Functional and Molecular Assessment of the Role of Toxic Shock Syndrome Toxin-1, Staphylococcal Enterotoxin A, and Staphylococcal Enterotoxin B in the Pathogenesis of Toxic Shock Syndrome

by

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ABSTRACT

Staphylococcus aureus produces exoproteins including toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin B (SEB) which have been implicated in causing an acute multisystem disease called toxic shock syndrome (TSS). These toxins belong to the family of proteins known as superantigens. Superantigens cause profound immunological disturbances which are believed to underly the mechanisms causing TSS upon infection with toxigenic strains of *S. aureus*.

Production of TSST-1, SEA and SEB are examples of variable genetic traits in that they are produced by some *S. aureus* isolates but not others. Certain toxin combinations are more prevalent than others. For example, SEA and TSST-1 are frequently co-produced among menstrual TSS isolates, whereas TSST-1 and SEB appear to rarely, if ever, be co-produced. The nature of these interactions on both functional and molecular levels was examinined in this thesis.

It was hypothesized that co-production of SEA and TSST-1 might yield a more virulent strain than production of TSST-1 alone, providing a putative explanation for why TSST-1^+/SEA^+ strains are associated with the majority of menstrual TSS cases. To test this hypothesis, a TSST-1^+/SEA^- derivative of a TSST-1^+/SEA^+ clinical strain was constructed by the method of plasmid integration. Introduction of the TSST-1^+/SEA^+ and TSST-1^+/SEA^- strains into a tampon-associated vaginal infection model of TSS and a D-galactosamine sensitized mouse model of lethal shock however did not demonstrate any detectable differences in lethality or morbidity between the strains. In addition, *in vitro*

analyses revealed that the culture filtrate from the SEA$^-$ mutant did not differ in its ability to induce TNFα secretion from rabbit splenocytes or stimulate T cell proliferation at low dilutions when compared to the TSST-1$^+$/SEA$^-$ strain. This suggests that production of SEA with TSST-1 in this strain does not increase the virulence of *S. aureus*.

The molecular basis of mutually exclusive production of TSST-1 and SEB in *S. aureus* was examined. Analysis of TSST-1$^+$ isolates indicated that 8 of 8 TSST-1$^+$ isolates had sequences homologous with *seb*, even though SEB was not produced. PCR analysis with primers that targeted various regions of *seb* failed to amplify the expected PCR product, suggesting that a full SEB coding sequence is not present. Hybridization of restriction fragments with TSST-1 and SEB genetic element probes suggested that *tst* was located in close proximity to SEB genetic element sequences on the chromosome. DNA similiarities between TSST-1$^+$ and SEB$^+$ isolates were also found upon hybridization with SEB genetic element probes. These observations could suggest that the SEB genetic element carries a preferred site of TSST-1 genetic element insertion. Upon insertion, the TSST-1 genetic element may interfere with the expression of SEB in as yet some unidentifed manner. Alternatively, the TSST-1 and SEB genetic elements may share a common point of insertion in the *S. aureus* chromosome linked to DNA sequences homologous to the SEB genetic element. Insertion of one genetic element carrying one of the toxin genes may inhibit the insertion of the other genetic element carrying the alternate toxin gene.

TABLE OF CONTENTS

Chapter 2. Materials and Methods

Chapter 7. Summary of Scientific Contributions and Future Avenues of Research

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

APC	antigen presenting cell
agr	accessory gene regulator
BHI	brain heart infusion
BSA	bovine serum albumin
Cm	chloramphenicol
Em	erythromycin
ET	electrophoretic type
δ-IFN	gamma-interferon
i.p.	intraperitoneal
IL	interleukin
LB	Luria-Bertani
LD	lethal dose
LFA	lymphocyte function antigen
MTSS	menstrual toxic shock syndrome
NMTSS	nonmenstrual toxic shock syndrome
PBS	phosphate buffered saline
PBS-T	PBS + .05% Tween 20
PBS-TW	PBS + .1% Tween 20
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
MLEE	multilocus enzyme electrophoresis
NZW	new zealand white
SDS	sodium dodecyl sulfate
SDS-Page	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCR	T cell receptor
TH	T helper
TNF	tumor necrosis factor
TNFa	tumor necrosis factor alpha
Tc	tetracycline
TSA	tryptic soy agar
TSB	tryptic soy broth
TSS	toxic shock syndrome
TSST-1	toxic shock syndrome toxin-1
SEA	staphylococcal enterotoxin A
SEB	staphylococcal enterotoxin B
SEC	staphylococcal enterotoxin C
sar	staphylococcal accessory regulator
sae	*S. aureus* exoprotein expression

