSST-1 and SEA may also bind to either or both of these molecules at low levels. A more likely reason to account for the difference in the shapes of the SEA and TSST-1 cross-competition curves is that SEA may have an additional binding site on HLA-DR other than the one it shares with TSST-1 and SEB. Pontzer et al. (1991) have confirmed our findings by analysing SEA and TSST-1 binding to both the human Raji

and the murine A20 (Ia^d) cell lines. The authors showed that both toxins bound similarly to A20 and that blocking of binding was observed only with homologous toxin, suggesting distinct binding sites. In the Raji cell line, however, SEA inhibited TSST-1 binding strongly, whereas TSST-1 could only minimally inhibit SEA binding. What suggested two distinct binding sites for SEA was the finding that the SEA peptide [SEA (1-45)] and ß-chain peptides of MHC class II molecules [$I\text{-}A_ß^b$ (65-86)] blocked SEA but not TSST-1 binding on both cell lines. The authors proposed that there was one binding site for SEA on A20 and two binding sites on Raji; one site involving the residue 1-45 region on SEA and the 65-85 region of MHC ß chain and the other site involving a different region on SEA and MHC class II ß-chain to which TSST-1 also binds. Recent work by Chintagumpala et al. (1991) also suggests that two or more binding sites may exist for SEA on HLA-DR. Their experiments showed the existence of two distinct binding sites for TSST-1 and SEB on the HLA-DR molecule, both of which SEA competes with effectively. When unlabeled SEB and TSST-1 were used to block ^{125}I-SEA binding, only 30% and 50% inhibition of binding, respectively, were observed. These investigators hypothesized that if there were two binding sites for staphylococcal toxins (one each for SEB and TSST-1), both of which are being shared by SEA, then a combination of TSST-1 and SEB should totally abolish radiolabeled SEA binding. Unexpectedly, this was not the case since the combination of toxins was only as effective as each individually, providing support of an alternative binding site for SEA on HLA-DR. Thus from their data, there appears to be at least three distinct binding sites for toxins on HLA-DR, one each for TSST-1 and SEB to which SEA also binds and a third site for SEA which is not bound by TSST-1 or by SEB (Chintagumpala et al., 1991). This hypothesis would explain why TSST-1 in our studies does not inhibit radiolabeled SEA binding as strongly as the converse. Unlabeled TSST-1 would only be expected to block binding of radiolabeled SEA at the site of overlap and vice-versa. Because of an additional site on HLA-DR for SEA, competition with excess TSST-1 would not be totally complete.

The reasons for multiple binding sites on HLA-DR for SEA are not immediately clear. The binding of two molecules of SEA per HLA-DR molecule may

explain why SEA is the most potent T cell mitogen of the staphylococcal toxins. Even more surprising is the possibility of three potential binding sites on a heterodimeric receptor composed of a 35 kd and 28 kd subunit, both of which are comparable to the size of the toxins themselves. The small size of the HLA-DR receptor indicates that the three toxin binding sites, although distinct, would tend to lie in close proximity to each other. The idea of three binding sites on HLA-DR for staphylococcal toxins may be related to the structural composition of the toxins. As mentioned in Chapter 1, the staphylococcal toxins can be divided into three major groups based on their amino acid composition. SEA, SED and SEE fall into one group, SEB and SECs belong to another while TSST-1 is distantly related to the first two groups. Since separate sites exist for TSST-1, SEA and SEB, then the ability of these toxins to engage a binding epitope on HLA-DR may be related to their conformation which, in turn, is dependent on their amino acid sequences. Therefore, for example, one might expect SEA, SED and SEE to compete effectively with each other for HLA-DR binding sites. Indeed, recent studies have shown that SED and SEE strongly compete with SEA for receptor binding sites (Chintagumpala et al., 1991).

What might be the biological consequence of staphylococcal enterotoxins binding to separate or overlapping epitopes? One consequence is that the binding of a particular epitope on MHC class II molecules may determine the toxins's ability to stimulate similar, although not identical V_β-specific sequences on T cells. For example, SEA, SED and SEE all recognize murine T cells bearing $V_{\beta 11}$, while the related SEB, SEC_1, SEC_2, and SEC_3 all recognize human $V_{\beta 12}$ (Marrack and Kappler, 1990). Another consequence may be the mode of activation of class II-bearing accessory cells by these toxins. As will be apparent in the next chapter, TSST-1, SEA and SEB appear to activate human monocytes by different signal transduction pathways (See et al., 1992b). Whether the differences in intracellular signalling between these toxins is related to the binding of unique epitopes on HLA-DR remains to be determined.

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Chapter 4

Phosphorylation Events In Human Monocytes Induced By TSST-1 And Staphylococcal Enterotoxins

4.1 Introduction

The previous chapter dealt with the characterization of the receptor for TSST-1 in human monocytes and how this toxin and the staphylococcal enterotoxins bind to overlapping epitopes on the HLA-DR receptor. In this chapter, the signal transduction events induced by TSST-1 and the staphylococcal enterotoxins will be examined.

It is well-known that cells respond to extracellular growth factors through activation of plasma membrane receptors specific for the polypeptide. This results in the generation of intracellular intermediates whose functions is to transmit and amplify the signals delivered by the original stimuli. The phosphorylation/dephosphorylation of cellular proteins is now considered an important mechanism by which intracellular activities such as changes in cell morphology, DNA synthesis, cell division, and the activation of metabolic pathways are regulated by external stimuli (Cohen, 1982; Exton, 1985; Krebs and Beavo, 1979). The enzymes responsible for the transfer of the ATP gamma phosphate to the serine, threonine, and tyrosine residues of protein substrates are known as protein kinases, whereas those that remove phosphates are known as phosphatases. Therefore, the phosphorylation state of a protein substrate depends upon the interplay between the activities of kinases and phosphatases, which themselves are regulated by phosphorylation and dephosphorylation mechanisms (Cohen, 1989). Examples of well-known protein kinases include protein kinase C (Nishizuka, 1986) and cAMP-dependent protein kinases (Rozengurt, 1981), which phosphorylate at serine/threonine residues, and growth factor receptors (eg. epidermal growth factor receptor) which possess intrinsic tyrosine kinase activity (Hunter and Cooper, 1985).

Although TSST-1 and the staphylococcal enterotoxins stimulate similar cellular responses in human monocytes, the mechanisms by which these toxins activate monocytes are poorly understood. Even less is known about the early intracellular events that mediate responses to these toxins. Neutrophils, platelets, and lymphocytes are among the blood cells that have been demonstrated to respond to a wide variety of agonists by altering the phosphorylation levels of key proteins (Andrews and Babior, 1983; Lyons et al., 1975; Patel and Miller, 1991). The phosphorylation of these proteins at serine, threonine, or tyrosine residues may have important ramifications since their modification may result in conformational changes leading to altered biological activity (Cohen, 1989). In this chapter, we have compared the protein phosphorylation patterns of human monocytes activated by TSST-1, SEA, SEB, and two other monocyte agonists, PMA and bacterial LPS, in order to investigate whether these agents share similar or dissimilar signal transduction pathways. Protein phosphorylation/dephosphorylation reactions were examined rather than identifying specific kinases activated by these factors for the following reasons: 1) protein phosphorylation/dephosphorylation reactions may reveal unsuspected relationships between factors with similar modes of action; and 2) the method has the potential for the identification and subsequent purification of interesting protein substrates.

Specific Aims

1) To examine the phosphorylation changes in human monocyte proteins in response to TSST-1 stimulation.
2) To compare phosphorylation patterns of human monocytes activated by TSST-1, staphylococcal enterotoxins, bacterial LPS, and PMA to determine whether their signal transduction pathways are similar or dissimilar.
3) To determine whether TSST-1 and staphylococcal enterotoxins stimulate tyrosine-specific phosphorylation of human monocyte cellular proteins.

4.2 Results

4.2.1 Phosphorylation Changes In Human Monocyte Cellular Proteins In Response To TSST-1 Activation

To characterize the early phosphorylation events associated with human monocyte activation by TSST-1, purified monocytes were labeled with ^{32}P-orthophosphate and then stimulated with the toxin for 15 min at 37°C. Cell lysates were prepared and phosphoproteins separated by two-dimensional gel electrophoresis. Radioactive spots in the autoradiographs were analysed by a computerized densitometry program for two-dimensional gels (Visage 110 Image System). The intensity of each spot (integrated absorbance units) was normalized against a reference internal standard shown as a boxed phosphoprotein in Figures 17 through 20. Changes in the level of phosphorylation of a phosphoprotein were then determined by comparing the normalized intensities of the spot of interest in the absence or presence of agonist. Dephosphorylation is indicated by a lower intensity of the phosphoprotein relative to the medium control; phosphorylation is indicated by a higher intensity of the phosphoprotein relative to the medium control. Results from 4 healthy donors revealed that exposure of human monocytes to 0.1 μg/ml TSST-1 stimulated the dephosphorylation of a number of phosphoproteins (representative changes indicated by arrows in Figure 17). A number of phosphoproteins remained unaltered, thereby serving as a negative control for the dephosphorylated proteins. The phosphorylation changes were dose-dependent since several more phosphoproteins became dephosphorylated at a TSST-1 concentration of 10 μg/ml (see arrows in Figure 17). Time course studies revealed that TSST-1-induced dephosphorylation of monocyte cellular proteins were detectable within 5 min after the addition of TSST-1 (Figure 18). Dephosphorylation in response to TSST-1 stimulation remained stable for at least 1 h (Figure 18). Phosphorylation changes induced by TSST-1 in human monocytes from 8 different healthy donors are summarized in Table IX. Six phosphoproteins (molecular weights of 16.5 kd, 17 kd, 17.5 kd, 20 kd, 29.0 kd and 65 kd) were consistently dephosphorylated by TSST-1. In contrast, a few phosphoproteins (eg.

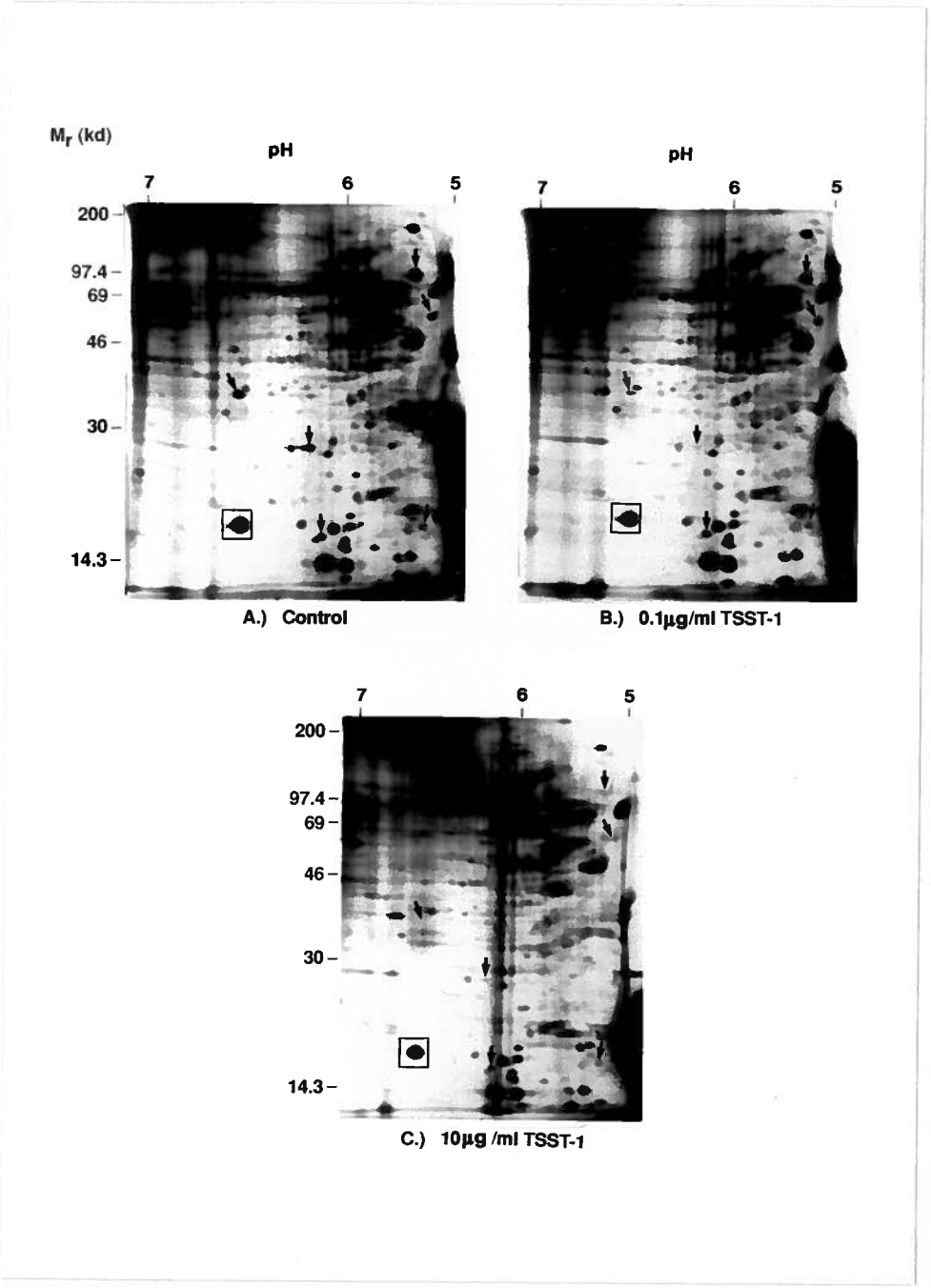

Figure 17. Phosphorylation events induced by TSST-1. Human monocytes (5 x 10^6 cells) were labeled with ^{32}P-orthophosphate and stimulated with TSST-1 for 15 min at 37°C. Cells were lysed and samples analysed by two-dimensional gel electrophoresis as described in Materials and Methods. Exposure of autoradiograms was adjusted according to the intensity of the boxed phosphoprotein internal reference standard. Downward arrows indicate representative dephosphorylations induced by each concentration of TSST-1 relative to negative controls. Results are representative of 4 experiments.

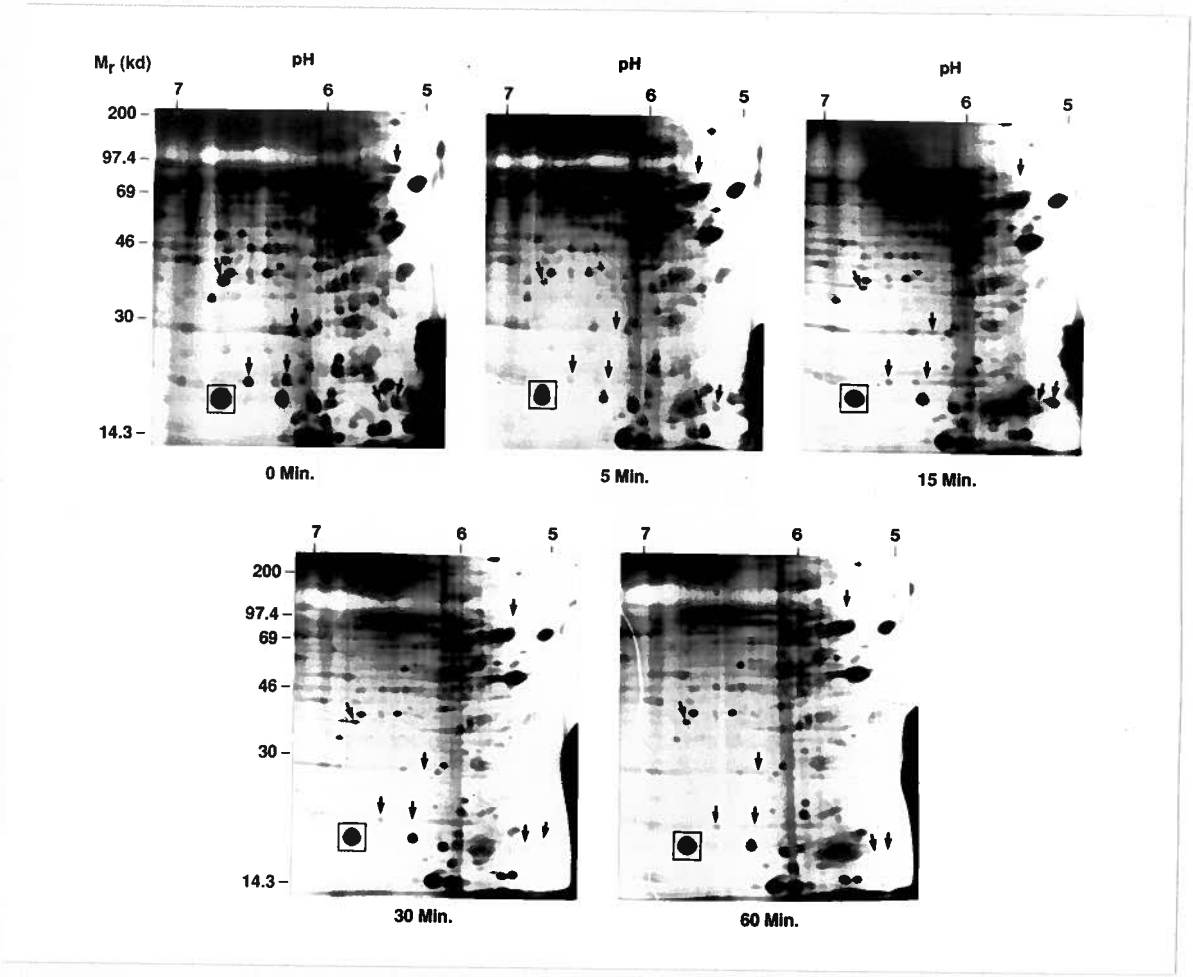

Figure 18. Time course of TSST-1-induced dephosphorylation of monocyte cellular proteins. ^{32}P-labeled monocytes (5 x 10^6 cells) were stimulated with 10 μg/ml TSST-1 for the indicated time periods at 37°C. Exposure intensity of autoradiograms was standardized according to the boxed phosphoprotein internal reference. Downward arrows indicate dephosphorylation of representative phosphoproteins relative to negative control at the indicated time. Results are representative of 3 experiments.

Table IX

Composite Analyses Of Protein Phosphorylation Changes Of Human Monocyte Cellular Proteins After Stimulation With TSST-1 Or Medium Control[a]

Phosphprotein M_r (kd)	pI	DIR[b]		P value[c]	Change in Phosphorylation[d]
		Control	TSST-1		
14.5	5.3	0.094 ± 0.017	0.159 ± 0.022	0.46	
14.5	5.4	0.405 ± 0.026	0.465 ± 0.026	0.13	
16.5	5.3	0.141 ± 0.051	0.082 ± 0.032	0.05	↓
17.0	5.2	0.266 ± 0.052	0.077 ± 0.057	0.005	↓
17.5	5.1	0.394 ± 0.088	0.158 ± 0.044	0.01	↓
20.0	6.3	0.025 ± 0.007	0.005 ± 0.002	0.07	
20.0	6.4	0.094 ± 0.019	0.031 ± 0.010	0.005	↓
20.0	6.6	0.094 ± 0.012	0.126 ± 0.052	0.48	
21.0	5.9	0.053 ± 0.036	0.070 ± 0.036	0.66	
22.0	5.9	0.189 ± 0.042	0.248 ± 0.047	0.46	
23.0	5.9	0.132 ± 0.025	0.117 ± 0.032	0.44	
29.0	6.3	0.145 ± 0.043	0.104 ± 0.020	0.42	
29.0	6.4	0.246 ± 0.107	0.074 ± 0.033	0.02	↓
35.0	6.7	0.251 ± 0.050	0.153 ± 0.041	0.07	
37.0[e]	5.3	0.154 ± 0.036	0.649 ± 0.193	0.80	
65.0	5.2	0.166 ± 0.066	0.053 ± 0.020	0.04	↓
95.0	5.3	0.271 ± 0.096	0.065 ± 0.018	0.07	
170.0	5.5	0.341 ± 0.068	0.118 ± 0.028	0.07	

[a] Human monocytes were stimulated with 10 μg/ml TSST-1 or RPMI 1640 medium for 15 min at 37°C. Results are the composite analyses of 8 donors.

[b] DIR = Densitometric intensity ratios calculated by quantifying each phosphoprotein by computerized densitometry and dividing the values for each spot by the value obtained for the boxed internal phosphoprotein standard. Results represent the mean ± SEM of phosphoproteins from 8 different donors.

[c] P values determined by paired student t test (two-tailed).

[d] ↓ = decrease in phosphorylation of phosphoprotein.

[e] Phosphorylation of 37 kd phosphoprotein increased in 4 of 8 donors and decreased in 4 of 8 donors after stimulation with TSST-1.

37 kd) were phosphorylated upon treatment of monocytes with TSST-1 in some donors while in others, these phosphoproteins were dephosphorylated.

4.2.2 Comparison Of Protein Phosphorylation Profiles Of Human Monocytes Stimulated With TSST-1, SEA, SEB, LPS and PMA

To determine whether other bacterial superantigens activate human monocytes in a similar manner, ^{32}P-labeled monocytes were stimulated with SEA or SEB for 15 min at 37°C. Compared with medium controls, 10 μg/ml TSST-1 again induced dephosphorylation of several monocyte cellular phosphoproteins (Figure 19). In contrast, a number of phosphoproteins (some indicated by arrows in Figure 19) were phosphorylated after SEA or SEB stimulation.

The phosphorylation profiles of TSST-1-stimulated monocytes were also compared with those of monocytes treated with two other agonists, bacterial LPS and PMA. As shown in Figure 20, many of the phosphoproteins dephosphorylated by TSST-1 were not affected in the same way by treatment of monocytes with LPS or PMA. In fact, a number of PMA-sensitive proteins were also responsive to LPS (Figure 20), possibly suggesting that these two agents may be activating monocytes by a similar mechanism.