LIST OF APPENDICES

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Chapter 1. BACKGROUND

1.1. Clinical, Epidemiologic and Microbiologic Features of Toxic Shock Syndrome

1.1.1. Historical Perspectives/Epidemiology/Prevalence

Although illnesses clinically resembling the manifestations observed in toxic shock syndrome (TSS) have been described since 1927 (Chesney *et al.*, 1984), the first documented case of TSS was portrayed by Dr. James Todd in 1978 while working at the Children's Hospital in Denver, Colorado (Todd *et al.*, 1978). Dr. Todd named the syndrome and first described its association with the organism *Staphylococcus aureus*. Shortly thereafter, many studies aimed at determining what risk factors may play a part in the etiology of the disease were carried out. As a result, in 1980, a positive association was found between TSS and tampon use among women during menstruation (Davis *et al.*, 1980; Shands *et al.*, 1980). In particular, it was noted that women using Rely^{R} tampons (distributed by Proctor and Gamble Co.) were at a greater relative risk than women using other tampon brands (Schlech *et al.*, 1982). At this time (September 1980) Rely^{R} was voluntarily removed from the market by the company.

TSS was listed as a nationally reportable disease by the United States Center for Disease Control (CDC) in 1982 (Chesney *et al.*, 1984). Using a passive-surveillance system, the number of reported cases of menstrual TSS (MTSS) was found to peak in 1980, followed by a significant decline thereafter (Broome, 1989). Whether the decrease was due to an actual lower incidence, or as a result of removal of Rely^{R} brand tampons from the market in conjunction with increased public awareness leading to changes in the number of women

using tampons, or their menstrually-related habits, is not known.

Although a positive association between TSS and tampon use during menstruation was made early on in the identification of this syndrome, cases of TSS in men, children, and nonmenstruating women have also been described (Silver, 1980; Reingold *et al.*, 1982; Bartlett *et al.*, 1982; Chesney *et al.*, 1984). Nonmenstrual TSS (NMTSS) is often associated with the presence of *S. aureus* in focal wound or soft tissue infections. The temporal trends from 1979-1986 are difficult to assess due to the low incidence rate during this time period (Broome, 1989). However in recent years, NMTSS is becoming more prevalent than MTSS (Chow, 1993).

The estimated prevalence of TSS is 0.22 to 1.23 cases per 100,000 (Chow, 1995). Recurrences are quite frequent (15-22%) (Chow, 1995). Today, TSS still remains a potentially life-threatening disease with a mortality rate of 3 to 7% (Chow, 1995).

1.1.2. Causative Agent of Toxic Shock Syndrome

Staphylococcal TSS is caused by various toxins elaborated by the organism *Staphylococcus aureus*. The toxins that are strongly implicated in causing the disease include toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB) and staphylococcal enterotoxin C (SEC).

S. aureus isolates associated with TSS are phenotypically and genotypically distinct from other *S. aureus* strains, as reviewed by See and Chow (1989). The most important differentiating factor of TSS-associated strains is the production of TSST-1. TSST-1 was found to be produced by over 90% of staphylococci isolated from TSS patients (Crass and Bergdoll, 1986a). In addition, Todd *et al.* (1984) showed that TSS-associated strains of *S.*