Table X illustrates phosphorylation/dephosphorylation changes induced by different agents on the same donor monocytes. Results show that TSST-1 and staphylococcal enterotoxins do vary in their phosphoprotein substrates. These differences are further exemplified in Table XI where increases or decreases in phosphorylation of several phosphoproteins relative to medium control were analysed by the sign test. In 3 of 4 donors studied, TSST-1 induced significantly more dephosphorylation than phosphorylation changes. However, in the same monocytes stimulated with SEA or SEB, 3 of 4 donors significantly showed more phosphorylation than dephosphorylation. PMA also stimulated the phosphorylation of a number of phosphoproteins, some of which were responsive to SEA or SEB in a similar manner (Table X and Table XI). Additionally, comparing the stimulation index [densitometric intensity ratio (DIR) for stimulator divided by DIR for the medium control] of the phosphoproteins shows that the patterns of phosphorylation

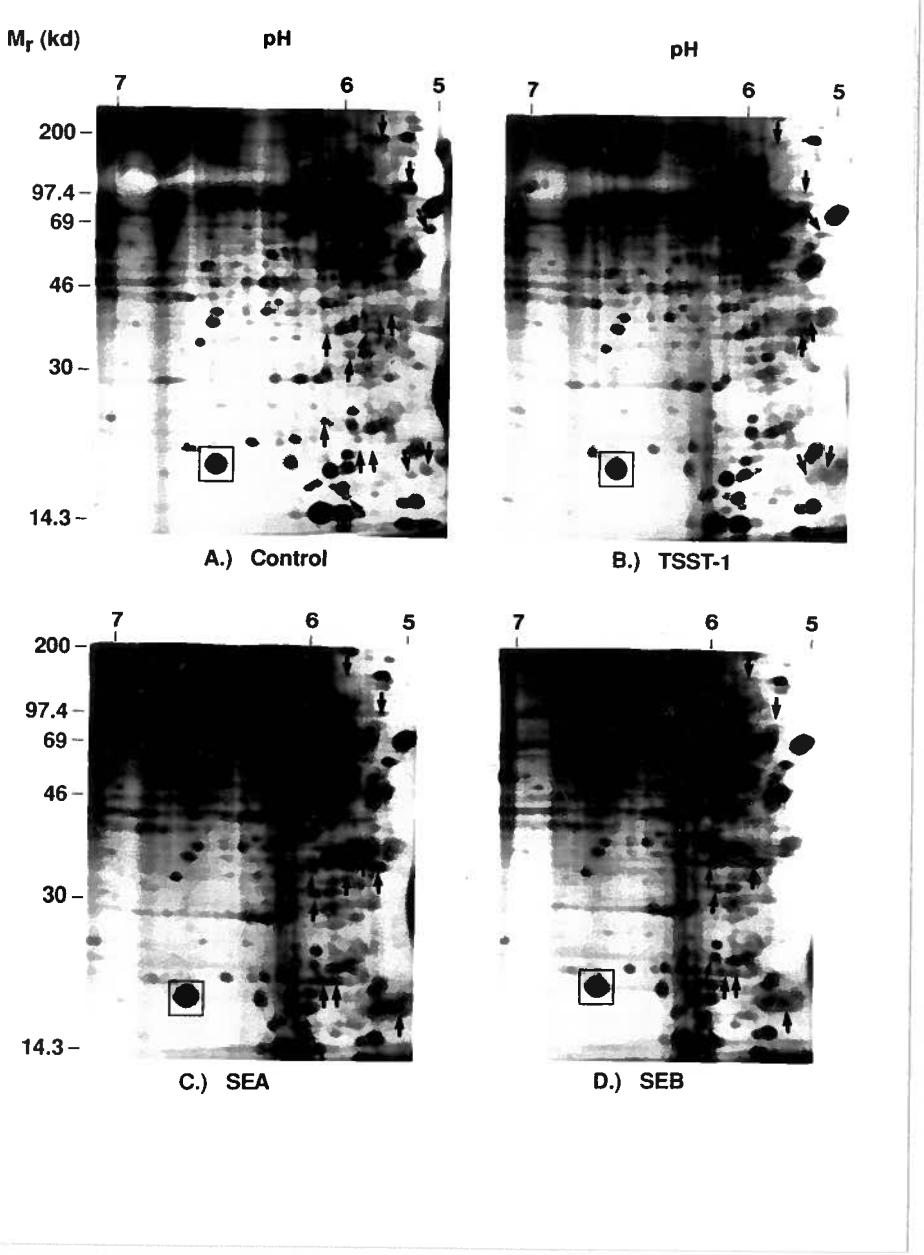

Figure 19. Analysis by two-dimensional gel electrophoresis of monocyte proteins phosphorylated or dephosphorylated during stimulation with TSST-1 or staphylococcal enterotoxins A and B. Human monocytes were treated with 10 μg/ml TSST-1, SEA, or SEB for 15 min at 37°C. Exposure intensity of autoradiograms was standardized according to the boxed phosphoprotein internal reference. Upward and downward arrows indicate representative increases and decreases in phosphorylation, respectively, for the specific stimulus. Large arrowheads indicate phosphorylation changes in common between TSST-1, SEA and SEB. Results are representative of 4 experiments.

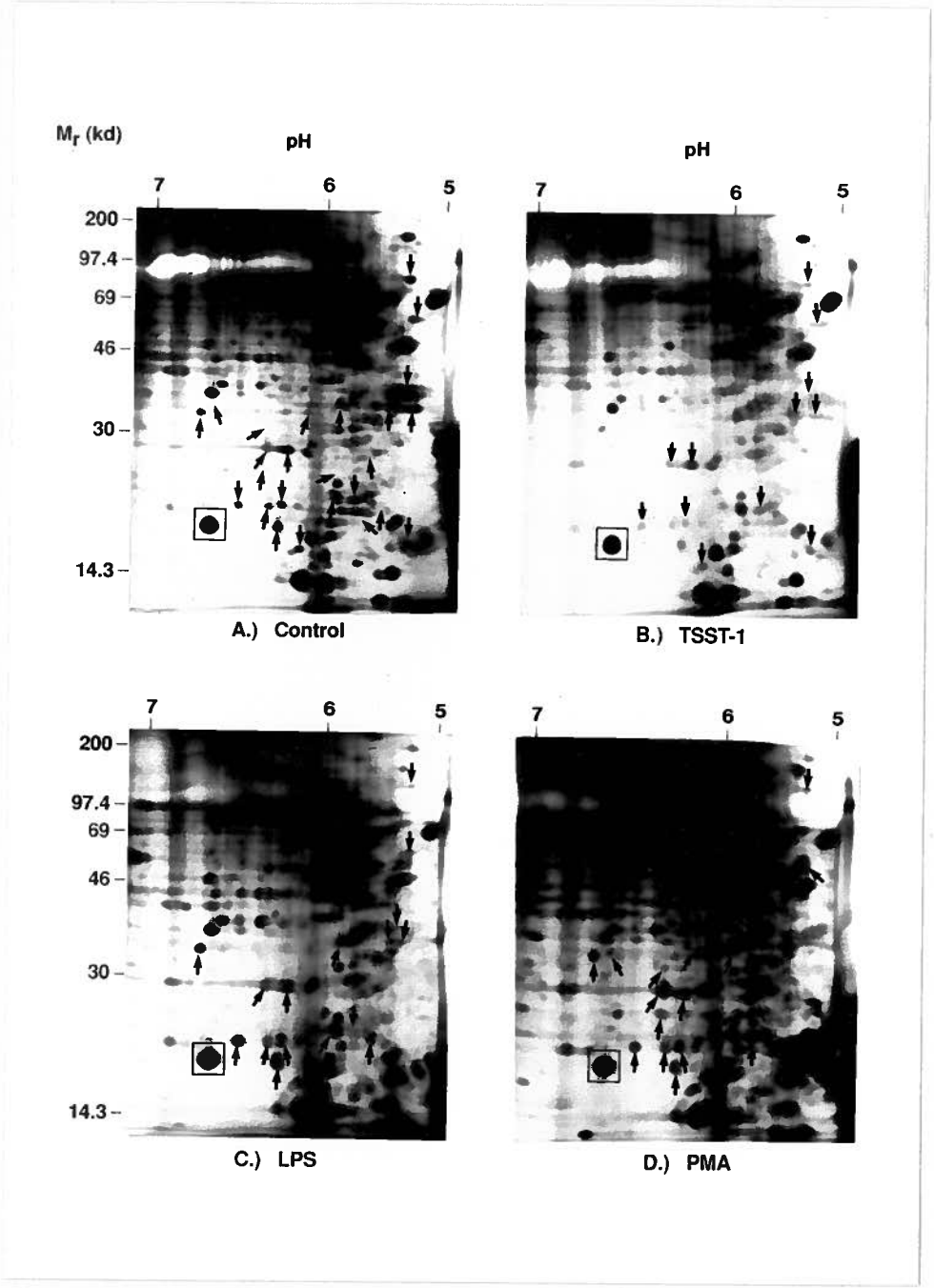

Figure 20. Comparison of phosphorylation or dephosphorylation events in human monocytes induced by TSST-1, E. coli 026:B6 LPS, or PMA. Cells were stimulated with 10 μg/ml TSST-1, 10 μg/ml LPS, or 250 ng/ml PMA for 15 min at 37°C. Exposure intensity of autoradiograms was standardized according to the boxed phosphoprotein internal reference. Representative increases or decreases in phosphorylation of proteins of stimulated monocytes are indicated by upward arrows and downward arrows, respectively. See Table X for a summary of phosphorylation changes. Results are representative of 3 experiments.

Table X

Analysis By Two-Dimensional Gel Electrophoresis Of Human Monocyte Protein Phosphorylation Levels After Stimulation With TSST-1, SEA, SEB, LPS, Or PMA[a]

Phosphoprotein M_r (kd)	pI	DI[b]					
		Control	TSST-1	SEA	SEB	LPS	PMA
14.5	5.3	0.254	0.103	0.233	0.184	0.203	0.618
14.5	5.4	0.371	0.591	0.738	0.488	0.203	0.724
16.5	5.3	0.111	0.034	0.137	0.143	0.528	0.185
17.0	5.2	0.544	0.107	0.526	0.571	0.594	0.206
17.5	5.1	0.599	0.322	0.588	0.634	0.626	0.193
20.0	6.4	0.049	0.015	0.197	0.082	0.153	0.160
20.0	6.6	0.073	0.016	0.128	0.133	0.218	0.206
21.0	5.9	0.072	0.023	0.084	0.054	0.119	0.240
22.0	5.9	0.137	0.167	0.121	0.152	0.195	0.219
23.0	5.9	0.145	0.133	0.176	0.166	0.167	0.175
24.0	5.8	0.001	0.001	0.001	0.001	0.002	0.125
29.0	6.3	0.385	0.220	1.250	0.580	0.462	0.551
29.0	6.4	0.251	0.063	0.422	0.357	0.229	0.216
32.0	5.3	0.268	0.061	0.214	0.274	0.135	0.195
32.0	5.4	0.218	0.032	0.132	0.196	0.043	0.033
34.5	5.9	0.052	0.043	0.867	0.651	0.032	0.755
34.8	5.8	0.007	0.005	0.652	0.473	0.544	0.743
35.0	6.7	0.460	0.270	0.910	0.641	0.385	0.331
37.0	5.3	1.063	0.073	0.234	0.976	0.095	0.897
65.0	5.2	0.331	0.071	0.379	0.397	0.230	0.819
95.0	5.3	0.147	0.038	1.450	0.067	0.021	0.041
170.0	5.5	0.544	0.032	0.087	0.072	0.034	0.584

[a] Monocytes from the same donor were stimulated with 10 μg/ml TSST-1, SEA, SEB, LPS, or 250 ng/ml PMA for 15 min at 37°C. Results represent a summary of Figure 20. Two-dimensional gels for SEA and SEB are not shown on Figure 20 but are summarized here.

[b] Densitometric intensity ratios (DIR) were calculated by quantifying each phosphoprotein by computerized densitometry and dividing the values for each spot by the value obtained for the boxed internal phosphoprotein standard.

Table XI

Comparison Of Phosphorylation Profiles Of Monocyte Cellular Proteins After Stimulation With TSST-1, SEA, or SEB

Agents Tested[a]	Phosphorylation Change[b]			
	Donor 1	Donor 2	Donor 3	Donor 4
TSST-1 vs Medium	NS	↓(0.0004)	↓(0.02)	↓(0.002)
SEA vs Medium	↑(0.06)	↑(0.08)	↑(0.06)	NS
SEB vs Medium	NS	↑(0.03)	↑(0.0001)	↑(0.002)
LPS vs Medium	NS	NS	-	-
PMA vs Medium	-	↑(0.02)	-	-

[a] Comparison of phosphorylation changes induced by 10 μg/ml TSST-1, SEA, SEB, LPS or 250 ng/ml PMA in human monocytes relative to medium controls. Cells were stimulated with the indicated agent for 15 min at 37°C.

[b] Phosphorylation changes, as analysed by computer densitometry, observed in 20 phosphoproteins after exposure to the indicated stimulus. Phosphoproteins were normalized against the internal reference standard and the densitometric intensity ratio compared in the presence or absence of the indicated stimuli. Upward arrows indicate overall increased phosphorylation while downward arrows indicate overall decreased phosphorylation of the scanned phosphoproteins relative to medium controls.

[c] Values in brackets represent p values obtained by the analysis of 20 phosphoproteins in the presence or absence of indicated stimulus by the sign test (two-tailed). NS, no significant difference in overall change for phosphorylation. -, not done.

induced by PMA and staphylococcal enterotoxins were not significantly different ($p > 0.5$, two-tailed sign test) while those induced by PMA and TSST-1 were significantly different ($p = 0.002$, two-tailed sign test).

4.2.3 Western Blot Analysis Of Tyrosine-Specific Phosphoproteins In Human Monocyte Cytosolic And Membrane Fractions With Anti-Phosphotyrosine Antibodies

To specifically investigate changes associated with tyrosine phosphorylation and dephosphorylation, tyrosine-containing phosphoproteins were detected by immunoblotting with the anti-phosphotyrosine mAb, 4G10. Initially, analysis of total monocyte cellular lysates by immunoblotting with 4G10 revealed no difference between untreated and TSST-1-treated cells when analysed by one-dimensional gel electrophoresis (data not shown). Therefore, cytosolic and membrane fractions from monocytes incubated with or without toxin were first separated and similarly analysed for tyrosine-specific phosphorylation. Results showed that TSST-1 but not SEA or PMA induced the tyrosine dephosphorylation of a 32 kd protein in the membrane fraction (indicated by arrow in Figure 21). PMA, in contrast, stimulated the tyrosine phosphorylation of a 40 kd and 99 kd phosphoprotein in the membrane fraction (indicated by arrows in Figure 21, lane 4). Interestingly, both TSST-1 and SEA induced the tyrosine phosphorylation of several phosphoproteins in the cytosolic fraction (molecular weights ranging from 18 kd to 35 kd as indicated by arrows in Figure 21). Although PMA also induced the tyrosine phosphorylation of several cytosolic proteins (molecular weights ranging from 15 kd to 35 kd), it had no effect on an 18 kd cytosolic protein (labeled "A" in Figure 21) which was tyrosine phosphorylated by TSST-1 and SEA. Conversely, a 22 kd cytosolic phosphoprotein (labeled "B" in Figure 21) that was tyrosine phosphorylated by TSST-1 and SEA was tyrosine dephosphorylated by PMA. Specificity of the anti-phosphotyrosine mAb was demonstrated by the abolition of immunoreactive bands in the presence of 2 mM phosphotyrosine (data not shown).

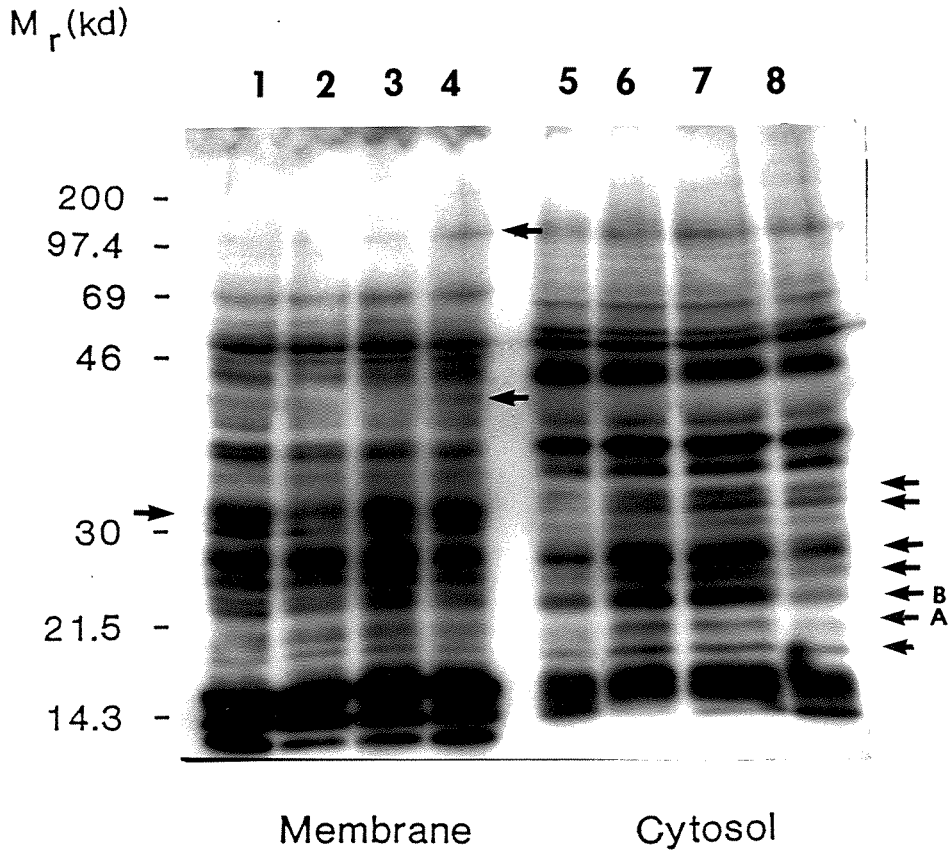

Figure 21. Tyrosine phosphorylation of monocyte subcellular fractions induced by TSST-1, SEA, or PMA. Human monocytes were treated with either RPMI 1640 medium control (lanes 1 and 5), 10 μg/ml TSST-1 (lanes 2 and 6), 10 μg/ml SEA (lanes 3 and 7) or 250 ng/ml PMA (lanes 4 and 8) for 5 min at 37°C before separation into cytosolic or membrane fractions as described in Materials and Methods. Cell fraction proteins were resolved using one-dimensional SDS-PAGE (14% gels), transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine mAb, 4G10. Protein-antigen complexes were detected with biotinylated goat anti-mouse IgG followed by incubation with ^{125}I-streptavidin. Proteins tyrosine phosphorylated or dephosphorylated by toxins or by PMA are indicated with dark arrows. Arrows marked "A" or "B" represent tyrosine phosphoproteins differentially affected by treatment of monocytes with toxins or PMA. Results are representative of 3 experiments.

4.3 Discussion

It is well-known that human monocytes are target cells for bacterial superantigens such as TSST-1 and the staphylococcal enterotoxins. However, the signal transduction mechanisms by which these superantigens activate human monocytes are poorly understood. As mentioned earlier, the reversible phosphorylation of proteins is an important mechanism by which intracellular events are controlled in eukaryotic cells and the phosphorylation state of a protein depends on the relative activities of the protein kinases or phosphatases at a given period (Cohen, 1989). Phosphorylation or dephosphorylation of proteins at serine, threonine, and tyrosine residues can trigger changes in protein conformation, thus altering their biological activity (Cohen, 1989). Furthermore, proteins can be phosphorylated at multiple sites; phosphorylation at a particular site may antagonize or amplify the effects of phosphorylation at other sites, or even the rates at which they are phosphorylated or dephosphorylated (Cohen, 1982).

In this study, early phosphorylation events were examined in TSST-1-, SEA- and SEB- treated human monocytes using a high resolution two-dimensional gel electrophoresis system. In view of the earlier observations that TSST-1 and staphylococcal enterotoxins bind to a common receptor but on distinct or overlapping epitopes on human monocytes and stimulate similar responses, it was of interest to determine whether these toxins share similar or dissimilar signal transduction pathways. Our data show that TSST-1 mainly induces the dephosphorylation of several monocyte cellular phosphoproteins (ranging in molecular weight from 16.5 kd to 170 kd) in a dose- and time-dependent manner. These dephosphorylation changes occur rapidly (ie. within 5 minutes after TSST-1 stimulation), suggesting that the toxin is affecting the turnover of phosphate groups rather than the degradation or down-regulation of the phosphoprotein itself. The dephosphorylation process does not appear to be readily reversible since phosphoproteins remained dephosphorylated for at least 1 h. These studies

indicate that rapid dephosphorylation of cellular phosphoproteins may represent an early and important event during the activation of human monocytes by TSST-1.

The present data also provide insight into the intracellular signal transduction pathways utilized by the related staphylococcal enterotoxins, SEA and SEB, during the activation of human monocytes. The level and time course of phosphorylation, and the similarities in phosphorylation substrates suggest that SEA and SEB may share common pathways of monocyte activation. However, the composite phosphorylation profiles revealed major differences between TSST-1 and the enterotoxins. Specifically, TSST-1 induced primarily dephosphorylation changes, while in the same donor monocytes, both SEA and SEB increased the phosphorylation of phosphoproteins. This finding was surprising considering that the staphylococcal enterotoxins share a number of biological properties with TSST-1 including TNF and IL-1 induction in human monocytes (Fast et al., 1989; Fischer et al., 1990; Ikejima et al., 1989; Parsonnet et al., 1985; Parsonnet and Gillis, 1988) and activation of T cell proliferation in a MHC class II-dependent manner (Fischer et al., 1989; Fleischer et al., 1989). Furthermore, TSST-1 and the staphylococcal enterotoxins both bind to the HLA-DR receptor on human monocytes (Fischer et al., 1989; Fraser, 1989; Scholl et al., 1989; See et al., 1992). TSST-1 does differ from enterotoxins in that it bears little structural similarity to these toxins and lacks a disulfide loop. Grossman et al. (1990) have reported that although the disulfide loop is important for T cell proliferation following induction by staphylococcal enterotoxins, its reduction and alkylation neither affect MHC class II binding nor monocyte stimulatory activity of these antigens. We have previously reported that TSST-1 and SEA bind to overlapping epitopes on the HLA-DR receptor on human monocytes (See et al., 1990; See et al., 1992) while others have demonstrated that TSST-1 and SEB bind to distinct epitopes on HLA-DR (Scholl et al., 1989). The differences in the overall composite phosphorylation patterns may be a reflection of the epitope specificity of the toxins for the HLA-DR receptor. It has been shown previously that MHC class II molecules play a role as signal transducers, particularly in B lymphocytes (Cambier and Lehman, 1989; Mooney et al., 1990). Studies have shown

that cross-linking MHC class II molecules on resting B cells results in a number of changes, including a rise in intracellular calcium, an increase in inositol trisphosphates, and tyrosine phosphorylation of cellular proteins (Lane et al., 1990; Mooney et al., 1990). Anti-HLA-DR mAbs, either in soluble form or covalently linked to Sepharose beads, were able to induce phosphorylation events in B lymphocytes (Mooney et al., 1990). Furthermore, anti-Ia antibodies have been found to induce protein kinase C translocation from the cytosol to the nucleus (Chen et al., 1987). Possibly, activation of a specific group of kinases or phosphatases may be coupled to binding of a particular epitope on HLA-DR by the staphylococcal toxins, resulting in different phosphorylation profiles. A molecule identified as the regulatory subunit of cAMP-dependent protein kinase has been shown to co-purify with I-A class II antigens (Newell et al., 1988). Whether cAMP-dependent protein kinases are involved in superantigen signal transduction in monocytes is highly speculative at this point. Whatever the mechanism of MHC class II signal transduction, it appears that binding of TSST-1 to a distinct epitope on HLA-DR results in the stimulation of unique kinases or phosphatases that differ from those activated by SEA or SEB, two enterotoxins whose epitope specificities on HLA-DR are more closely related to each other as demonstrated by receptor cross-competition studies (Fraser, 1989). Alternatively, the dissimilarity in the functional effects of TSST-1 and the staphylococcal enterotoxins may reflect unique properties of the toxins themselves rather than the functional heterogeneity of MHC class II epitopes (Moseley and Huston, 1991).

Our results also show that numerous monocyte cellular proteins are phosphorylated in response to LPS and the protein kinase C activator, PMA (Fig. 20 and Table X). However, the phosphorylation changes induced by LPS and PMA are different from those induced by TSST-1. Differences in phosphorylation profiles between TSST-1 and LPS are not unexpected since we will see in the next chapter that LPS and TSST-1 differ in their requirement for T lymphocytes for the production of cytokines (See et al., 1992). This suggests that the signal transduction pathways for monocyte activation by TSST-1 and LPS are different.

In addition to examining serine and threonine phosphorylation, tyrosine phosphorylation of monocyte cellular proteins was analysed by Western blotting. Phosphotyrosine proteins represent only 0.03% of all phosphorylated amino acids in the cell with phosphoserines and phosphothreonines accounting for the remainder (Cohen, 1982). However, the fact that the phosphotyrosine content of a cell can increase up to 10-fold during transformation by RNA tumour viruses and that binding of many known ligands to their respective receptors results in tyrosine phosphorylation suggest that tyrosine phosphorylation is an important signal transduction mechanism for mediating cellular responses (Cohen, 1982; Hunter, 1987). The present study shows that TSST-1 and SEA activated tyrosine phosphorylation of several cytosolic proteins in human monocytes. The phosphorylation process was rapid, occurring within 5 min of toxin stimulation. Additionally, our data suggest that TSST-1- and SEA-induced tyrosine phosphorylation did not appear to involve protein kinase C, since a different set of monocyte cellular proteins was tyrosine phosphorylated by PMA. Furthermore, several proteins that were tyrosine phosphorylated or dephosphorylated in response to PMA did not respond to TSST-1 or SEA. Recently, increased tyrosine phosphorylation of a number of proteins from a murine macrophage cell line in response to E. coli LPS has been reported (Weinstein et al., 1991). Tyrosine phosphorylation of these macrophage proteins by LPS also appears to be independent of protein kinase C. In this study, the effects of LPS on tyrosine phosphorylation of human monocyte cellular proteins were not examined.

At present, neither the identity of the monocyte cellular proteins dephosphorylated or phosphorylated by TSST-1, SEA or SEB, nor the kinases and/or phosphatases involved are known. The phosphorylation results of Figures 17 through 20 as well as the tyrosine phosphorylation profiles in Figure 21 indicate that TSST-1 may be activating a number of serine/threonine and tyrosine phosphatases and kinases. TSST-1 does not appear to stimulate protein kinase C activity in the early stages of monocyte activation since the phosphorylation profiles of PMA- and TSST-1-stimulated monocytes are quite different. However, this does not preclude the role of protein kinase C in downstream events of

monocyte activation by this toxin. The lack of protein kinase C involvement in the early stages of monocyte activation by TSST-1 does contrast with results observed in human T cells. TSST-1 has been shown to stimulate protein kinase C activity as well as to induce inositol phospholipid turnover within 2 minutes after the addition of the toxin to purified T cells (Chatila et al., 1988).

Staphylococcal enterotoxins, on the other hand, may phosphorylate human monocyte cellular proteins by activating various kinases (serine/threonine or tyrosine) or by blocking the activity of phosphatases or both. Although there were marked differences between the composite phosphorylation profiles of TSST-1 and the staphylococcal enterotoxins, the similarities in biological properties (eg. cytokine induction) do suggest that subsequent downstream intracellular signals eventually converge to produce the same biological response. Therefore, these phosphorylation studies should be taken as to reflect early events in monocyte activation, which may be quite different from later events. Nevertheless, it is intriguing that binding of the same receptor on monocytes by these toxins result in different phosphorylation profiles.

In summary, our data provide strong evidence that the biological effects of TSST-1, SEA or SEB on human monocytes may be mediated through modulating the activities of protein kinases and/or phosphatases. The relevance of these phosphorylation changes to specific biological effects will not be known until the phosphoprotein substrates or their respective kinases or phosphatases are further characterized. Moreover, additional studies will be required to determine how these toxins activate human monocytes by different mechanisms yet still induce the same biological responses.

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Chapter 5

Mechanism Of Cytokine Induction In Human Peripheral Blood Monocytes By TSST-1

5.1 Introduction

As mentioned in Chapter 1, TSST-1 and the staphylococcal enterotoxins are strong inducers of cytokines such as IL-1, TNF, IFN-τ, and IL-2 from human peripheral blood mononuclear cells. The induction of these cytokines by TSST-1 may have important relevance in TSS pathogenesis since toxic shock-like symptoms (eg. fever, hypotension, etc.) are observed in rabbits administered with purified TNF and/or IL-1 (Ikejima et al., 1989; Okusawa et al., 1988) as well as in human cancer patients receiving IL-2 therapy (Rosenberg et al., 1988). Furthermore, the pathogenesis of Gram-negative septic shock is now known to be mediated by TNFα released by endotoxin-responsive macrophages (Beutler and Cerami, 1988).

Since these cytokines are considered important in the mode of action of TSST-1, an understanding of the stimulatory mechanisms of their production is essential. Although monocytes and macrophages are thought to be the major producers of TNF and IL-1 in response to TSST-1, little is known about the involvement of other cell types (eg. T lymphocytes) in cytokine induction. Most reports describing cytokine production by TSST-1 have used either unfractionated human peripheral blood mononuclear cells or plastic adherence-purified monocytes (de Azavedo et al., 1988; Fast et al., 1989; Parsonnet and Gillis, 1988). In the latter instance, the purity of human monocytes is usually 70 to 90% (Rosenberg et al., 1981) and the potential of other contaminating cells supporting cytokine production cannot be ruled out. The use of highly purified cell fractions in cytokine studies becomes even more critical since two reports have recently demonstrated that SEA induction of IL-1 and TNF secretion in human monocytes requires the presence of T lymphocytes (Fischer et al., 1990; Gjorloff et al., 1991). Whether this phenomenon is unique to SEA or common to other toxins like TSST-1 will be a focus of this chapter. Additionally, as will be examined in this

section, the purity of the TSST-1 preparation may determine whether T cells are required for induction of cytokines in human monocytes by TSST-1.

This chapter will also investigate how T cells contribute to cytokine production by human monocytes stimulated with TSST-1. The interaction between these cells may occur by direct cell-cell contact or by the release of soluble mediators such as IFN-τ. T lymphocytes have previously been shown to secrete IFN-τ in response to TSST-1 stimulation (Jupin et al., 1988; Micusan et al., 1989). This lymphokine is known to act on macrophages by enhancing microbicidal and tumoricidal activity as well as upregulating HLA-DR antigen expression in these cells (Becker, 1984; Nathan et al., 1983). In addition, the secretion of TNF and IL-1 by blood monocytes is higher when these cells are primed with IFN-τ prior to LPS stimulation (Beutler and Cerami, 1988). A possible reason for the requirement of T cells in TSST-1-induced cytokine production by monocytes might be their secretion of IFN-τ, which then might prime monocytes for TSST-1 activation.

Aside from analysing the role of soluble mediators, the role of the lymphocyte function-associated antigen-1 (LFA-1) adhesion molecule in cytokine induction by TSST-1 will also be examined. In general, stimulation of T lymphocytes occurs as a result of interaction of the specific TCR with the MHC class II antigen complex. This interaction is additionally stabilized by the binding of either the CD4 molecule or CD8 molecule to class II or class I antigens, respectively (Meuer et al., 1982; Meuer et al., 1982). Further adhesion is mediated through the binding of CD2 to the lymphocyte function-associated antigen-3 (LFA-3) and the binding of LFA-1 to its counterpart receptors, intercellular adhesion molecule-1 (ICAM-1) and ICAM-2 (Berzins et al., 1988; Schwartz et al., 1989; Spits et al., 1986; Springer, 1990). The interaction of an antigen with the TCR is coordinated with increased cell-cell adhesiveness and this is important for T cell immune recognition by the antigen (Dustin and Springer, 1989). LFA-1, commonly found on T lymphocytes and antigen-presenting cells, increases its avidity for ICAM-1 and ICAM-2 following activation of the TCR (Dustin and Springer, 1989). Therefore, there is the possibility that monocyte-T cell interaction in response to TSST-1

stimulation may be mediated in part by the LFA-1 molecule. In this study, the effects of LFA-1 on cytokine production by TSST-1-activated human monocytes will be addressed using antibodies directed against the alpha and beta chain of LFA-1.

Specific Aims

1) To determine whether T lymphocytes are required for the production of IL-1 and TNF by monocytes in response to TSST-1 stimulation.
2) To examine whether the T cell requirement for cytokine production in human monocytes stimulated with TSST-1 can be replaced by IFN-τ.
3) To determine whether TSST-1-stimulated cytokine production requires direct cell contact between monocytes and T lymphocytes.
4) To determine the role of the adhesion molecule, LFA-1, in mediating monocyte-T cell interaction necessary for cytokine production.
5) To determine whether the anti-HLA-DR mAb, L243, can block induction of cytokines by TSST-1.
6) To compare a commercial TSST-1 preparation with our own highly purified TSST-1 in the stimulation of cytokine secretion from human monocytes in the presence or absence of T lymphocytes.

5.2 Results

5.2.1 Assays For The Measurement Of TNF And IL-1 Levels In Culture Supernatants Of Cells Stimulated With TSST-1

TNF levels were measured by a combination of the L929 murine fibroblast cytotoxicity assay and an ELISA method which I developed specifically for TNFα. The quantitation of IL-1ß was determined by an IL-1ß-specific ELISA which I developed in a similar manner to the TNFα ELISA. The specificity of the L929 fibroblast cytotoxicity assay was demonstrated by using goat anti-human TNFα or -ß antibodies to neutralize the activity. TSST-1 itself showed no cytotoxic effects on L929 cells (data not shown). A typical standard curve for the bioassay

is given in Figure 22. A strong correlation was found between the L929 cytotoxicity assay and the TNFα ELISA (Spearman rank correlation coefficient = 0.84, $p < 0.01$). The ELISAs for TNFα and IL-1ß were sensitive down to 62 pg/ml (Figures 23 and 24) and demonstrated no cross-reactivities to heterologous cytokines, as described in Materials and Methods.

5.2.2 Requirement Of T Cells For Cytokine Production By Human Monocytes Stimulated With TSST-1

TSST-1 purified to homogeneity from the culture supernatants of S. aureus MN8 by preparative isoelectric focusing and chromatofocusing were used in these studies. To determine whether T lymphocytes play a role in cytokine secretion, highly purified human monocytes (>90%), alone or with T lymphocytes (1:1 ratio), were incubated with TSST-1 for 24 h at 37°C. Results show that no TNF activity could be detected in monocytes or T cell cultures incubated for 24 h with 10 μg/ml TSST-1 (Figure 25). However, when 10 μg/ml TSST-1 was added to a 1:1 mixture of monocytes and T cells, a significant increase in TNF cytotoxic activity was observed compared with RPMI 1640 medium controls (Figure 25). The nature of the TNF cytotoxic activity was investigated using antibodies directed against the alpha or beta form of TNF. The cytotoxic activity in culture supernatants of TSST-1-stimulated monocytes and T cells was neutralizable by preincubation with antibodies to TNFα but not to TNFß prior to testing in the bioassay (Figure 25). Comparable results were also found using an ELISA to measure TNFα levels in culture supernatants. Figure 26 shows that TSST-1 did not induce TNFα secretion into the medium unless T cells were added to monocytes in a 1:1 ratio. In contrast, LPS was able to stimulate TNFα release from human monocytes in the absence of T cells (Figure 26). The TNFα activity was neutralized by incubating LPS with polymyxin B sulphate (Figure 26). Dose-response studies showed that TSST-1 was very potent in stimulating TNFα secretion from monocytes in the presence of T lymphocytes (Figure 27). As little as 0.1 ng/ml of toxin was active in inducing cytokine secretion. Time course studies show that stimulation of monocytes alone for up to 72 h with 10 μg/ml TSST-1 did

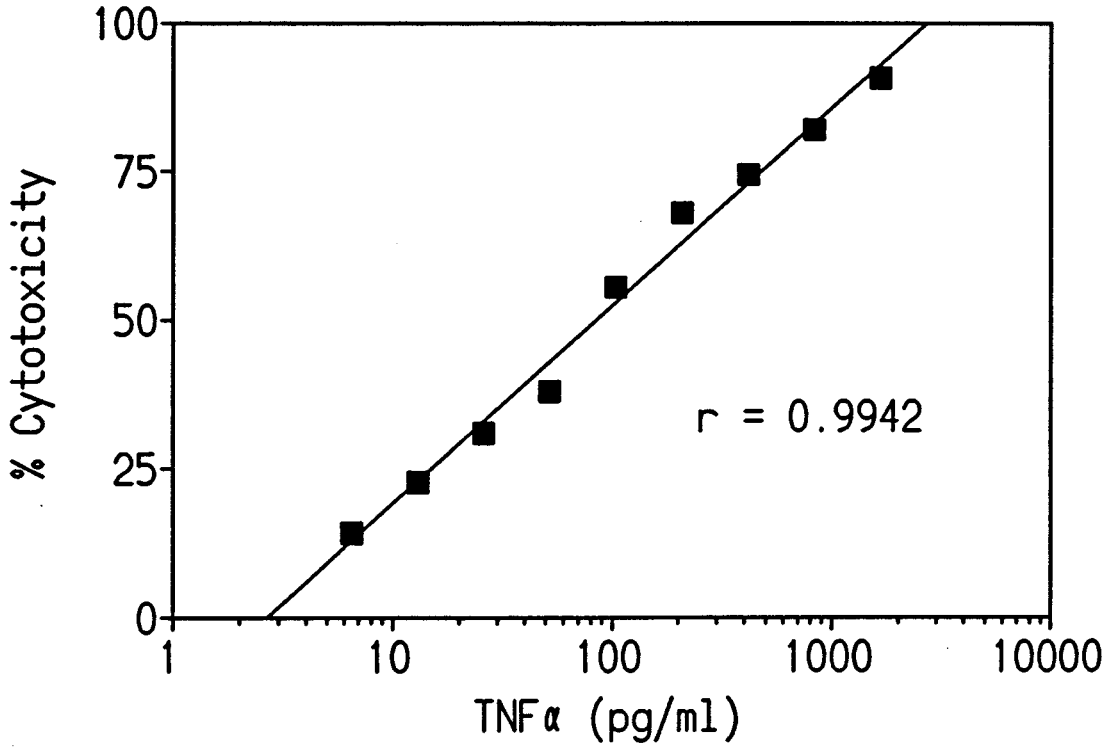

Figure 22. Representative standard curve of L929 fibroblast cytotoxicity assay for TNF. Cells were treated with human TNFα for 20 h at 37°C as decribed in Materials and Methods in Chapter 2. The correlation coefficient (r) of the assay is 0.9942.

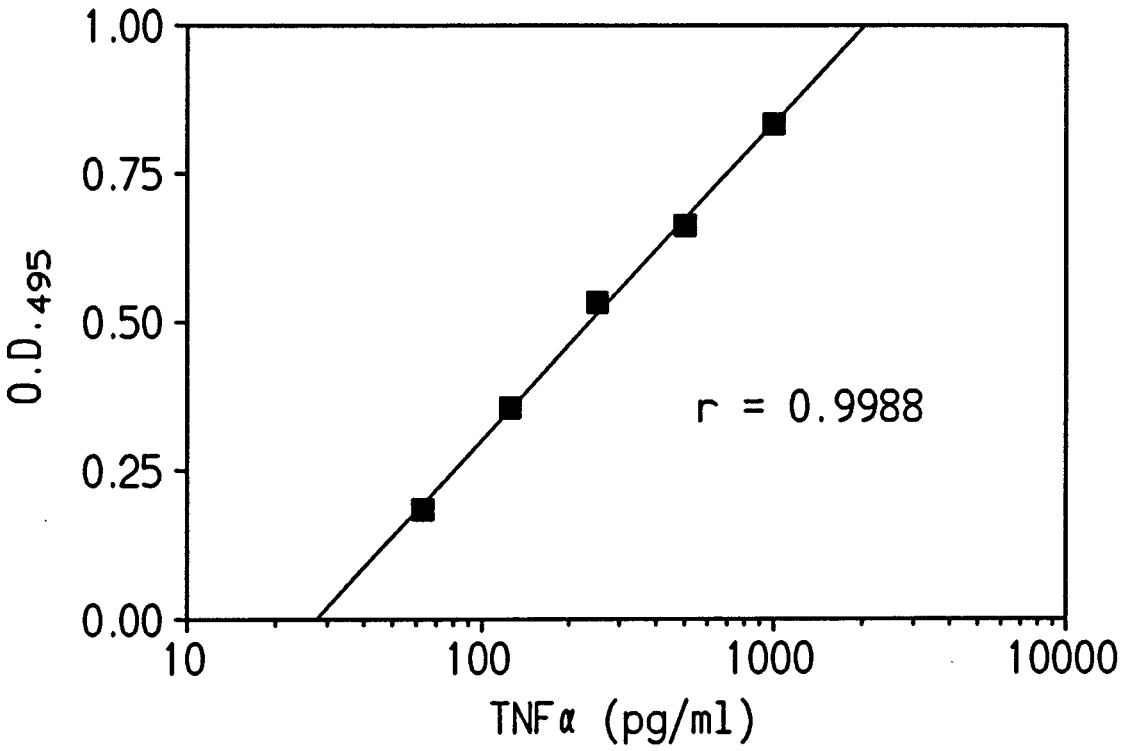

Figure 23. Representative standard curve for developed TNFα ELISA. Correlation coefficient (r) of assay was 0.9988.

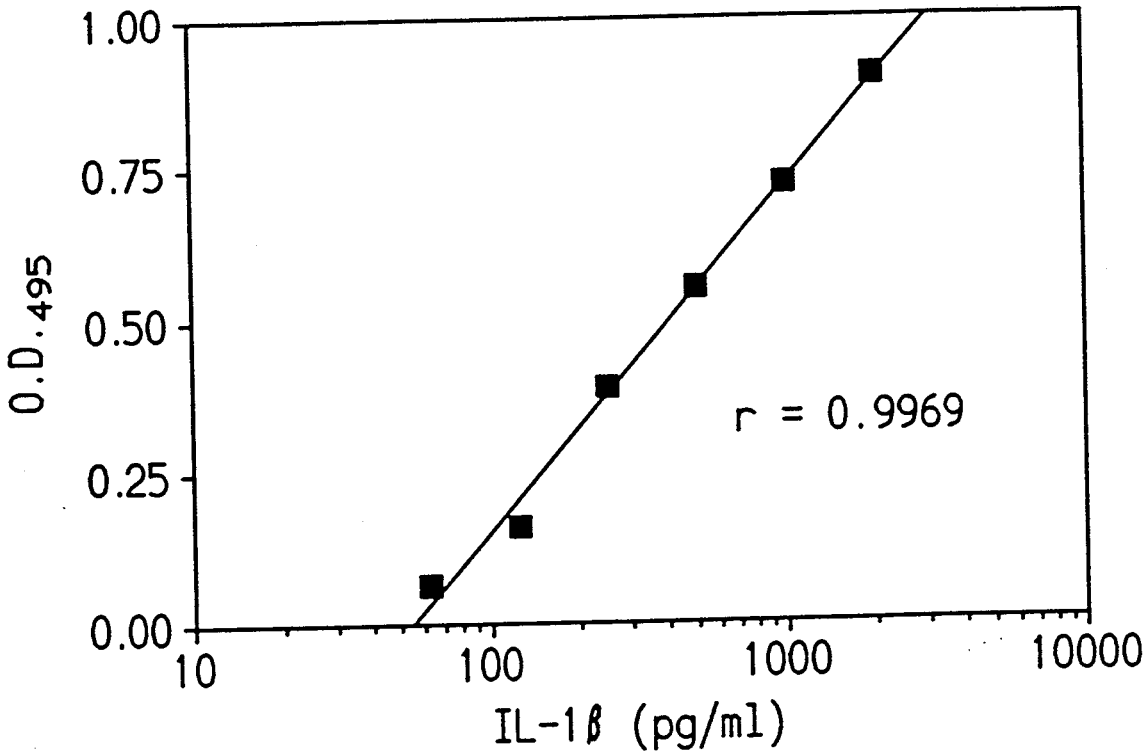

Figure 24. Representative standard curve for developed IL-1ß ELISA. Correlation coefficient (r) was 0.9969.

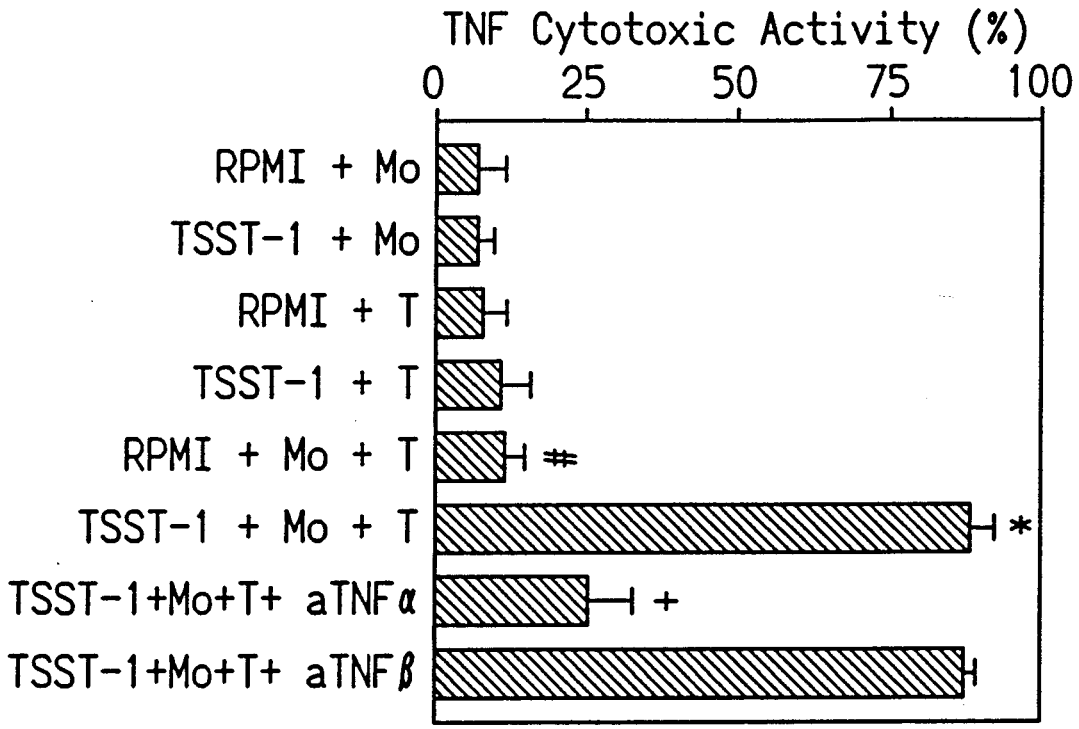

Figure 25. Production of TNF by human monocytes and T cells in response to TSST-1. Human monocytes (Mo, 1 x 10^6 cell/ml) or T cells (T, 1 x 10^6 cells/ml), alone or co-cultured 1:1, were incubated with 10 μg/ml toxin for 24 h at 37°C. Supernatants were collected and assayed for TNF by the L929 fibroblast cytotoxicity assay. For neutralization experiments, supernatants were incubated with 10 μg/ml goat anti-human TNFα (aTNFα) or anti-human TNFß (aTNFß) for 2 h prior to assay for TNF activity. The results represent the mean ± SEM for 6 separate donors. TNF production induced by TSST-1 in the presence of monocytes and T cells (*) is of significance compared with RPMI controls (#) [$P < 0.01$, one-tailed Wilcoxon's signed rank test]. (+) indicates neutralization of activity by anti-TNFα is of significance compared to TSST-1 positive control levels (*) [$P < 0.05$, one-tailed Wilcoxon's signed rank test].

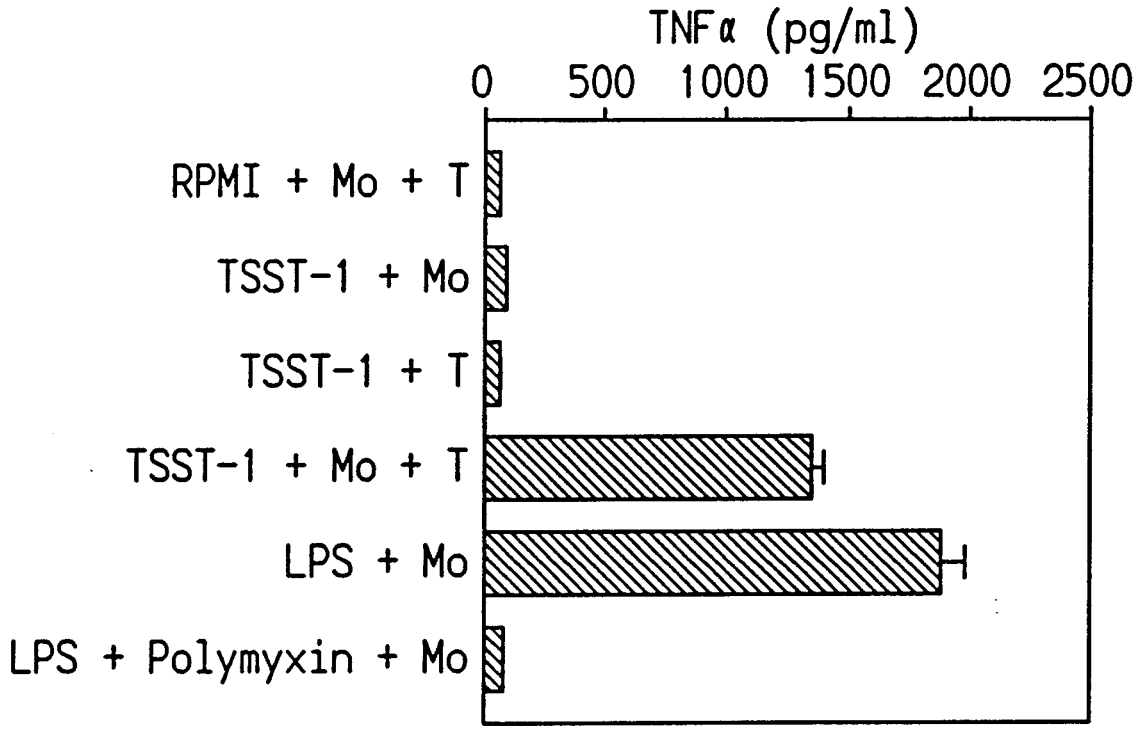

Figure 26. Comparison of TSST-1- and LPS-induced TNFα secretion by human monocytes and T cells. Monocytes (1 x 10^6 cells/ml) or T cells (1 x 10^6 cells/ml), alone or co-cultured 1:1, were incubated with 10 μg/ml TSST-1 for 24 h at 37°C. Monocytes alone were also incubated with 1 μg/ml E. coli 026:B6 LPS in the absence or presence of 10 μg/ml polymyxin B sulphate. TNFα levels in culture supernatants were measured by ELISA as described in Materials and Methods. Results (mean ± SEM) are from one representative donor studied in triplicate experiments.

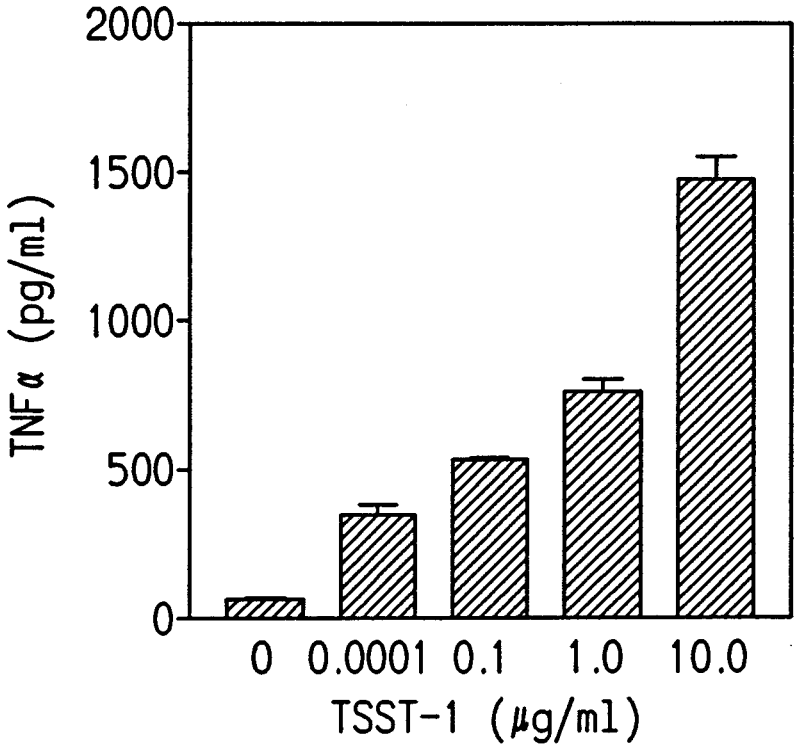

Figure 27. The effect of different concentrations of TSST-1 on TNFα secretion. Human monocytes and T cells, co-cultured 1:1, were stimulated with TSST-1 for 24 h at 37°C. TNFα levels in culture supernatants were measured by TNFα ELISA. Each bar represents the mean ± SEM for one of three representative donors.

not induce the secretion of TNFα whereas LPS was able to do so at 24 h (Figure 28).

To determine whether induction of another cytokine, IL-1ß, also involved both cell types, monocytes or T cells, alone or mixed 1:1, were incubated with TSST-1 for 24 h. Results indicate that IL-1ß induction by TSST-1 occurred only in the presence of both cell types, and that monocytes stimulated with TSST-1 in the absence of T lymphocytes could not produce this cytokine (Figure 29). Furthermore, the ratio of T cells:monocytes seemed to be important in TSST-1-stimulated IL-1ß and TNFα production since a T cell:monocyte ratio of 0.1:1 or a monocyte:T cell ratio of 0.1:1 considerably reduced cytokine production by these cells (data not shown).

5.2.3 The Role Of IFN-τ In TSST-1-Induced Cytokine Production By Human Blood Monocytes

The hypothesis that IFN-τ might substitute for the requirement of T cells was addressed directly by stimulating human monocytes with IFN-τ prior to TSST-1 activation or indirectly by adding anti-IFN-τ antibodies to monocyte/T cell cultures in the presence of TSST-1. Figure 30 shows that pretreatment of human monocytes with 200 U/ml IFN-τ for 24 h at 37°C did not induce extracellular TNFα production after TSST-1 stimulation. However, in the same experiment, priming of monocytes with IFN-τ did enhance TNFα secretion in response to LPS stimulation (Figure 30). IFN-τ alone did not induce TNF immunoreactivity in the culture supernatants. Furthermore, when 10 μg/ml rabbit anti-human IFN-τ was added to TSST-1-stimulated monocyte/T cell cultures, no inhibition of TNFα levels was noted compared with positive controls (Figure 31). Instead, a borderline increase in TNFα secretion was observed, possibly due to uptake of immune complexes of IFN-τ and its antibody by monocytes.

5.2.4 Role Of Monocyte-T Cell Contact In Cytokine Induction By TSST-1

To further investigate the nature of the monocyte/T cell interaction for cytokine induction by TSST-1, experiments were performed to determine whether

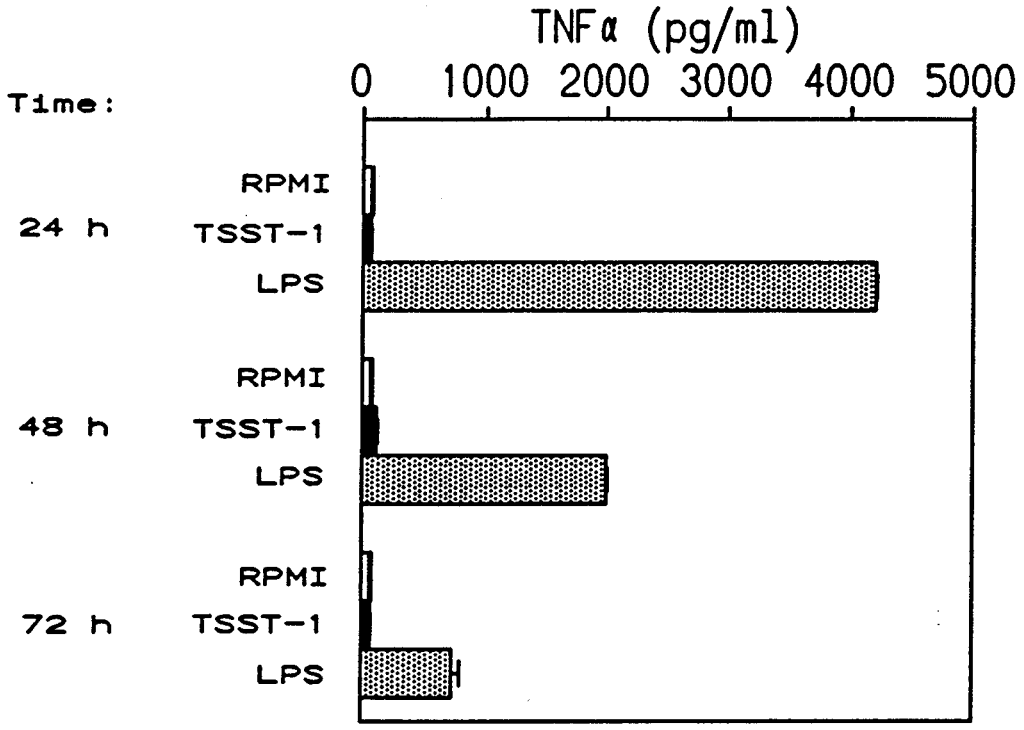

Figure 28. Kinetics of TNFα secretion by TSST-1- or LPS-treated human monocytes alone. 1 x 10^6 monocytes were exposed to 10 μg/ml TSST-1 or 1 μg/ml E. coli 026:B^6 LPS. Culture supernatants were harvested at the indicated times and assayed for TNFα by ELISA. No TNFα was detected when monocytes were exposed to RPMI 1640 medium alone. Each bar represents the mean ± SEM of triplicate experiments for one representative donor.

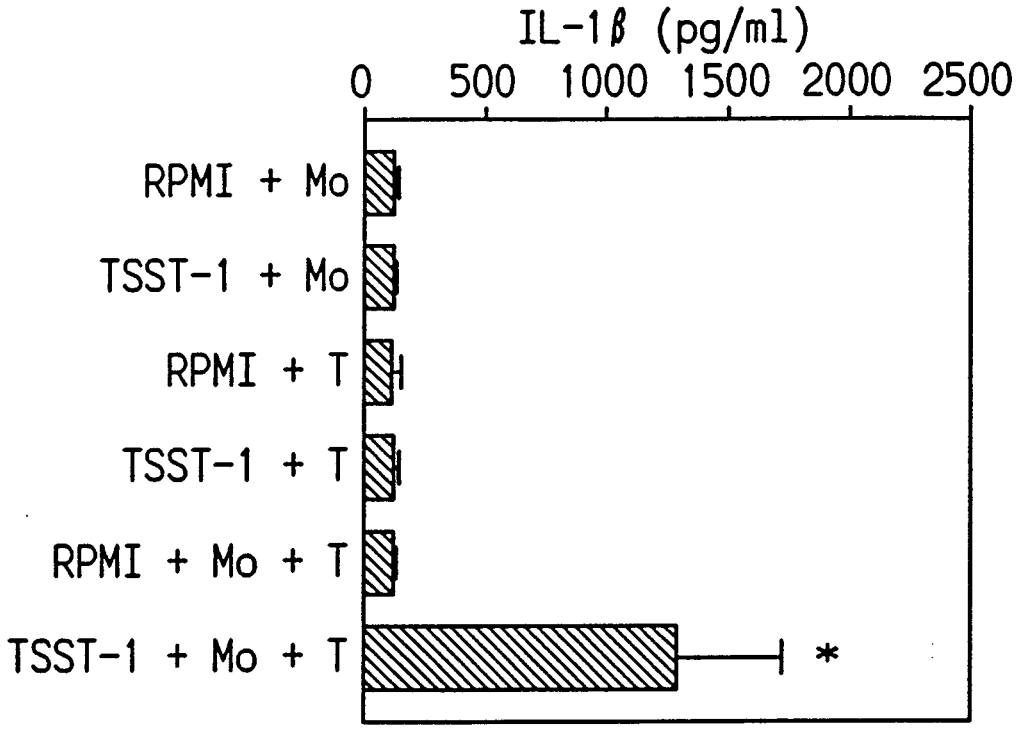

Figure 29. Induction of IL-1ß in human monocytes by TSST-1 in the presence or absence of T cells. Monocytes (1 x 10^6 cells/ml), co-cultured with T cells in a 1:1 ratio, were stimulated with 10 μg/ml TSST-1 for 24 h at 37°C. Supernatants were assayed for IL-1ß by an ELISA as described in Materials and Methods in Chapter 2. (*) indicates significant difference compared to RPMI 1640 medium control ($P < 0.01$, one-tailed Wilcoxon's signed rank test). Results represent the mean ± SEM for 7 separate donors.

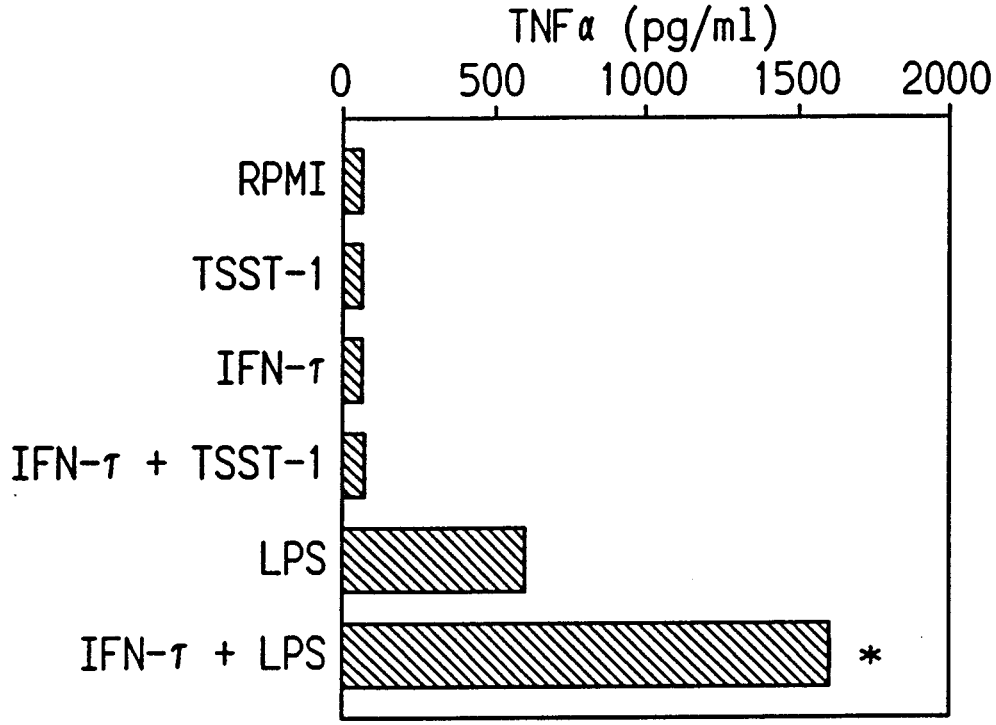

Figure 30. Effect of IFN-τ treatment on TNFα secretion by TSST-1- or LPS-treated human monocytes. 1 x 10^6 monocytes were incubated with or without 200 U/ml IFN-τ for 24 h at 37°C. Cells were washed extensively with RPMI 1640 medium and then exposed to 10 μg/ml TSST-1 or 1 μg/ml E. coli 026:B6 LPS for 24 h at 37°C. Culture supernatants were then harvested and assayed for TNFα by ELISA. (*) denotes significant difference ($P < 0.05$, one-tailed Wilcoxon's signed rank test) compared to LPS alone. Results are representative of two separate experiments.

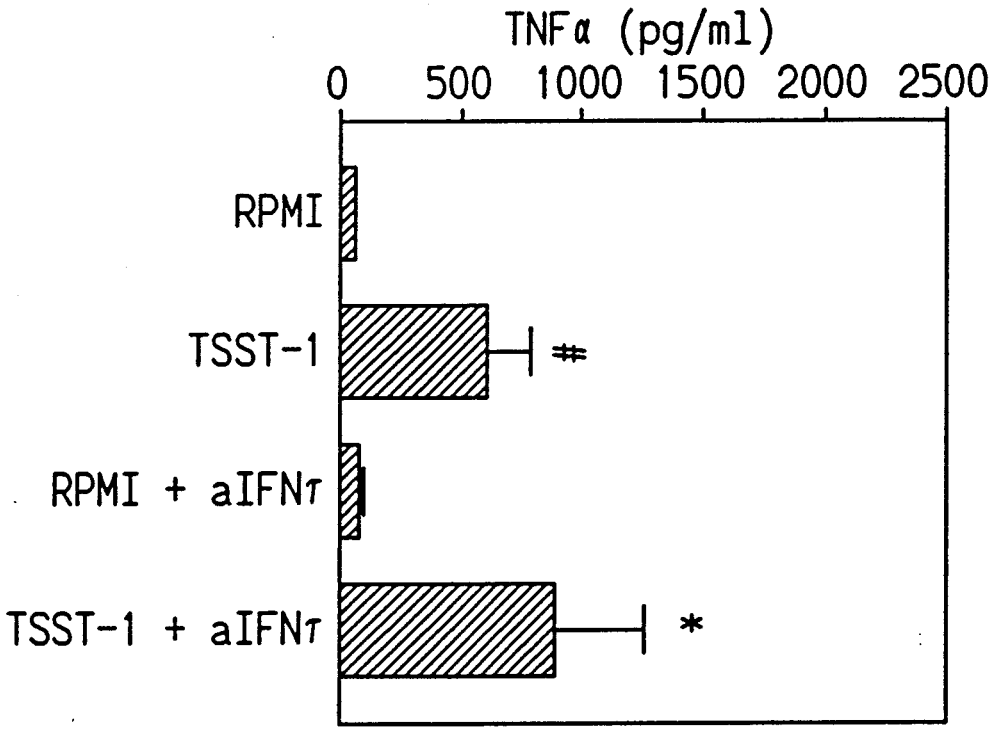

Figure 31. Effect of anti-IFN-τ antibodies on TSST-1-induced TNFα secretion by co-cultures of monocytes and T lymphocytes. A 1:1 ratio of monocytes and T cells (1 x 10^6 cells each) was preincubated with 10 μg/ml rabbit anti-human IFN-τ antibodies for at least 1 h prior to the addition of 10 μg/ml TSST-1 for 24 h at 37°C. Each bar represents the mean ± SEM for 5 separate donors. (*) denotes a borderline significant increase in TNFα levels (P = 0.06, one-tailed Wilcoxon's signed rank test) compared to TSST-1 controls (#).

cell-cell contact was necessary. A Transwell chamber insert was used to physically separate monocytes from T cells but still allow the diffusion of TSST-1 and soluble cytokines through its 0.45 μm membrane. Results show that TSST-1 did not induce TNFα release when monocytes were physically separated from T cells (Figure 32). The abolition of cytokine secretion occurred regardless of whether monocytes were placed in the upper or lower chamber away from the T lymphocytes (data not shown). In contrast, monocytes in contact with T cells produced large amounts of TNFα, indicating that contact between these cell types was required for cytokine secretion (Figure 32).

5.2.5 Effect Of Cell Fixation On The Induction Of TNFα Secretion By TSST-1

Although the double chamber culture experiments indicated that contact between monocytes and T cells was important, there was still the possibility that metabolic activity of one cell type might be important for the production of cytokines by the other cell type. To address this question, either monocytes or T cells were metabolically inactivated by paraformaldehyde-fixation and reconstituted with viable counterpart cell types. Results show that fixing either monocytes or T cells almost completely abolished TSST-1-induced TNFα secretion by the viable cells (Figure 33), suggesting that in addition to monocyte-T cell contact, both cell types must also be metabolically active for TNFα production to occur.

5.2.6 Role Of The Lymphocyte Function-Associated (LFA-1) Adhesion Molecule In Mediating TSST-1-Induced Monocyte-T Cell Interaction

The role of the LFA-1 adhesion molecule in cytokine production by TSST-1-stimulated blood cells was addressed by using mAbs directed against the LFA-1 alpha chain (anti-CD11a) or the LFA-1 beta chain (anti-CD18). Before stimulation with TSST-1, co-cultures of monocytes and T cells were incubated for 2 h at 37°C with 10 μg/ml anti-CD11a or anti-CD18. Anti-CD14 mAb was used as a negative control. After toxin stimulation, the culture supernatants were assayed for TNFα or IL-1ß by ELISA. Figure 34 shows the induction of TNFα in human monocytes and

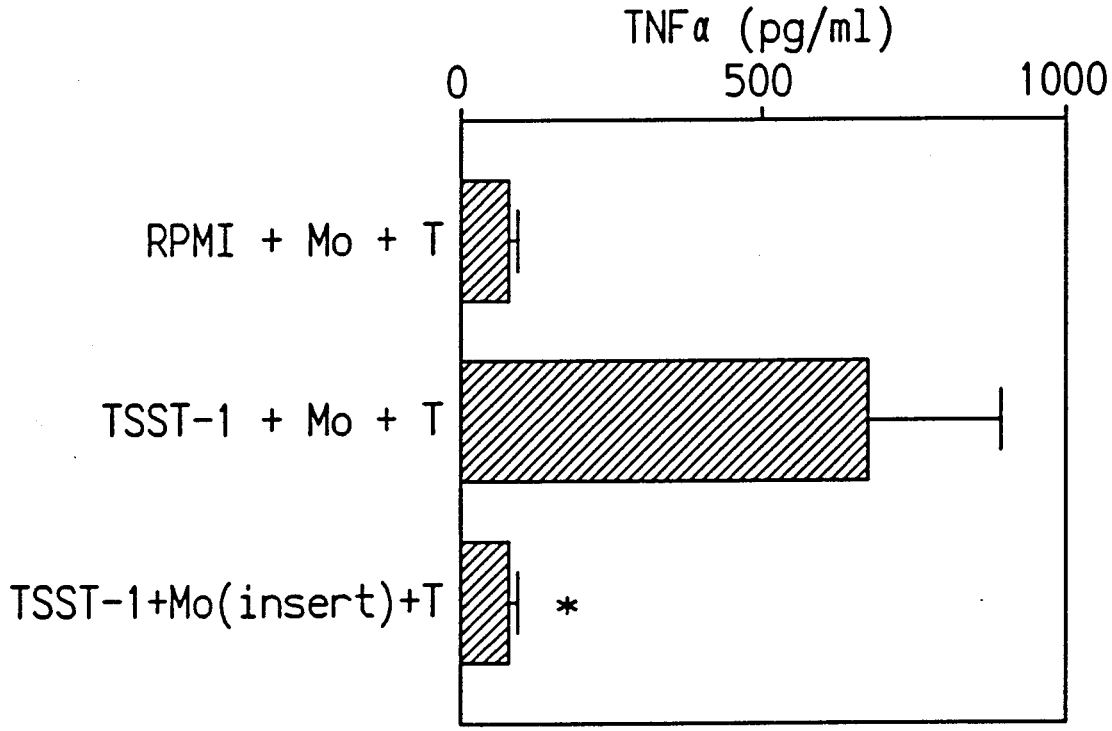

Figure 32. Requirement for monocyte-T cell contact for the induction of TNFα by TSST-1. Monocytes and T cells, mixed together in a 1:1 ratio, or separated by a Transwell insert containing a 0.45 μm membrane were cultured with 10 μg/ml TSST-1 for 24 h at 37°C. Supernatants were assayed for TNFα by ELISA. Each bar represents the mean ± SEM for 5 separate donors. (*) indicates significant difference ($P < 0.05$, one-tailed Wilcoxon's signed rank test) compared with supernatants of unseparated monocytes/T cells stimulated with TSST-1.

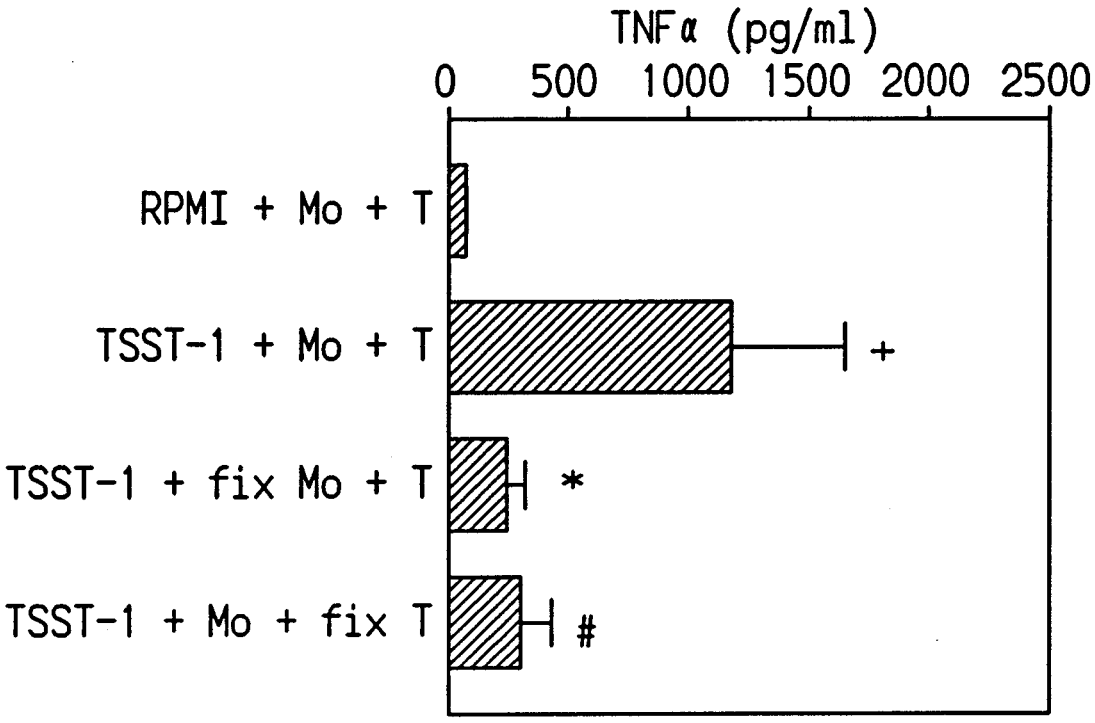

Figure 33. Effect of fixation of human monocytes or T cells in the induction of TNFα by TSST-1. Monocytes or T cells were metabolically inactivated by fixing with 4% paraformaldehyde and added in a 1:1 ratio (1×10^6 cells/ml) to viable T cells or monocytes, respectively. Cells were stimulated with 10 μg/ml TSST-1 for 24 h at 37°C and the supernatants assayed for TNFα by ELISA. Results represent the mean ± SEM for 7 donors. Significant difference in TNFα levels were found for TSST-1/fixed monocytes/T cells (*) and positive control (+) ($P < 0.01$, one-tailed Wilcoxon's signed rank test), and for TSST-1/monocytes/fixed T cells (#) and positive control (+) ($P < 0.01$, one-tailed Wilcoxon's signed rank test).

T lymphocytes with or without anti-LFA-1 and control mAbs among 12 donors studied. TSST-1 in the absence of mAb induced TNFα levels of 1210 ± 212 pg/ml (mean ± SEM). Anti-CD11a or anti-CD18, each at 10 μg/ml, significantly inhibited TNFα secretion compared with TSST-1 alone (612 ± 176 pg/ml, $p<0.0025$; and 830 ±185 pg/ml, $p<0.02$, respectively). The control mAb, anti-CD14, had no effect (1460 ± 269 pg/ml). The combination of anti-CD11a and anti-CD18 was also inhibitory compared with TSST-1 alone (507 ± 118 pg/ml, $p<0.005$), but the percent inhibition was not significantly greater than either mAb alone (54.3 ± 8.5% inhibition, vs 48.9 ± 4.6% and 31.4 ± 8.7%, respectively). Figure 35 shows the induction of IL-1ß with or without anti-LFA-1 and control mAbs among 7 donors studied. TSST-1 in the absence of mAb induced IL-1ß levels of 2039 ± 912 pg/ml. Anti-CD11a and anti-CD18, each at 10 μg/ml, significantly inhibited IL-1ß secretion compared with TSST-1 alone (468 ± 183 pg/ml, $p<0.025$; and 545 ± 205 pg/ml, $p<0.025$, respectively). The control mAb, anti-CD14, again had no effect (1277 ± 557 pg/ml, $p = 0.18$). The combination of anti-CD11a and anti-CD18 also significantly inhibited IL-1ß secretion compared with TSST-1 alone (226 ± 63 pg/ml, $p<0.025$); furthermore, the percent inhibition was significantly greater than with either mAb alone [77.8 ± 5.3% inhibition, vs. 66.6 ± 7.6% ($p<0.05$, one-tailed) and 56.4 ± 10.9% ($p<0.05$, one-tailed), respectively]. Anti-CD 11a or anti-CD18 mAb in the absence of TSST-1 did not induce the release of either cytokine (data not shown).

5.2.7 Role Of The HLA-DR Antigen In TSST-1-Induced Cytokine Production

The anti-HLA-DR mAb L243, which was shown in Chapter 3 to block binding of TSST-1 to the HLA-DR receptor on human monocytes as well as inhibit TSST-1-stimulated T cell proliferation, was preincubated with co-cultures of monocytes and T cells for 2 h at 37°C prior to TSST-1 stimulation. Surprisingly, there was no inhibition of TNFα secretion by L243 even at concentrations of 100 μg/ml of the mAb (Figure 36). L243 in the absence of TSST-1 did not stimulate TNFα secretion from the monocyte-T cell cultures (Figure 36).

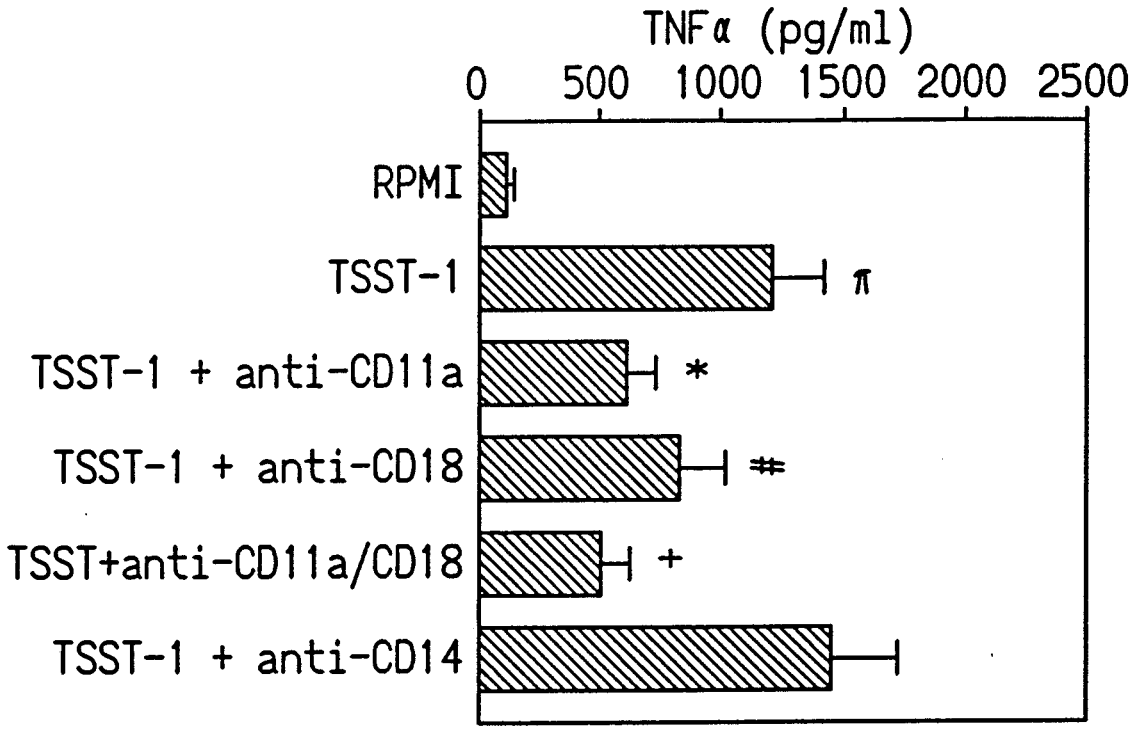

Figure 34. Effect of mAb to LFA-1 on TNFα release by TSST-1-stimulated human blood cells. A 1:1 culture of monocytes and T cells (1 x 10^6 cells) in RPMI 1640 medium was treated with 10 μg/ml of either anti-CD11a, anti-CD18, both, or the control mAb anti-CD14 for 2 h at 37°C before stimulation with 10 μg/ml TSST-1 for 24 h at 37°C. Culture supernatants were harvested and TNFα levels measured by ELISA. Each bar represents the mean ± SEM for 12 donors. Significant differences, as determined by the one-tailed Wilcoxon's signed rank test, were found for anti-CD11a (*) ($p < 0.002$), anti-CD18 (#) ($P < 0.02$), anti-CD11a/anti-CD18 (+) ($P < 0.005$) compared with TSST-1 control (π).

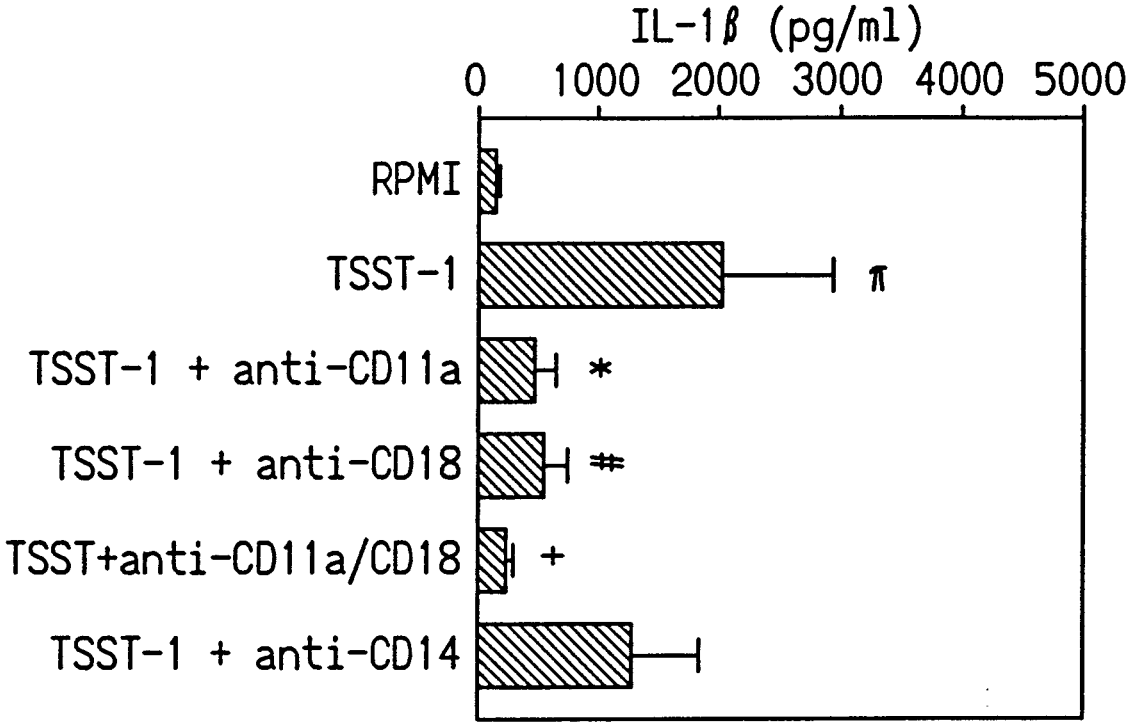

Figure 35. Effect of anti-LFA-1 mAbs on IL-1ß secretion by TSST-1 stimulated blood cells. A 1:1 culture of monocytes and T cells (1 x 10^6 cells) in RPMI 1640 medium was incubated with 10 μg/ml of various mAbs for 2 h before stimulation with 10 μg/ml TSST-1. Culture supernatants were assayed for IL-1ß by ELISA. Results represent the mean ± SEM for 7 donors. Significant differences, as determined by the one-tailed Wilcoxon's signed rank test, were found for anti-CD11a (*) ($P < 0.025$), anti-CD18 (#) ($P < 0.025$), and anti-CD11a/anti-CD18 (+) ($p < 0.025$), compared with TSST-1 control (π).

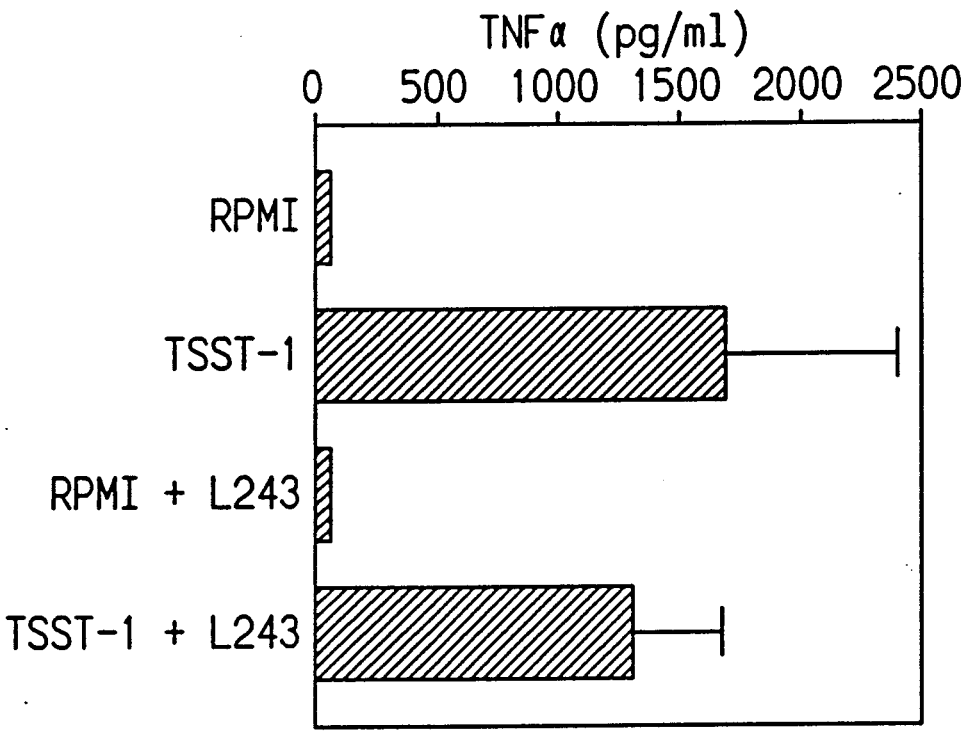

Figure 36. Effect of L243 mAb on TNFα induction in human monocytes and T cells by TSST-1. Monocytes co-cultured with T cells were pretreated with 100 μg/ml anti-HLA-DR mAb for at least 1 h prior to the addition of 10 μg/ml TSST-1 for 24 h at 37°C. TNFα levels in culture supernatants were determined by ELISA. No significant difference was found for TSST-1-treated monocyte/T cells in the presence or absence of L243.

5.2.8 Morphologic Changes In Monocytes Exposed To TSST-1 In The Presence Of T Lymphocytes

Monocytes co-cultured with T lymphocytes in RPMI 1640 medium did not show any signs of unusual morphologic changes (Figures 37A). However, when TSST-1 was added to the co-cultures for 24 h, monocytes began to differentiate into macrophages after 24 h. The differentiated cells, with their elongated shapes, resembled fibroblasts in morphology (Figure 37B). T lymphocytes did not appear to be affected. The morphologic changes of monocytes remained stable for up to a week after the removal of TSST-1 and replacement with RPMI 1640 medium alone (data not shown). No morphologic changes were noted in monocytes alone treated with TSST-1 (Figures 37C). LPS-stimulated monocytes did show some signs of differentiation in the absence or presence of T cells (Figures 37D and 37E, respectively), but the changes were not as prominent as those observed in TSST-1-stimulated monocytes in the presence of T cells. Of note, the TSST-1-induced morphologic changes in monocytes were absent when these cells were physically separated from T lymphocytes by Transwell chambers (data not shown), suggesting that contact with T lymphocytes may be important for the process.

5.2.9 Comparison Of A Commercial Preparation With Our Own Highly Purified TSST-1 In The Induction Of Cytokine Secretion In Human Blood Monocytes

Commercial preparations of TSST-1 (Toxin Technology, Madison, WI) are commonly used to analyse cytokine production in human blood monocytes. We have observed that these preparations commonly contain multiple protein impurities as demonstrated by silver staining of SDS-PAGE gels or by immunoblotting with either pooled normal human sera or with rabbit antisera to MN8 culture filtrate. To determine whether the impurities themselves might stimulate cytokine production, our own highly purified in-house TSST-1 was tested in parallel with the commercial preparation. As shown in Table XII, in contrast to our TSST-1 stock, commercial TSST-1 induced TNFα release from monocytes in the absence of T lymphocytes. When commercial TSST-1 was further purified to homogeneity by chromatofocusing and then incubated with monocytes alone, no TNFα activity was

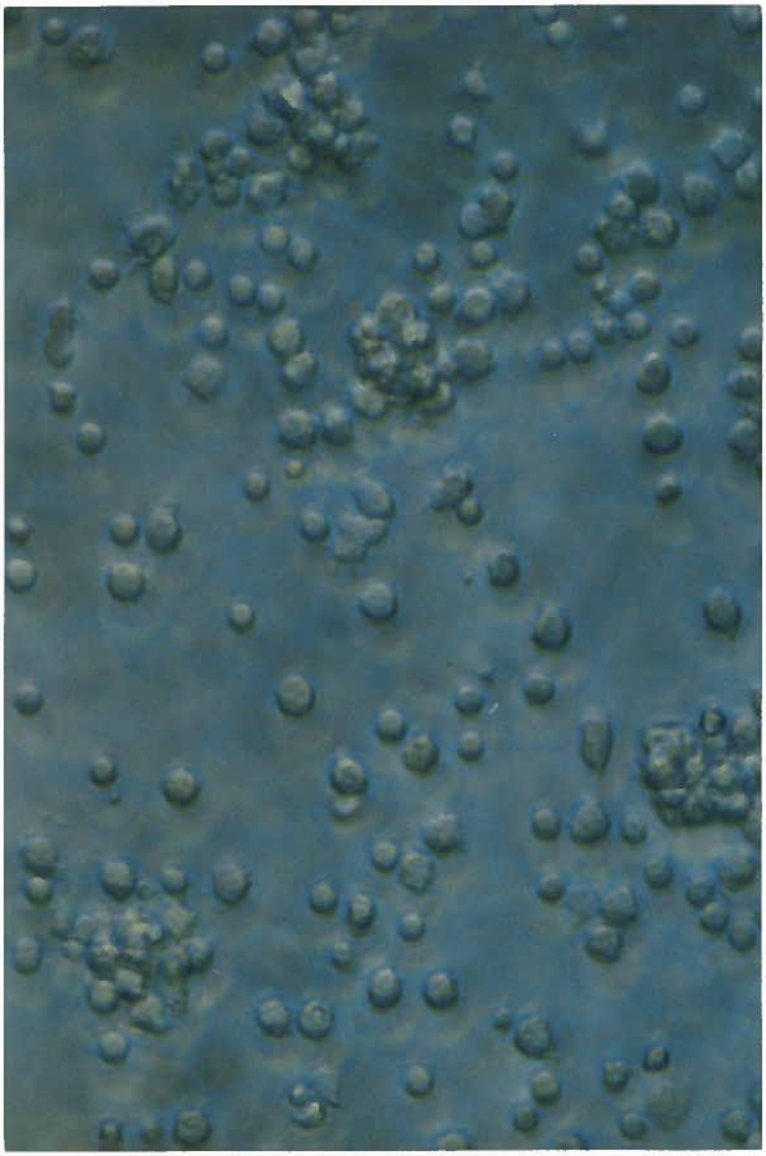

Figure 37A. Co-culture of human monocytes and T lymphocytes in RPMI 1640 medium. Cells were cultured in a 1:1 ratio (1 x 10^6) for 24 h at 37°C (400x magnification).

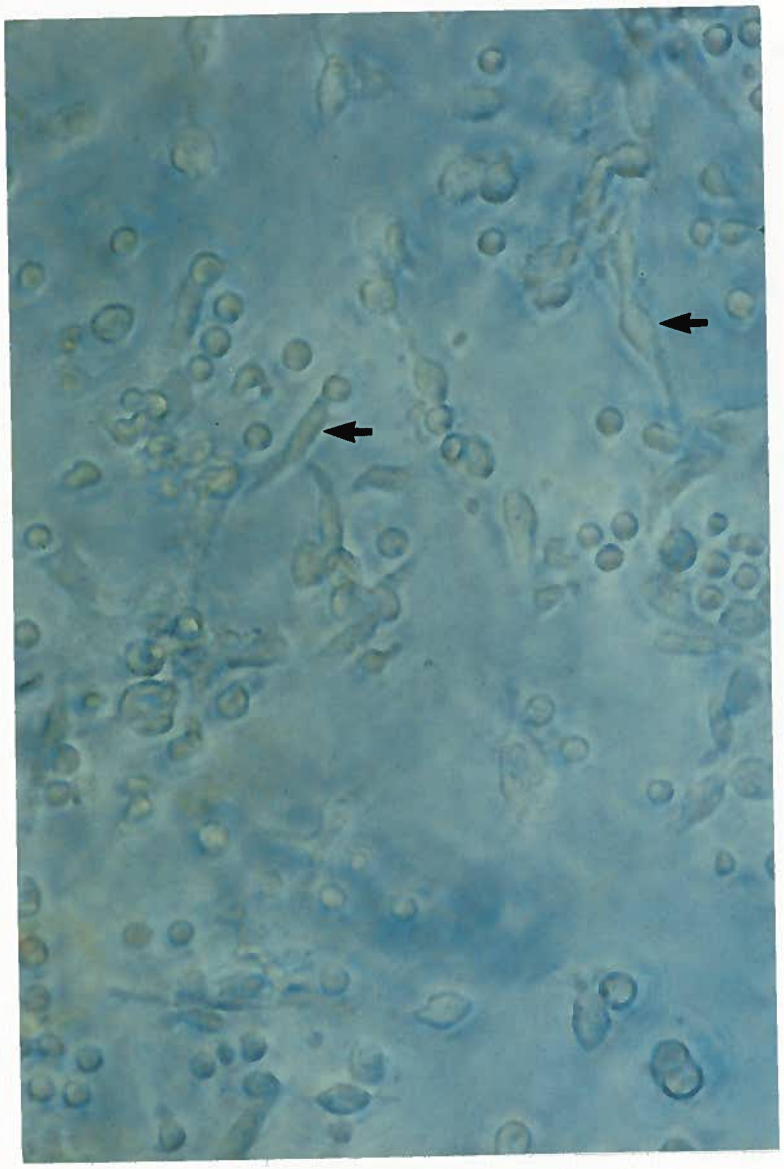

Figure 37B. Co-culture of human monocytes and T lymphocytes stimulated with TSST-1 . A 1:1 ratio (1 x 10^6) of cells were stimulated with 1 μg/ml TSST-1 for 24 h at 37°C. Arrows indicate differentiated monocytes after TSST-1 stimulation (400x magnification).

Figure 37C. Human monocytes alone stimulated TSST-1. 1 x 10^6 monocytes were treated with 1 μg/ml TSST-1 for 24 h at 37°C (400x magnification).

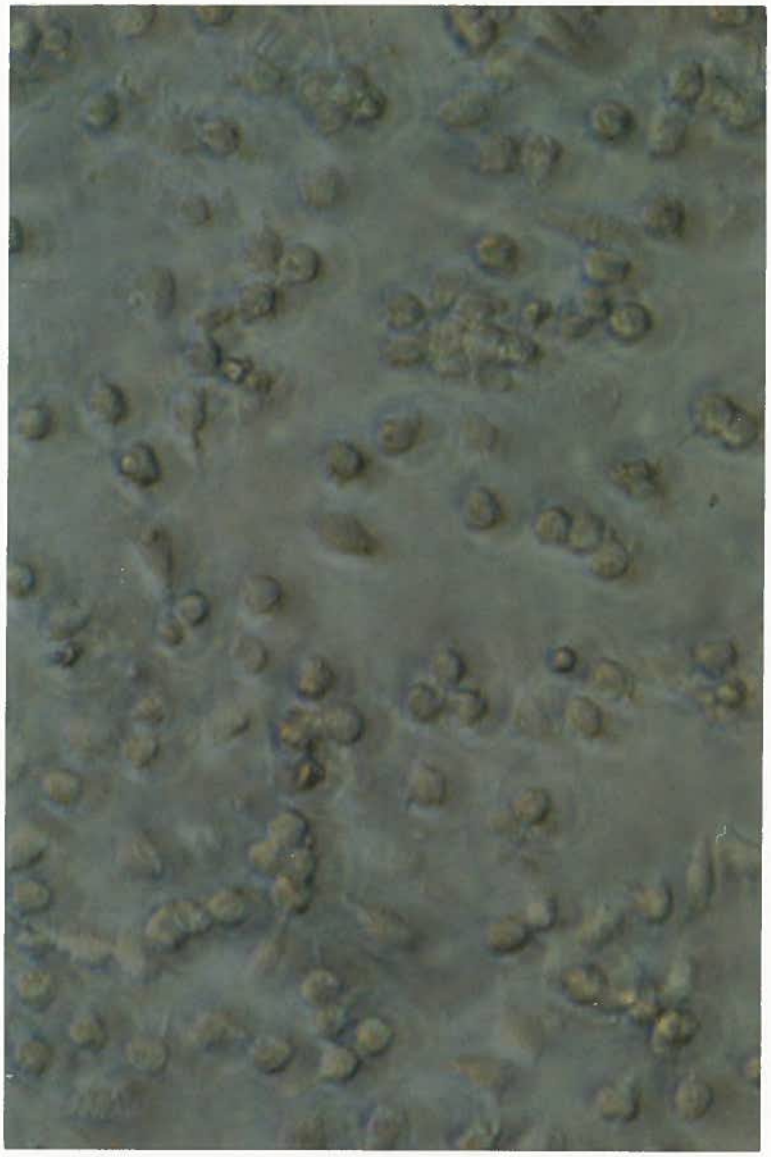

Figure 37D. Human monocytes alone stimulated E. coli LPS. 1 x 10^6 monocytes were stimulated with LPS for 24 h at 37°C (400x magnification).

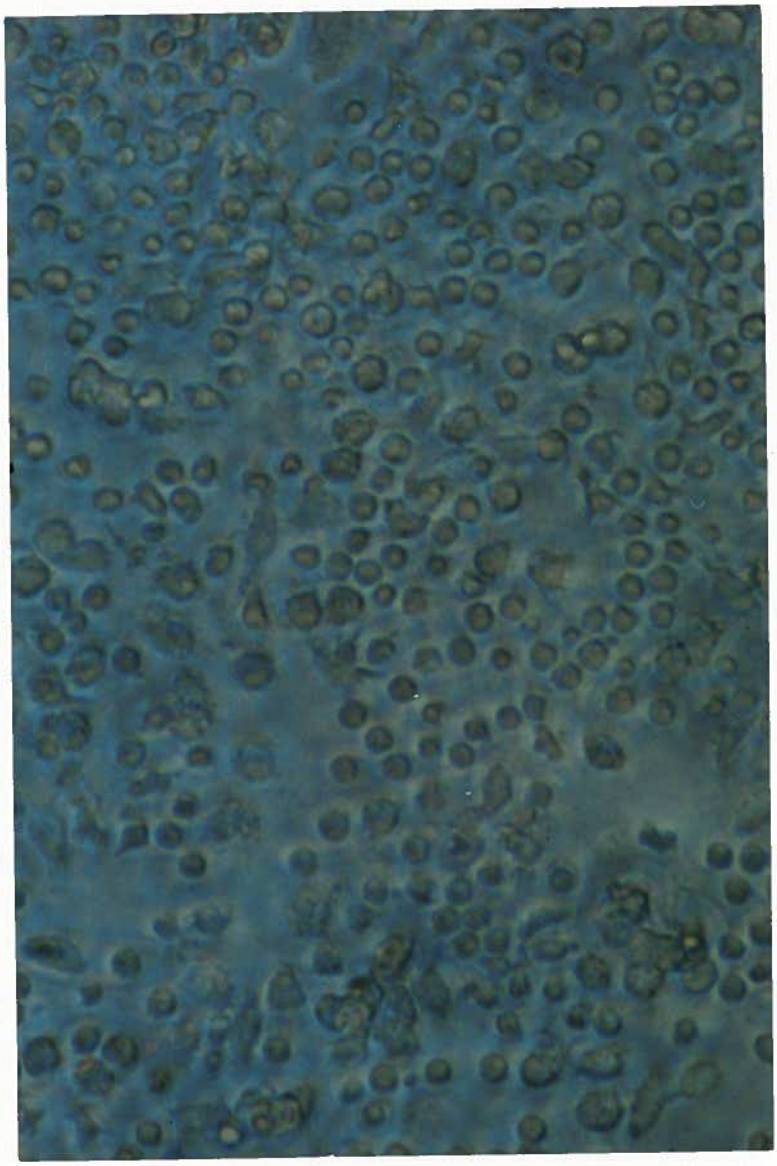

Figure 37E. Co-culture of human monocytes and T lymphocytes stimulated with E. coli LPS. A 1:1 ratio (1 x 10^6) of cells were stimulated with LPS for 24 h at 37°C (400x magnification).

Table XI

TNF Production By Human Monocytes And T Cells Stimulated With Various TSST-1 Preparations

Stimulus and Cells[a]	TNFα (pg/ml)[b]	
	Donor 1	Donor 2
RPMI + Mo	<62	<62
RPMI + Mo + T	<62	<62
TSST-1 (AWC) + Mo	<62	<62
TSST-1 (AWC) + T	<62	<62
TSST-1 (AWC) + Mo + T	1500	3700
TSST-1 (TT) + Mo	1400	1900
TSST-1 (TT) + Mo + T	1500	ND
Purified TSST-1 (TT) + Mo	<62	<62
Purified TSST-1 (TT) + Mo + T	2600	3500

[a] Mo = monocytes; T = T lymphocytes; RPMI = RPMI 1640 medium, TSST-1 (AWC), in-house TSST-1; TSST-1 (TT) = commerial TSST-1 from Toxin Technology, Inc.; ND = not determined; purified TSST-1 (TT) = Toxin Technology TSST-1 purified to homogeneity by chromatofocusing.

[b] TNFα quantitated by ELISA (lower sensitivity limit, 62 pg/ml). Values represent the mean of duplicate determinations.

found. Like our TSST-1 stock, the further purified commercial TSST-1 induced TNFα activity only when T lymphocytes were added to the monocyte culture (Table XII). Two impurity peaks (as determined by immunoblot staining after SDS-PAGE) contained within the commercial TSST-1 preparation were eluted from the chromatofocusing column. Only the first impurity peak was able to activate human monocytes alone to produce TNFα (Table XII). Preliminary evidence indicates that the impurity in this fraction is a 42 kd staphylococcal exoprotein (data not shown).

5.3 Discussion

The results in this chapter show that induction of the cytokines TNFα and IL-1ß by TSST-1 requires the presence of both monocytes and T lymphocytes. Using highly purified TSST-1 and fractionated human peripheral blood mononuclear cells, our data show that cultures of monocytes or T cells alone, stimulated with TSST-1 for up to 72 h, were unable to produce TNFα or IL-1ß. However, when both cell types were co-cultured together, significant cytokine production following TSST-1 stimulation was observed after 24 h. Our findings are in agreement with those observed with another superantigen, SEA. Fischer et al. (1990) reported that TNFα and TNFß production by SEA require the presence of both cell types. Using an intracytoplasmic staining technique for TNF-producing cells, they showed that both monocytes and T lymphocytes were actively producing TNFα in response to SEA stimulation. T lymphocytes were further shown to produce TNFß in the presence of autologous monocytes, although with later kinetics than that observed with TNFα (Fischer et al., 1990). In a later study by the same group, SEA-induced production of IL-1α and IL-1ß in human monocytes was also found to be dependent on the presence of T lymphocytes (Gjorloff et al., 1991).

These findings suggest that monocytes alone are not sufficient to initiate pathogenesis of TSS. It has been reported that TSST-1 is able to induce TNFα and IL-1ß production in monocytes in the absence of T cells (Fast et al., 1989; Jupin

et al., 1988; Parsonnet et al., 1985; Parsonnet and Gillis, 1988). The discrepancy between our results and those of other laboratories may lie in the use of unfractionated peripheral blood mononuclear cells for cytokine studies or in the purity of monocytes obtained by plastic adherence. It has been reported that the purity of monocytes obtained through the latter procedure ranges from 70% to 90% monocytes as determined by fluorescence activated cell sorting using the monocyte-specific mAb 63D3 (Rosenberg et al., 1981). Even a few contaminating T lymphocytes may be sufficient to support monocyte production of cytokines induced by these toxins (Fischer et al., 1990). Furthermore, as will be discussed later, the purity of TSST-1 used in cytokine studies also may affect whether T cells are required for TNF and IL-1 production by human monocytes.

The subset of T cells (eg. $CD4^+$ or $CD8^+$) required for the production of cytokines by human monocytes was not examined in this study. Presumably, T cells interacting with TSST-1 bound on the surface of MHC class II-bearing monocytes possess $V_{\beta 2}$ sequence on their TCR (Choi et al., 1989). It has been demonstrated in other studies that the $CD4^+$ but not $CD8^+$ subset supported SEA-induced TNFα production in human monocytes (Fischer et al., 1990). Further analysis of the $CD4^+$ cells showed that the $CD45RO^+$ memory T cell but not the $CD45RA^+$ naive T cell subpopulation was involved in SEA-stimulated TNFα production in monocytes (Fischer et al., 1990). The differences in the ability of these cell subpopulations to support TNF production was partly attributed to differences in IFN-τ production between the cell types (Fischer et al., 1990). Interestingly, although SEA-induced IL-1 production in monocytes was also supported by the $CD4^+$ $45RO^+$ subtype, CD4 $45RA^+$ and $CD8^+$ T cells were also involved to a smaller extent. This suggests that TNF and IL-1 production may be independently regulated by multiple pathways (Gjorloff et al., 1991).

The predominant TNF produced by TSST-1-stimulated monocyte-T cell cultures after 24 h was the alpha form as determined by ELISA and by neutralization of TNF cytotoxic activity with anti-human TNFα. Very little TNFβ was detected during this period. At 72 h, however, very little TNF cytotoxic activity was inhibited with anti-human TNFα antibodies (data not shown). It has been reported that SEA-

induced TNF cytotoxicity at 24 h is due to the alpha form and the cytotoxic effect observed at 72 h was the result of TNFß (Fischer et al., 1990). The same study showed that TNFß was produced predominantly by $CD4^+$ T cells (Fischer et al., 1990). These results suggest that TNFα is produced by monocytes initially after TSST-1 stimulation followed by the production of TNFß by T lymphocytes after 24 h. Although studies were not done here to determine which cell type was responsible for TNFα production, presumably monocytes were the major source of this cytokine. However, there is evidence that T lymphocytes may be contributing to the total TNFα pools in the supernatants as well. Work by others have shown that TNFα can be produced by T lymphocytes when induced by stimuli such as mitogenic lectins, phorbol esters with ionomycin, or antibodies against the TCR receptor or CD28 (Cuturi et al., 1987; Steffen et al., 1988; Sung et al., 1988; Thompson et al., 1989). Recent data indicate that the subpopulation of T cells predominantly involved in TNFα production after stimulation with a combination of anti-CD28 and PMA were memory T cells (CD45RO isoform) and not naive T lymphocytes (CD45RA isoform) (von Fliedner et al., 1992). Using an intracytoplasmic staining technique for TNFα, Fischer et al. (1990) also noted that in addition to monocytes, T lymphocytes were also able to produce TNFα in response to SEA stimulation. Therefore, in our studies, the TNFα detected after TSST-1 stimulation may be derived from either monocytes or T lymphocytes.

The nature of the monocyte-T cell interaction for cytokine production was investigated in this study. It has been previously mentioned that TSST-1 and other bacterial superantigens bind to nonpolymorphic regions of MHC class II molecules and together, these proteins are recognised by T cells bearing the appropriate $V_ß$ TCR sequences (Choi et al., 1989; Fleischer et al., 1989; Kappler et al., 1989). This would suggest that cytokine production occurs because of monocyte-T cell contact or as a result of lymphokines released by T cells. One possible lymphokine is IFN-τ, which has been shown to upregulate TNF and IL-1 production in LPS-stimulated monocytes or macrophages (Beutler and Cerami, 1988) and also to be released by T lymphocytes in response to TSST-1 stimulation (Jupin et al., 1988; Micusan et al., 1989). Others have previously shown that several

cytokines such as IL-2, IL-4, IL-6, IL-7 and IFN-τ, alone or in combination, could not replace the T cell requirement for TNFα or IL-1 production by monocytes stimulated with SEA (Fischer et al., 1990; Gjorloff et al., 1991). In our study, consistent with these observations, rabbit anti-human IFN-τ antibodies were ineffective in abolishing TNFα production by monocytes/T cells after TSST-1 induction. Furthermore, the priming of human monocytes overnight with IFN-τ prior to the addition of TSST-1 did not induce TNFα production in the absence of T cells. These results strongly indicate that the signal transduced by T cells to monocytes does not consist entirely of IFN-τ and that physical contact or possibly other soluble mediators may be required for TSST-1-induced TNFα production. This hypothesis is supported by our experiments in which Transwell culture chambers were used to physically separate monocytes from T cells but still allow the diffusion of soluble molecules through a filter. The abolition of TNFα production by monocytes or T cells using these chambers strongly indicates a requirement for monocyte-T cell contact.

Morphologic changes were noted in TSST-1-stimulated monocytes in the presence of T cells. Monocytes appeared to transform into macrophages with a morphology similar to that of fibroblasts. Analogous morphologic changes have been observed in B cells stimulated with anti-class II antibodies (Cambier and Lehmann, 1989; St.-Pierre and Watts, 1991). The presence of these elongated macrophage-like cells was not seen when TSST-1-treated monocytes were separated from T cells by Transwell culture chambers, suggesting that the morphologic changes may be initiated as a result of contact with T cells. It is also possible that factors released by TSST-1-activated T lymphocytes may contribute to the morphologic changes observed in monocytes. The reasons for the morphologic changes in monocytes are speculative at this stage. One possibility is that morphologic changes increase the surface area on the accessory cell for presentation of TSST-1 to T lymphocytes, although this needs to be confirmed with inhibitors of cytoskeletal elements such as cytochalasins.

Besides cell contact, it appears that metabolic activity of T cells is important since the addition of paraformaldehyde-fixed T cells to viable

monocytes resulted in a significant decrease in cytokine production. The reduction in cytokine production by viable monocytes may represent the loss of additional signals from T cells as a result of fixation. One possible explanation is that T cell-derived soluble mediators are lost when cells are fixed prior to activation. TSST-1 has been shown to induce the release of several lymphokines including IFN-τ (Jupin et al., 1988), IL-2 (Uchiyama et al., 1986), and TNFß (Anderson et al., 1989). These cytokines, alone or in combination, may be important for production of cytokines by monocytes. Alternatively, fixation of T cells may result in loss of T cell proliferative activity or the loss of antigen-processing capability, both of which may affect the ability of T cells to provide signals to monocytes. The possibility also exists that fixation of monocytes or T cells could destroy the receptors or epitopes crucial for activation of cytokine production. However, the same fixation procedure has been used to show that fixed monocytes in the presence of SEA can support activation of T lymphocyte proliferation (Fischer et al., 1989). Furthermore, in our own studies, monoclonal antibodies were able to recognize the CD2 and CD3 markers on fixed T cells as determined by immunofluorescence studies (data not shown). Despite this, the loss of receptor mobility after fixation (St. Pierre and Watts, 1991) may impede contact between monocytes and T cells and does represent another potential explanation for the decrease in cytokine production.

Recently, Lands et al. (1991) showed that induction of IL-1 by anti-CD3 requires two signals, direct contact between monocytes and activated T cells and a soluble T cell lymphokine. They reported that monocytes in contact with viable or fixed anti-CD3-stimulated T cells showed rapid induction of IL-1α- and -ß mRNAs and the production of cell-associated but not extracellular IL-1. Further experiments showed that a soluble T cell lymphokine was involved in the secretion of IL-1 since only viable T cells or the supernatants of anti-CD3-activated T cells resulted in the detection of extracellular IL-1. Our own results suggest that induction of cytokine secretion by TSST-1 may also occur as a result of monocyte-T cell interaction. We have shown in Chapter 4 that TSST-1 is able to stimulate dephosphorylation events in human monocytes in the absence of T cells.

This indicates that TSST-1 does initially activate monocytes, but additional signals from T lymphocytes, either in the way of cell contact, lymphokine production, or T cell proliferation are required for monocyte production and secretion of IL-1 or TNF to occur. One possible mechanism of cytokine induction by TSST-1 is that the toxin activates IL-1 and TNF mRNA transcription and translation in monocytes in the absence of T cells. Recent data indicate that TSST-1 can stimulate IL-1ß and TNFα mRNA production in human monocytes alone (Trede et al., 1991). However, the secretion of cytokines by monocytes may not occur until T cell-derived signals are received. There is now strong evidence that cytokine production and secretion are independent events (Bakouche et al., 1992) and it is possible that TSST-1-mediated T cell participation may be acting at either level. Further work is needed to determine at what point T cells are required for cytokine induction by TSST-1.

The requirement of T cells for TSST-1 induction of cytokine production in human monocytes is in contrast to that observed with bacterial LPS. It has been shown that the physiological responses to LPS may be dictated by its ability to bind to a 60 kd serum glycoprotein, LPS binding protein (LBP) (Tobias et al., 1986; Wright et al., 1990). Interaction of the LPS-LBP complex with the CD14 receptor on human monocytes results in the secretion of TNFα from these cells (Wright et al., 1990). Thus, the lack of the T cell requirement for LPS induction of TNFα secretion from monocytes suggests that TSST-1 and LPS activate monocytes by different signaling mechanisms. Further support that TSST-1 and LPS activate monocytes by dissimilar signal transduction pathways comes from the phosphorylation studies reported in Chapter 4. Whereas dephosphorylation events were primarily noted with TSST-1-stimulated monocytes, an increase in phosphorylation of proteins was consistently observed with LPS-treated cells. The differences may be a result of binding to different receptors on human monocytes by TSST-1 and LPS (HLA-DR and CD14, respectively). Nevertheless, the induction of IL-1 and TNF secretion by these two agents indicates that there may be a downstream convergence of signals, perhaps at the level of cytokine gene transcription. Synergism between TSST-1 and LPS has been reported by other

investigators. Specifically, higher levels of IL-1 were produced when TSST-1-stimulated macrophages were initially primed with LPS, although the authors did note that the converse was not found (Beezhold et al., 1987). In similar studies, Henne et al. (1991) showed that pretreatment of mice with TSST-1 12 h prior to LPS exposure resulted in a 20-fold enhancement of serum TNF levels compared to those receiving LPS alone. In light of the finding that TSST-1 enhances host susceptibility to lethal endotoxic shock (Schlievert et al., 1982), one cannot ignore the fact that the two agents may interact synergistically with each other to induce shock. Perhaps some of the signals normally provided by T cells during TSST-1 stimulation of monocytes are bypassed when the cells are treated with LPS. More work will be needed to decipher the role of LPS in TSST-1-mediated shock.

The role of the adhesion molecule, LFA-1, in the induction of cytokine production by TSST-1 was examined in light of our earlier results showing that contact between T lymphocytes and monocytes is important for the induction of cytokines by TSST-1. The present study demonstrates that the LFA-1 molecule on human T cells and on monocytes plays a major role in TSST-1-induced cytokine secretion. Monoclonal antibodies directed against either the CD11-alpha chain or the CD18-beta chain of LFA-1 were highly effective in blocking TSST-1-induced IL-1ß and TNFα secretion by co-cultures of monocytes and T cells. In contrast, the anti-CD14 mAb did not inhibit secretion of either cytokine. The mechanisms by which LFA-1 mAbs inhibit cytokine production are not clear. Presumably, the inhibition is likely the result of disruption of cell-cell adhesion via LFA-1/ICAM-1 or LFA-1/ICAM-2 on monocytes and T lymphocytes. This disruption may subsequently impair the production of IL-1 and TNF in monocytes by preventing additional stimulatory signals from T cells, perhaps via either T cell-derived lymphokines or via T cell membrane-associated molecules. Mourad et al. (1990) have reported that binding of TSST-1 to MHC class II antigen activates the adhesion function mediated by the LFA-1 molecule. In their study, they found that mAbs to the α-chain or the ß-chain of the LFA-1 heterodimer inhibited TSST-1-induced aggregation between Ia^+ antigen-presenting cells and T lymphocytes. Furthermore, a patient defective in the expression of LFA-1 did not show evidence

of cell aggregation in response to TSST-1 (Mourad et al., 1990). Other studies have also demonstrated the role of adhesion molecules in cytokine induction in response to other external stimuli. Monoclonal antibodies to the intercellular adhesion molecule, ICAM-1, have been shown to block IL-1 and TNF release from phytohemagglutinin-activated human peripheral blood mononuclear cells (Geissler et al., 1990). However, in their study, the authors postulated that the ICAM-1 mAb itself may be directly suppressing cytokine release at the molecular level. Additionally, the participation of adhesion-promoting proteins in cytokine production has been directly demonstrated by using immobilized LFA-1, LFA-3, and CD2 mAbs to trigger TNF and IL-1 release from purified human monocytes (Couturier et al., 1990; Webb et al., 1990). A role for the LFA-1 molecule in superantigenic toxin activation of T lymphocyte proliferation has been documented by Mittrucker et al. (1992). In their study, mAb to LFA-1 alone or CD2 alone was relatively ineffective in inhibiting the T cell proliferative response to SEB when presented by accessory cells with high levels of ICAM-1 and LFA-3 expression. However, using accessory cells with low expression of LFA-3, mAb to LFA-1 but not to CD2 was highly inhibitory. Recently, Fischer et al. (1992) reported that T cell activation by SEA is also dependent on the expresssion of ICAM-1 molecule on accessory cells. Mouse L cells cotransfected with both MHC class II HLA-DR and ICAM-1 stimulated $CD3^+$ T cells at 100-fold lower concentrations than single transfected cells. The role of ICAM-1 was even more profound when using a transfectant with lower HLA-DR density since cotransfection of ICAM-1 allowed responses down to picomolar concentrations of SEA (Fischer et al., 1992). These results suggest that presentation of superantigens to the TCR by MHC class II may not be sufficient for T cell activation. Further adhesive interactions mediated by accessory molecules such as CD2 or LFA-1 may be required, particularly when expression of MHC class II on the accessory cell is low (Fischer et al., 1992; Mittrucker et al., 1992). The importance of adhesion molecules in mediating other biological effects of staphylccoccal exotoxins has also been examined by Dohlsten et al. (1991). These investigators found that ICAM-1 expression on target cells plays a major role in SEA-dependent cell-mediated cytotoxicity. Specifically,

they observed that SEA-stimulated human cytotoxic T lymphocytes (CTL) were more potent in killing HLA-DR-transfected murine L cells if they co-expressed the ICAM-1 molecule as well. The cytotoxic effect on HLA-DR/ICAM-1-expressing cells could be blocked by anti-CD11a or anti-CD18 mAbs, indicating that interaction of LFA-1 on CTL with target cell ligand ICAM-1 was essential (Dohlsten et al., 1991). Interestingly, in our studies, a mAb to ICAM-1 (mAb 84H10, AMAC Inc., Westbrook, ME) had no effect on cytokine induction by TSST-1 in 3 donors studied. The lack of inhibition does not necessarily indicate that ICAM-1 does not play a major role in blocking cytokine induction by TSST-1. One explanation for the lack of inhibition may be that the ICAM-1 epitope recognized by the mAb does not block interactions with LFA-1 or, alternatively, when ICAM-1 sites are blocked, LFA-1 may be binding to its second counterpart receptor, ICAM-2 (Dustin, 1990).

Whether the role of adhesion molecules is primarily to facilitate TCR recognition of the MHC class II-toxin complex by promoting cell-cell contact is not clear at this point. There is now strong evidence that the LFA-1α subunit may have transmembrane signaling properties such as inducing phosphoinositol hydrolysis and increases in intracellular calcium (Dustin, 1990; Pardi et al., 1989). Recent work by Fischer et al. (1992) suggests that adhesion molecules themselves may provide additional stimulatory signals to T cells. The authors found that recombinant ICAM-1 immobilized on a plastic surface was able to strongly costimulate SEA-induced T lymphocyte activation by HLA-DR-transfected cells. These results imply that costimulatory signals provided by ICAM-1 to the T cell can be delivered independently from the TCR signal (Fischer et al., 1992). More work will certainly be needed to address the role of LFA-1/ICAM-1-mediated activation of monocytes and T cells by TSST-1.

It is surprising that in 4 separate donors, the addition of the anti-HLA-DR mAb, L243, did not abolish TNFα production by monocyte/T cell cultures stimulated with TSST-1. Earlier work described in Chapter 3 and by other laboratories have demonstrated that the L243 mAb strongly blocks binding of TSST-1 to the HLA-DR receptor in human monocytes (Fischer et al., 1989; Norton et al., 1990; Scholl et al., 1990; See et al., 1992). In addition, this mAb also abrogates monocyte-

dependent T cell activation and B cell production of immunoglobulins induced by TSST-1 and other staphylococcal exotoxins (Moseley et al., 1991; Mourad et al., 1989; Scholl et al., 1990; See et al., 1992). There is the possibility that binding of TSST-1 to HLA-DR on monocytes and subsequent activation of T cell proliferation is unrelated to the production of TNFα. Support for this argument comes from the findings of Grossman et al. (1990) where they showed that monocyte stimulatory activity in response to SEA and SEB could be dissociated from that of T cell proliferation. The authors found that reduction and alkylation of the cysteines forming the disulfide loop of SEA and SEB abolished T cell mitogenic activity but not the TNFα-inducing activity in monocytes. Alternatively, induction of TNFα by TSST-1 may involve other MHC class II molecules such as HLA-DQ and HLA-DP, with which L243 does not cross-react. Although antibodies to HLA-DP and HLA-DQ were not tested for their ability to block cytokine induction in this study, there is evidence to indicate that these other MHC class II molecules may play a role. It has been shown that in the presence of both I-E and I-A haplotypes on antigen-presenting cells (APC) of recombinant congenic mice strains, presentation of SEB to a murine T-cell receptor $V_{\beta 8+}$ T cell clone is dominated by I-E. However, in the absence of I-E, some APC bearing I-A alleles can present SEB as effectively as those expressing both I-A and I-E (Robinson et al., 1991). Therefore, in this study, it is possible that when HLA-DR receptors on monocytes are blocked by the L243 mAb, HLA-DQ and HLA-DP may play a more prominent role in TNFα induction by TSST-1. Finally, TSST-1 induction of TNFα in monocytes and T cells may be mediated through receptors other than MHC class II molecules, such as the LFA-1 adhesion molecule as indicated earlier.

The TSST-1 preparations used in these cytokine studies were homogeneous as indicated by silver staining of SDS-PAGE gels and by immunoblotting with pooled normal human sera or with rabbit antisera to S. aureus MN8 culture filtrate. We have previously reported that commercial TSST-1 from Toxin Technology contains multiple protein impurities (Rosten et al., 1989). This is exemplified in Figure 3, lane 1. As shown in Table XII, the commercial TSST-1 preparation was able to induce TNFα production in monocytes alone wheras our own preparation required

both monocytes and T lymphocytes. One possibility for the discrepancy is that impurities present in the commercial TSST-1 preparation are themselves inducing cytokine production in monocytes alone. This was shown directly by purifying the commercial TSST-1 preparation to homogeneity using chromatofocusing and showing that further purified commercial TSST-1 was not able to induce cytokine production in human blood monocytes unless T cells were added. Furthermore, one protein impurity fraction obtained from the chromatofocusing column was able to stimulate TNFα secretion from monocytes alone. Preliminary evidence indicates that a protein, 42 kd in size, may be responsible for inducing TNFα in monocytes alone. These results emphasize that for cytokine studies, more stringent methods of purity assessment of TSST-1 preparations must be used if the role of TSST-1 in TSS pathogenesis is to be further clarified.

Having determined some of the mechanisms by which human peripheral blood mononuclear cells release cytokines in response to TSST-1 stimulation, the next question is how they relate to TSS pathogenesis. It is well known that cytokines in small quantities are of benefit to the host in defending against infections and diseases (Beutler and Cerami, 1988; Marrack and Kappler, 1990). Overproduction of these cytokines can also be detrimental to the host. Fever, weight loss, shock and death are some of the features seen when IL-1 and TNF are produced in abundance (Beutler and Cerami, 1988; Ikejima et al., 1989; Okusawa et al., 1988). Although it is now widely accepted that release of cytokines play a major role in the pathogenesis of TSS, there is some debate as to whether monocytes or T lymphocytes are the primary source of cytokines induced by TSST-1. On the one hand, monocytes and macrophages are thought to be important since these cells have previously been shown to produce large amounts of TNF and IL-1 after TSST-1 stimulation (Fast et al., 1989; Parsonnet et al., 1985; Parsonnet and Gillis, 1988; Parsonnet, 1989). The combined action of these two mediators may explain many of the characteristic features seen among TSS patients (Ikejima et al., 1989; Okusawa et al., 1988; Parsonnet, 1989). On the other hand, others believe that TSS occurs as a result of overstimulation of T lymphocytes by TSST-1 with the subsequent release of massive amounts of lymphokines (eg. IL-2, TNFß,

IFN-τ) by these cells (Marrack and Kappler, 1990; Miethke et al., 1992). The release of these lymphokines by T cells in response to TSST-1 stimulation has been well-documented (Jupin et al., 1988; Micusan et al., 1986; Micusan et al., 1989). The symptoms of TSS patients resemble that of cancer patients receiving IL-2 therapy or transplant recipients receiving anti-CD3 mAb treatment (Miethke et al., 1992; Rosenberg et al., 1988). The interaction of bacterial superantigens with the V_β sequences of the TCR means that a higher proportion of T cells are activated compared with that of conventional antigens (Marrack and Kappler, 1990). For example, it has been estimated that 10% of all $\alpha\beta^+$ human T cells are stimulated as a result of TSST-1 interacting with $V_{\beta 2}$-bearing T cells (Marrack and Kappler, 1990). Furthermore, mice which lack T cells but contain functional class II-bearing macrophages, are not affected by doses of SEB that would kill normal mice (Marrack et al., 1990). Miethke et al. (1992) recently used D-galactosamine-sensitized mice to study the lethal toxicity mediated by SEB. These investigators found that treatment of mice with cyclosporine A, a reagent known to suppress lymphokine secretion by T cells, conferred protection against the lethal effects of SEB. Moreover, D-galactosamine-sensitized SCID mice, known to lack T and B cells, were also resistant to the lethal toxicity of SEB. The SCID mice were sensitive to LPS, indicating that macrophage reactivity was functional. Reconstitution of D-galactosamine-sensitized SCID mice with T cells, however, restored the SEB-mediated toxicity. In these studies, the authors did show that TNFα and TNFβ were important since anti-TNFα/β mAbs conferred protection to the mice (Miethke et al., 1992). Based on these studies, the investigators hypothesized that T cell-dependent lymphokines were important in TSS.

Our own findings demonstrating that both monocytes and T cells are required for the induction of cytokine synthesis by TSST-1 suggest that both cell types are important for TSS pathogenesis. Interaction between monocytes and T cells (eg. via cell contact, soluble mediators, T cell proliferation, etc.) is important for IL-1 and TNF production to occur in response to TSST-1 stimulation. The requirement of both cell types for cytokine production is consistent with the results of others who advocate the importance of either T lymphocytes or

monocytes in TSS pathogenesis. The absence of SEB toxicity in mice lacking T cells may be because murine macrophages are unable to respond to this superantigen without the help of T cells. Similarly, cyclosporine-treated mice may be protected from SEB since the T cell-derived signals (possibly lymphokines) needed to activate macrophages are absent, even though the macrophage-T lymphocyte contact component is present. Consequently, TSST-1-mediated interaction of monocytes with T cells results in the release of IL-1, TNF, and IL-6 from monocytes or macrophages as well as the secretion of IL-2, TNFα/ß, and IFN-τ from T cells. Additionally, IL-1 is known to induce its own gene expression and synthesis (Dinarello et al., 1987) as well as stimulate the production of TNFα (Ikejima et al., 1990). TNFα also induces synthesis of IL-1 (Dinarello et al., 1986). These positive feedback loops contribute significantly to the large pool of circulating cytokines. A consequence of the release of such large amounts of mediators is the manifestation of shock in TSS patients or in animal models of the disease. Therefore, a major difference between LPS and TSST-1-induced shock is that the former is mediated primarily by macrophages/monocytes, and the latter by both macrophages/monocytes and T cells. Similarities do exist in LPS- and TSST-1-mediated shock in that common mediators such as TNF and IL-1 are important in their pathogenesis (Miethke et al., 1992).

In conclusion, our results suggest that cytokine production involves more than binding of TSST-1 to its receptor on human monocytes alone. Monocyte contact with metabolically active T cells (mediated in part by the LFA-1 adhesion molecule) is also required. Therefore, mechanistically, TSST-1 does differ from LPS in their target cells even though the clinical features of shock mediated by both agents are very similar.

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Chapter 6

The Role Of Protein Kinases In TSST-1-Induced Secretion Of IL-1 And TNF By Human Blood Monocytes And Lymphocytes

6.1 Introduction

This chapter examines the signal transduction mechanisms by which TSST-1 induces cytokine secretion from human blood monocytes and T lymphocytes. Signal transduction pathways consist of a series of reactions that mediate the cellular response to extracellular stimuli. The interaction of agonists with their respective receptors results in the generation of a group of cytosolic compounds known as the second messengers, which include diacylglycerol, inositol trisphosphate, calcium, cAMP and cGMP. These second messengers serve to transmit and amplify the extracellular signal by activating cytosolic protein kinases. These kinases then phosphorylate specific cellular proteins that are instrumental in effecting a biological response (Nishizuka et al., 1984; Nishizuka, 1988). As described in chapter 4, TSST-1 appears to activate human monocytes by dephosphorylating several cellular phosphoproteins. Whether the changes in the phosphorylation state of these proteins is related to cytokine induction or secretion has not been determined. By using various protein kinase inhibitors, the signal transduction pathways involved in the secretion of TNFα and IL-1ß by human monocytes and T cells has been investigated.

Specific Aim

1) To examine the effects of protein kinase inhibitors on TSST-1-mediated secretion of the cytokines, IL-1 and TNF, by human blood monocytes and T lymphocytes.

6.1.1 The Effect Of Protein Kinase Inhibitors On Cytokine Secretion Induced By TSST-1

To determine the second messenger pathways involved in cytokine induction and secretion by TSST-1, various protein kinase inhibitors were tested. Human monocytes, co-cultured 1:1 with T cells, were stimulated with 10 μg/ml TSST-1 in the presence or absence of the following inhibitors of protein kinases: H7 (protein kinase C), HA1004 (cAMP- and cGMP- dependent kinases), and genistein (tyrosine kinases). As shown in Figure 38, secretion of TNFα was strongly suppressed in the presence of 25 μM H7 and 50 μM genistein (92% and 83% inhibition, respectively). The inhibitory effect did not appear to be attributed to alteration in cell viability as judged by trypan blue staining. Furthermore, HA1004, an inhibitor of cAMP- and cGMP-dependent kinases but not of protein kinase C, did not affect TSST-1-induced secretion of TNFα as determined by ELISA. In addition, monocyte/T cell co-cultures treated with genistein for 24 h were still able to produce high levels of TNFα after washing cells free of the inhibitor and stimulating the cells with LPS for 24 h (data not shown). Finally, the concentrations of inhibitors used in these experiments are well within the range used in a number of other monocyte studies (Bakouche et al., 1992; Kovacs et al., 1988; Nezu et al., 1990; Taniguchi et al., 1989).

The effect of the same kinase inhibitors on IL-1ß secretion by co-cultures of human monocytes and T cells was evaluated using an ELISA specific for IL-1ß. Figure 39 shows that TSST-1-induced IL-1ß levels were strongly suppressed by H7, HA1004, and genistein (82%, 75% and 87%, inhibition respectively). All kinase inhibitors in medium alone did not induce TNFα or IL-1ß release (data not shown). Interestingly, the morphological changes in monocytes, observed in the presence of T lymphocytes and TSST-1 (section 5.2.8 of Chapter 5), were abolished by the presence of the kinase inhibitors (data not shown).

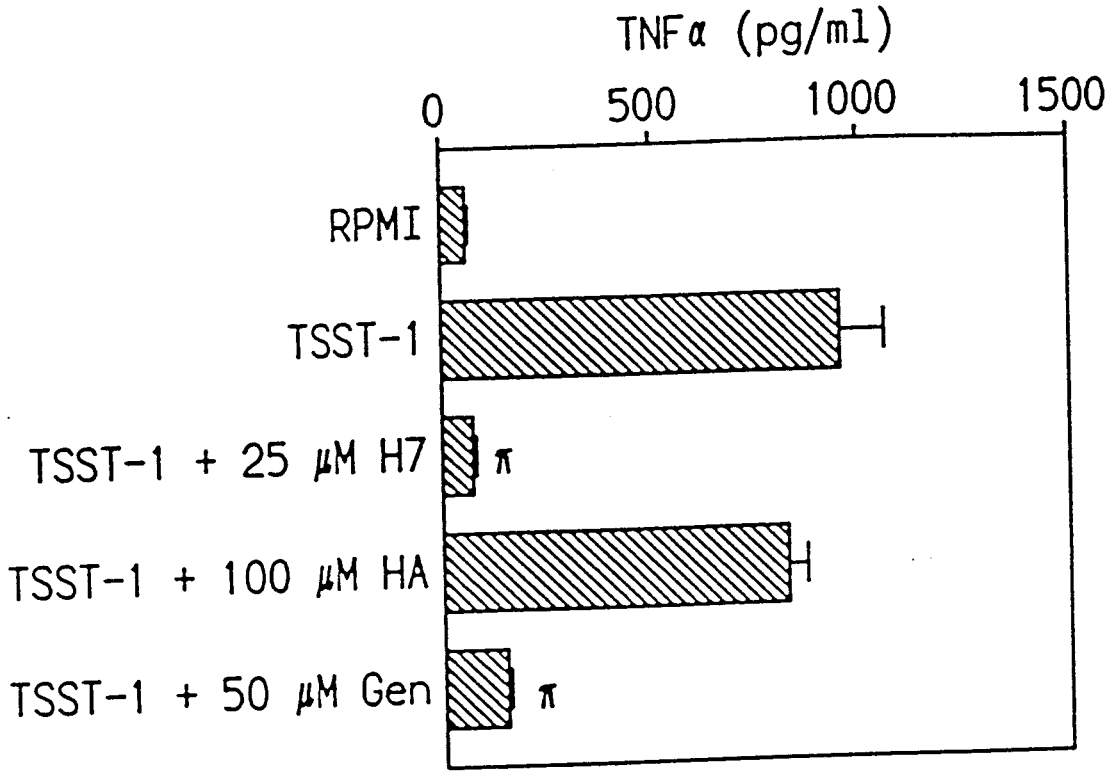

Figure 38. Effect of kinase inhibitors on TSST-1-induced TNFα secretion by human monocytes (1 x 10^6 cells) co-cultured 1:1 with T lymphocytes in RPMI 1640 medium. Cells were pretreated with 25 μM H7, 100 μM HA1004, or 50 μM genistein for 15 min prior to the addition of 10 μg/ml TSST-1 for 24 h at 37°C. TNFα levels in culture supernatants were determined by ELISA. The results represent the mean ± SEM for 12 donors. (π) indicates significant difference ($P < 0.001$) compared with TSST-1 control.

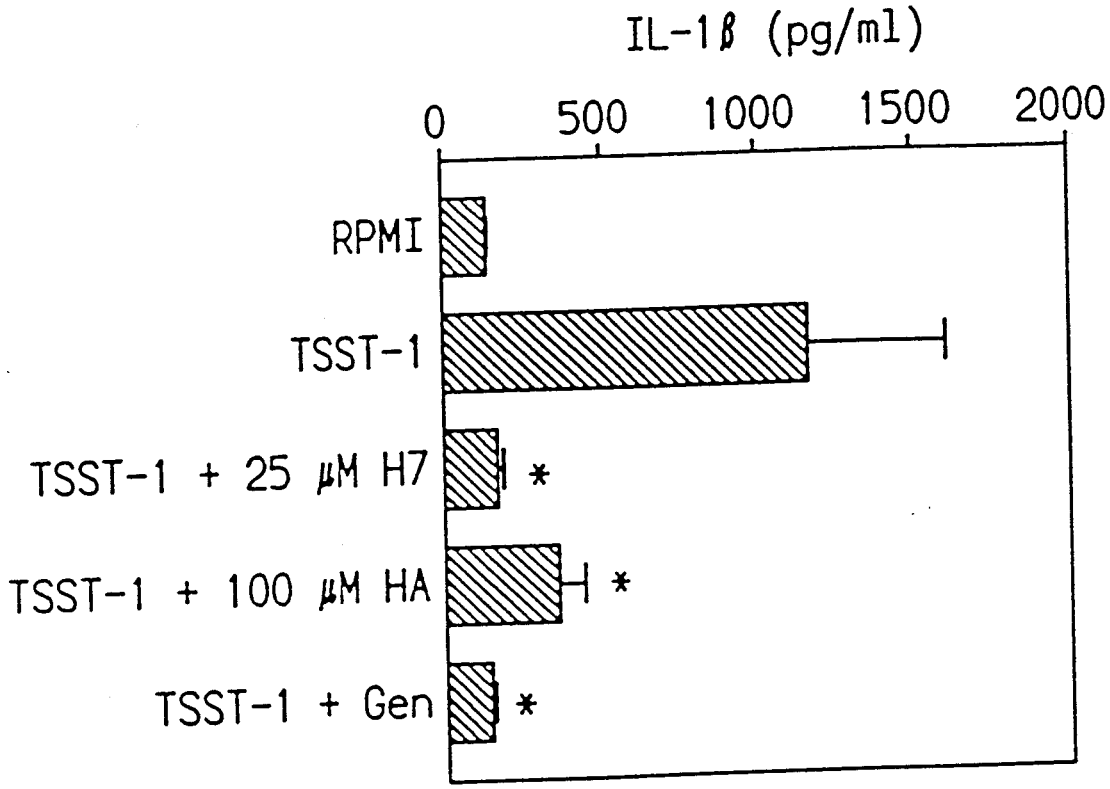

Figure 39. Effect of kinase inhibitors on TSST-1-induced IL-1ß secretion by human monocytes (1 x 10^6 cells) co-cultured 1:1 with T lymphocytes in RPMI 1640 medium. Cells were pretreated with 25 µM H7, 100 µM HA1004, or 50 µM genistein for 15 min prior to the addition of 10 µg/ml TSST-1 for 24 h at 37°C. Culture supernatants were assayed for IL-1ß by ELISA. The results represent the mean ± SEM for 11 donors. (*) indicates significant difference ($P < 0.05$) compared with TSST-1 control.

6.2 Discussion

The present study shows that inhibitors of second messenger pathways effectively block TSST-1-induced cytokine release from co-cultures of monocytes and T cells. Furthermore, our studies suggest that induction of TNFα and IL-1ß secretion by TSST-1 is differentially regulated. Both TNFα and IL-1ß secretion were strongly inhibited by H7, suggesting a role for protein kinase C in cytokine production or secretion by monocyte/T cell co-cultures after toxin stimulation. Protein kinase C is an enzyme responsible for phosphorylating a range of cellular proteins at serine or threonine residues (Nishizuka, 1988; Nishizuka et al., 1984). The phosphorylation of these proteins results in their activation for a wide variety of cellular functions including cell proliferation, protein secretion and exocytosis, and gene expression (Nishizuka, 1988; Nishizuka et al., 1984). H7 inhibits protein kinase C by binding to the catalytic fragment of the enzyme (Hidaka et al., 1984). The role of protein kinase C has also been implicated in gene expression and protein production by murine macrophages in response to LPS stimulation. Kovacs et al. (1988) have shown that in murine peritoneal macrophages, IL-1α, IL-1ß, and TNFα mRNA expression is inhibited in a dose-dependent manner by H7 during stimulation with LPS. Furthermore, they showed that the secretion of these cytokines by macrophages was inhibited when H7 was added after LPS stimulation, suggesting that post-translational events were also influenced by protein kinase C. Taniguchi et al. (1989) also reported that secretion of IL-1 by LPS-stimulated human monocytes was significantly inhibited by H7. Recently, in contrast to the work of Kovacs et al. (1988), Bakouche et al. (1992) showed that protein kinase C plays a major role in IL-1 secretion but not its production following LPS stimulation. Using LPS-treated human monocytes, they showed that the protein kinase C inhibitors, H7 and staurosporine, did not block the production of intracellular IL-1α or IL-1ß protein, indicating protein kinase C was not involved in this process. However, the inhibitors were strongly effective in suppressing extracellular release of IL-1, therefore suggesting that IL-1 secretory processes were highly dependent

on protein kinase C. The authors further showed that regulation of IL-1 production was independent of its secretion in LPS-stimulated monocytes by using LPS incorporated into multilamellar liposomes (MLV-LPS). Whereas free LPS activated monocytes by causing the translocation of protein kinase C from cytosol to the membrane, the production of IL-1α and ß and the secretion of IL-1, the MLV-LPS was only able to stimulate IL-1 production. MPV-LPS did not stimulate protein kinase C translocation or IL-1 secretion (Bakouche et al., 1992).

The inhibition of TSST-1-induced cytokine secretion by H7 appears to contradict the results obtained in Chapter 4, where TSST-1 and PMA were found to induce different phosphorylation profiles in human monocytes. Therefore, the question arises as to whether protein kinase C is actually involved in monocyte activation by TSST-1. In the cytokine experiments, we were examining the effects of H7 on IL-1ß and TNFα secretion by monocyte/T cell co-cultures whereas in the phosphorylation studies, we were studying the early signal transduction events in monocytes alone after the addition of TSST-1. In the former case, H7 may be inhibiting protein kinase C activation not only in monocytes, but also in T cells. TSST-1 has been reported to stimulate protein kinase C translocation from the cytosol to the plasma membrane in T lymphocytes (Chatila et al., 1988). It is possible that H7 is interfering with TSST-1-stimulated protein kinase C activation in T lymphocytes which, in turn, may prevent monocytes from receiving additional signals from T cells necessary for cytokine secretion. Alternatively, the differences in phosphorylation profiles in monocytes alone induced by TSST-1 and by PMA may reflect the fact that protein kinase C stimulation is not required for early events such as transcriptional activation of cytokine genes (Bakouche et al., 1992). However, protein kinase C activity may be involved in downstream processes such as cytokine secretion as has been previously documented for LPS (Bakouche et al., 1992). Future studies must address the mechanism by which H7 is inhibiting cytokine secretion as well as the target cells (ie. monocytes, T cells, or both) this protein kinase C inhibitor is affecting.

Cytokine secretion induced by TSST-1 appears to involve more than one second messenger pathway. An inhibitor of tyrosine kinases was also effective in

suppressing both TNFα and IL-1ß levels. To our knowledge, inhibition of IL-1ß and TNFα by the tyrosine kinase inhibitor, genistein, either in response to TSST-1 or LPS has not been reported. As demonstrated in Chapter 4, TSST-1 and the staphylococcal enterotoxins induce the tyrosine phosphorylation of several monocyte cellular phosphoproteins. Whether the tyrosine phosphorylation of these monocyte cellular proteins in response to TSST-1 stimulation represents the initial signals that eventually result in cytokine production is not immediately clear. Further characterization of the tyrosine kinases and their substrate proteins are required to address this question.

Our data also suggest that the induction of TNFα and IL-1ß secretion by TSST-1 may be differentially regulated. The cAMP- and cGMP-dependent protein kinase inhibitor, HA1004, strongly suppressed IL-1ß but not TNFα release in human monocyte/T cell cultures stimulated with TSST-1. Differential inhibition of IL-1 and TNF gene expression and protein production has also been reported for LPS-stimulated murine macrophages. Kovacs et al. (1988) have shown that while protein kinase C and calmodulin-dependent pathways were involved in the induction of IL-1 gene expression and production by LPS, only protein kinase C was involved in TNFα expression and secretion. Additionally, work by Bakouche et al. (1992) demonstrated that LPS presented to human monocytes in multi-lamellar vesicles resulted in the production and secretion of IL-1α but not IL-1ß. These findings imply that LPS and possibly TSST-1 can activate the production of one cytokine without the concomitant production of the other (Bakouche et al., 1992, Kovacs et al., 1988).

The relationship between the various kinases in TSST-1-induced IL-1ß and TNFα secretion is not known from the present study. It is possible that there are multiple independent pathways by which TSST-1 can induce the secretion of IL-1ß or TNFα. However, a more likely mechanism is the involvement of "cross-talk" between the different kinases. In general, activation of serine/threonine kinases occurs in parallel with tyrosine kinases. Perhaps the early events in cytokine production or secretion involve one group of protein kinases but subsequent downstream responses may be mediated by another group. Interestingly, we have

observed that the induction of TNFα secretion in human monocytes by PMA is suppressed both by H7 and genistein, therefore suggesting the involvement of multiple kinases. However, to decipher the role of each kinase along the pathway, other studies such as examination of IL-1ß and TNFα mRNA gene expression must be performed in parallel.

One general concern about the use of these inhibitors is that their specificity varies, depending on the concentrations used. For example, H7 can also inhibit both cAMP- and cGMP-dependent kinases but has the greatest activity towards protein kinase C (K_i = 6.0 μM) (Hidaka et al., 1984). However, the use of a range of kinase inhibitors with overlapping specificities may allow one to make some general conclusions. For all the inhibitors used in our experiments, concentrations were selected on the basis of their specificity towards the particular kinase at its reported K_i (Hidaka et al., 1984). The concentrations of kinase inhibitors used were not different from those used by others on human monocytes (Bakouche et al., 1992; Imamura et al., 1990; Taniguchi et al., 1989). Moreover, HA1004 inhibited IL-1ß but not TNFα secretion, further demonstrating its specific action on cAMP- and cGMP-dependent kinases.

In summary, our results indicate that TSST-1-induced cytokine secretion by human peripheral blood mononuclear cells is controlled by various second messengers. TNFα secretion is suppressed by inhibitors of both protein kinase C and tyrosine kinases. In contrast, IL-1ß release is blocked by inhibitors of cAMP- and cGMP-dependent protein kinases as well as by inhibitors of protein kinase C and tyrosine kinases, suggesting that the two cytokines are differentially regulated in response to TSST-1 induction.

6.3 References

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Chapter 7

Summary, Perspectives And Future Research Directions

7.1 Summary Of Thesis Work

The objective of this thesis was to focus on the mechanism of human blood monocyte activation by TSST-1. The monocyte has been considered a key target cell for TSST-1 although, as demonstrated in this thesis, T lymphocytes also play an important role. By dissecting the pathways involved in monocyte activation by TSST-1, we can learn more about the biological properties of this superantigen and determine how they differ from those of a conventional antigen. We systematically examined TSST-1-monocyte interaction from the initial binding of the toxin to its receptor to its final biological effect, the induction of cytokine release. For obvious reasons, there are still many issues to be resolved and some of these will be addressed in section 7.3. The main findings of this thesis are summarized as follows:

1) TSST-1 bound to a single class of high affinity receptors on human peripheral blood monocytes. Approximately 32,000 receptors were found per cell (K_d = 32 nM). Chemical cross-linking studies showed that the receptor consists of a 35 kd and 28 kd subunit.

2) TSST-1 and SEA competed for the same receptor but bound to overlapping epitopes as demonstrated by cross-competition binding studies. None of the other staphylococcal enterotoxins could compete for TSST-1 binding sites. Inhibition of TSST-1 and SEA binding to human monocytes by the anti-HLA-DR mAb, L243, provided strong evidence that the receptor is the MHC class II HLA-DR antigen. Based on these studies and those of others which demonstrate overlapping receptor epitopes for SEA and SEB (Fraser, Nature 339:221-223, 1989), and separate epitopes on HLA-DR for TSST-1 and SEB

(Scholl et al., J. Immunol 143:2583-2588, 1989), we propose that SEA occupies a binding site within HLA-DR that partially overlaps with both TSST-1 and SEB. The binding site for TSST-1 and SEB, in contrast, appear to be topographically distinct.

3) The L243 mAb blocked TSST-1- and SEA-induced monocyte-dependent T cell proliferation, suggesting that binding of these toxins to HLA-DR on accessory cells is a prerequisite for T cell activation.

4) As determined by two-dimensional gel electrophoresis of ^{32}P-labeled monocyte cellular proteins, TSST-1 induced the dephosphorylation of several phosphoproteins in a time- and dose-dependent manner. In contrast, neither SEA nor SEB induced this dephosphorylation pattern, but instead, stimulated the phosphorylation of a different set of proteins, suggesting that the early signal transduction events initiated in human monocytes by SE are different from those of TSST-1.

5) Protein phosphorylation profiles for other monocyte agonists such as bacterial LPS and the protein kinase C activator, PMA, were distinct from those generated by TSST-1, suggesting that these agents differ in their mechanism of monocyte activation. The similarity in phosphorylation patterns induced by LPS and PMA indicates that LPS may be activating human monocytes by a protein kinase C-dependent mechanism.

6) The use of an anti-phosphotyrosine mAb in Western blot analysis showed that TSST-1 and SEA stimulated the phosphorylation of several common cytosolic proteins (molecular weights ranging from 18 kd to 35 kd) in human monocytes. On the other hand, PMA did not induce the tyrosine phosphorylation of several of these cytosolic proteins, suggesting that protein kinase C is not involved in the induction of tyrosine phosphorylation by TSST-1 or SEA.

7) TSST-1-stimulation of TNFα and IL-1ß release requires the presence of both monocytes and T lymphocytes. Monocytes alone could not produce extracellular cytokines in response to TSST-1 stimulation. This is in contrast to E. coli LPS where T cells are not required for the induction of IL-1 and TNF release from human monocytes. The phosphorylation and cytokine studies add support to the hypothesis that TSST-1 and LPS activation of human monocytes occur by distinct signaling mechanisms.

8) The role of T lymphocytes in the induction of cytokine secretion by TSST-1 in monocytes was examined. IFN-τ alone did not substitute for the T cell requirement, as indicated by the following evidence: a) priming of monocytes alone with this lymphokine followed by stimulation with TSST-1 did not result in cytokine production, and b) the addition of anti-human IFN-τ antibodies to TSST-1-treated monocyte/T cell co-cultures did not block cytokine production. Direct contact between monocytes and T cells, however, was found to be important since the separation of these two cell types by chamber inserts (equipped with a 0.45 μm membrane) abolished cytokine production induced by TSST-1. Interaction of monocytes with T cell membrane-associated molecules is not sufficient since metabolic inactivation of T cells or monocytes also blocks TSST-1-induced cytokine secretion. This result suggests that activated T lymphocytes and monocytes are required for cytokine secretion.

9) TSST-1-mediated monocyte/T cell interaction also involved the LFA-1 adhesion molecule since mAbs to the alpha and beta subunit of LFA-1 significantly reduced both IL-1ß and TNFα secretion.

10) The functional relevance of protein kinases in cytokine induction by TSST-1 was explored in monocyte/T cell co-cultures. IL-1ß secretion was suppressed by inhibitors of protein kinase C (H7), tyrosine kinases (genistein) and cAMP- and cGMP-dependent kinases (HA1004). In contrast,

secretion of TNFα was blocked by only H7 and genistein, suggesting that induction of the secretion of these two cytokines may be differentially regulated.

7.2 Hypothesis Of TSS Pathogenesis-An Update

Since 1987, the year in which this thesis work began, an explosion of information has added to our understanding of TSS pathogenesis. Although TSST-1 is considered to be the major etiologic agent in this disease, there is little doubt that staphylococcal enterotoxins also play a major role, particularly where TSST-1-negative S. aureus isolates are involved. With the knowledge accumulated during the 5 years of studying TSS pathogenesis, a hypothesis will be proposed in this section with emphasis on the causation of menstrual TSS.

Presumably, the process begins with the colonization of a toxin-producing S. aureus isolate in the host, either at an urogenital site (menstrual TSS) or at other focal sites in the body (nonmenstrual TSS). The clonality and phenotypic relatedness (eg. reduced hemolytic activity, propensity to produce TSST-1, tryptophan auxotypy) of vaginal TSST-1-producing S. aureus isolates suggest that these isolates may have the unique ability to colonize the genital tract as opposed to the skin, nasal mucosa, or other sites. However, colonization of the genital tract by a TSST-1-producing S. aureus isolate is not sufficient to cause TSS since about 10% of women normally harbor this toxin-positive microoganism. (Davis et al., 1982). Other factors such as physiological pH, pO_2, pCO_2, protein levels, calcium and magnesium concentrations, etc. are also important for the growth of S. aureus and TSST-1 production in the genital milieu (Todd et al., 1987). There is evidence that apart from aeration, menstrual blood appears to provide the necessary constituents required for TSST-1 production by urogenital TSS-associated S. aureus isolates (Todd et al., 1987). The unique in vivo environment during menstruation, coupled with the ability of tampons to raise the pO_2 in an otherwise anaerobic vagina (Mahoney Jr, 1988), may be adequate for in

vivo production of TSST-1 during menstruation. The ability of TSST-1 to circulate in blood and interact with immune blood cells depends on the presence of pre-existing immunity. The lack of neutralizing antibodies to TSST-1 may predispose an individual to the effects of this toxin (Notermans et al., 1983). In the absence of pre-existing immunity, circulating TSST-1 and/or staphylococcal enterotoxins bind to HLA-DR (and to HLA-DQ and HLA-DP to a lesser extent) on human blood monocytes or on B lymphocytes and initiate a series of events which result in the activation of a number of kinases and phosphatases with the subsequent phosphorylation and/or dephosphorylation of a number of cellular proteins. The ability of TSST-1 and staphylococcal enterotoxins to bind to monocytes also depends on the allelic forms of HLA-DR present in a particular individual (Herman et al., 1990). Aside from activating monocyte protein kinases and phosphatases, the binding of staphylococcal exotoxins to HLA-DR on monocytes and B lymphocytes also allows the presentation of these superantigens to T lymphocytes as shown in Figure 40. Specifically, the MHC class II-toxin complex on monocytes recognizes the complementary TCR V_β sequences on both $CD4^+$ and $CD8^+$ T lymphocytes, resulting in the proliferation of a higher proportion of this cell population compared with conventional antigens (Herman et al., 1991; Marrack and Kappler, 1990). Furthermore, unlike conventional antigens, superantigens do not require processing, do not occupy the MHC antigen-binding groove, and show no MHC class II restriction (Herman et al., 1991; Marrack and Kappler, 1990). The interaction between the V_β sequence of TCR and MHC class II-superantigen complex is additionally strengthened by the interaction of adhesion molecules (eg. LFA-1 and ICAM) present on T lymphocytes and accessory cells. The binding of these exotoxins to different or overlapping epitopes on MHC class II HLA-DR (Chintagumpala et al., 1991; Fraser, 1989; Scholl et al., 1989; See et al., 1990) as well as the stimulation of different V_β subsets of the T cell TCR (Marrack and Kappler, 1990) suggest the potential for additive or even synergistic effects between these toxins. This may be particularly true for TSST-1 and SEA, which are commonly expressed together in vaginal isolates of TSS S. aureus isolates (Chang et al., 1991). Interaction of monocytes with T cells through the V_β-TSST-1-HLA-DR

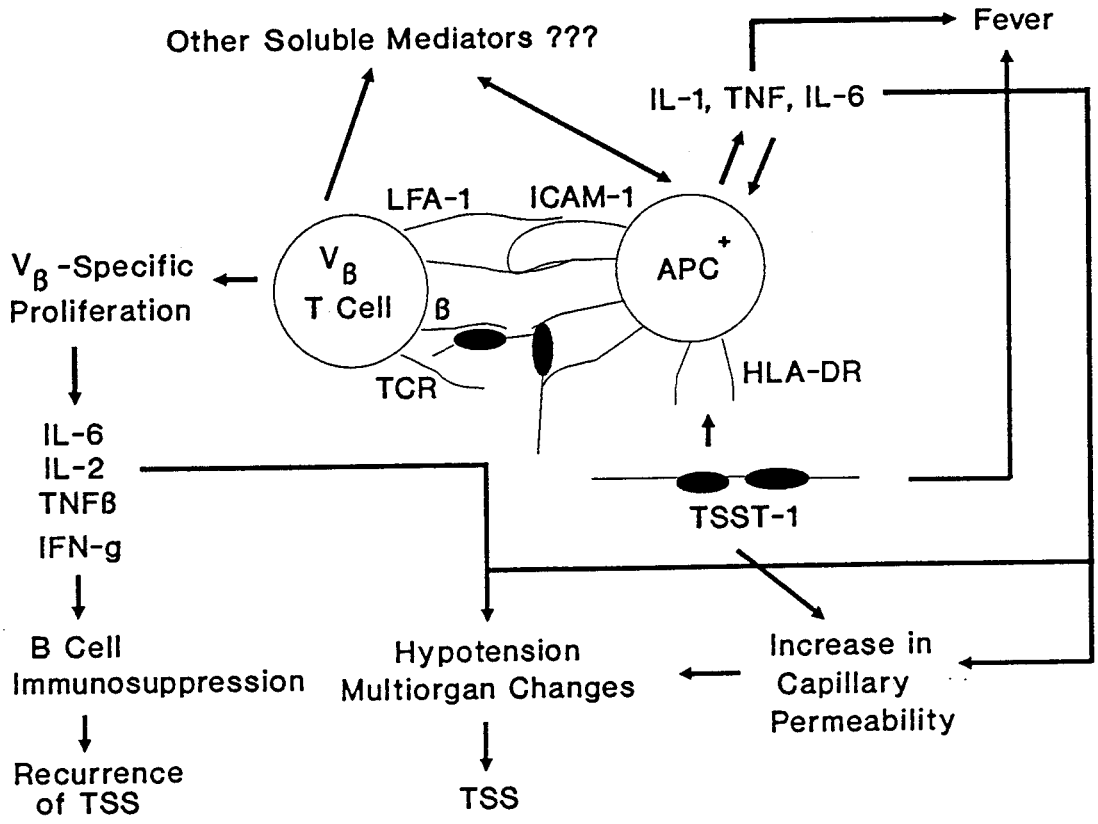

Figure 40. Updated Hypothesis of TSS Pathogenesis. Abbreviations: APC = antigen presenting cell; IFN-g = interferon-γ.

trimolecular complex results in a cascade of events leading to cytokine production by both cell types. Initial binding of TSST-1 to the HLA-DR receptor on human monocytes results in activation of monocytes as evidenced by dephosphorylation changes. However, secretion of cytokines like IL-1 and TNF does not occur in these cells unless there is direct cell contact with metabolically active T lymphocytes. Similarly, TSST-1 activation of T lymphocyte proliferation and secretion of lymphokines (eg. IL-2, TNFß, and IFN-τ) is also dependent on cell contact between T lymphocytes and MHC class II-bearing accessory cells. With the subsequent activation of monocytes and T lymphocytes, the massive release of cytokines from both cells contributes to the fever, hypotension, respiratory distress, shock and multisystem organ failure phenomena seen among TSS patients.

Finally, although the mechanism by which these toxins act is clearer than before, the question still remains as to how these bacterial superantigens may benefit the invading microorganism in the host. The stimulation of the host immune system may initially seem like a self-defeating process for the bacteria. However, it has been postulated that the overstimulation of the host immune cells and activation of massive cytokine release may create such a chaos within the host itself that the pathogen may escape detection. (Herman et al., 1991; Marrack and Kappler, 1990). How this chaos allows bacteria to escape detection by the immune system is not yet known. Moreover, as pointed out in Chapters 1 and 3, the immunosuppressive properties of these bacterial toxins plus the ability of these exoproteins to direct cytotoxic T lymphocytes against MHC class II^+ antigen-presenting cells may further confer a survival advantage to the bacteria (Herman et al., 1991). Therefore, evolutionary pressure may favor selection of bacteria like S. aureus capable of producing superantigens that can bind to a large array of MHC class II molecules (Herman et al., 1991). This might be facilitated by the fact that toxins such as TSST-1 may be encoded on a transposon (Kreiswirth et al., 1989).

In summary, as indicated in Chapter 1 of this thesis, TSS appears to be a multifactorial disease. Host factors, exposure to toxin-producing isolates of S. aureus, optimal toxin production in vivo, and immunogenetics (eg. HLA-DR type)

all play a major role in the susceptibility of an individual to TSS. The accumulated clinical, epidemiological, immunologic, and microbiological data strongly suggest that the pathogenesis of this disease represents a complex interaction of these and possibly other yet undefined factors. As outlined in the next section, more work will need to be done to further our understanding of the interaction of this amazing toxin with the human immune system.

7.3 TSS-Future Research Directions

Although the picture of TSS pathogenesis has become clearer in the last 5 years, there is still much to learn about the role of TSST-1 in this disease. The work in this thesis has opened up new avenues of research relating to the mechanism of action of this toxin. With respect to protein phosphorylation, the specific kinases/phosphatases and their phosphoprotein substrates that mediate monocyte responses to TSST-1 and staphylococal enterotoxins need to be characterized. These studies might also provide some clues as to why SEA and SEB induce different patterns of protein phosphosphorylation than TSST-1. Further studies on the phosphoproteins commonly dephosphorylated by TSST-1 may also prove interesting. Studies in Chapter 5 indicate a role for T lymphocytes in cytokine production by human monocytes. It would be interesting to analyse the phosphorylation patterns of monocyte cellular proteins in the presence and absence of T cells. ^{32}P-labeled monocytes could be incubated with or without unlabeled T lymphocytes and the phosphorylation profiles of monocyte cellular proteins examined after TSST-1 stimulation as described previously.

T cells may influence cytokine production by human monocytes at the level of gene transcription or of protein secretion. There is strong evidence, at least with LPS, that these two processes are independent of one another. In our study, direct contact between monocytes and T cells may affect either or both of these processes. More work is required to define the stage at which TSST-1-mediated cytokine production is affected by monocyte-T cell contact. Moreover, the role of LFA-1 in cytokine production needs to be defined. Besides facilitating TCR

recognition of the MHC class II-toxin complex, LFA-1 may play a role in transmembrane signalling. Finally, fixation of monocytes or T cells indicates that, besides cell contact, metabolic activity of both cells are important. Perhaps other as yet unidentified soluble mediators are also important for cytokine production in response to TSST-1 stimulation. The use of antibodies to different factors (eg. IL-2, IL-3, TNFß etc.) may help to identify which, if any, of these are important in the induction of cytokines by TSST-1.

As shown in Chapter 6, protein kinases and/or phosphatases appear to play an important role in monocyte activation. Whether these kinases are acting at the level of gene transcription or at the level of post-transcription/translation processes needs to be defined. These proposed studies will help to shed light on the mechanism of action of TSST-1 and other bacterial superantigens.

To conclude, I have attempted to clarify some of the mechanisms by which bacterial superantigens interact with human blood immune cells, particularly monocytes. Further studies will not only enhance our understanding of the role of bacterial superantigens in diseases such as TSS, but will also provide important clues to future preventative strategies in bacterial toxin-mediated diseases such as staphylococcal and streptococcal TSS.

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