aureus were also more likely to produce staphylococcal enterotoxins than non-TSS strains. Altemeier *et al.* (1982) found that the majority of TSS-associated *S. aureus* isolates belonged to phage group 1, and were lysed by phages 29 and 52. Other investigations have confirmed these findings (Kreiswirth *et al.*, 1984; Marples and Wieneke, 1993). In addition, TSS-associated *S. aureus* strains were less likely to carry plasmids (Todd *et al.*, 1984), exhibited resistance to arsenate, cadmium and pencillin (Barbour, 1981; Todd *et al.*, 1984; Kreiswirth *et al.*, 1984), were less likely to produce pigment (Todd *et al.*, 1984), exhibited bacteriocin susceptibility (Todd *et al.*, 1984), were more proteolytic for the substrates hemoglobin (Barbour, 1981) and casein (Todd *et al.*, 1984; Todd *et al.*, 1985), and have reduced hemolytic activity (Barbour, 1981; Schlivert *et al.*, 1982; Todd *et al.*, 1984). Vaginal TSS isolates were also less likely to produce lipase and nuclease (Schlievert *et al.*, 1982). TSS-associated *S. aureus* strains have also been found to be more lethal in rabbit models of TSS (Scott *et al.*, 1983; Arko *et al.*, 1984; Melish *et al.*, 1989). Collectively these observations suggest that *S. aureus* strains associated with TSS are phenotypically distinct from other *S. aureus* strains.

A. Carriage Rate of Staphylococci in the Population

Although in general staphylococci are found on the skin, skin glands and mucous membranes of warm-blooded animals, certain staphylococcal species colonize specific anatomical sites (Howard and Kloos, 1987). *S. aureus* is generally found in the anterior nares. Jacobson *et al.* (1984) observed a *S. aureus* nasal carriage rate of 31% in 770 patients admitted to a hospital in Utah. The carrier rate for TSS-associated protein (or TSST-1) positive strains in this study was 6.6%. In a separate study, Martin *et al.* (1982)

found a 26.9% nasal carriage rate and a 10.3% vaginal carriage rate in 145 young women. Studies by Guinan *et al.* (1982) and Chow *et al.* (1983) found vaginal carriage rates of 9.2 and 6.8% in 808 and 495 women respectively, indicating that *S. aureus* is not typically part of the vaginal microflora. In the latter study, 2.6% of the women were colonized with TSST-1 producing strains of *S. aureus*. A close association was found between nasal and vaginal carriage in the study by Martin *et al.* (1982), in that 60% of the women with vaginal staphyloccoci also had nasal carriage. This rate of nasal carriage was significantly higher in comparison to nasal carriers whose vaginal cultures were negative for *S. aureus*. This study provides support for the suggestion that MTSS may be mediated by the deposition of *S. aureus* in the vagina through the tampon, which may have become contaminated by the woman's own hands (Mortimer, 1982). Consistent with this is the observation made by Linnemann *et al.* (1982) that *S. aureus* isolates obtained from nasal and genital cultures of the same individual were of the same phage type, suggesting a clonal relationship. In a similiar manner, nasal carriage of *S. aureus* may be an important factor dictating predisposition to NMTSS through infection of surgical wounds by autoinoculation (Mortimer, 1982). These studies provide a basis for estimating the percentage of the population that is potentially at risk for developing TSS.

1.1.3. **Clinical Spectrum and Diagnosis of Toxic Shock Syndrome**

The pathological manifestations of TSS can be primarily divided into four distinctive features, as summarized by Chesney (1989). First, TSS can be readily characterized by the rapidity of its onset in an otherwise healthy individual. Upon initial presentation, symptoms of fever, chills, malaise, headache, myalgias, muscle tenderness, vomiting and diarrhea

commonly exist. The second unique feature of TSS is the rapid onset of hypotension resulting from a loss of vascular tone and the massive leakage of fluid from the intravascular to the interstitial space. In fact TSS has been often referred to as a 'capillary leak syndrome'. The hypotension observed in TSS patients leads in part to the pathological effect that is observed in almost every organ system. The involvement of almost every organ system is the third distinctive aspect observed in TSS patients. Finally, TSS is associated with various dermatologic sequalae. Upon presentation, TSS patients may exhibit a scarlatinform rash, that 10-21 days after onset of the disease may result in full thickness desquamation, in particular of the palms of the hands and soles of the feet. Nonpitting edema and petechiae may be observed in conjunction with inflammation of mucous membranes, particularly of the oropharynx, conjunctiva and vagina (Chow, 1983). In addition, a 'strawberry tongue' has been described in over 25% of one patient group (Chow, 1983).

Recently Kain *et al.* (1993) attempted to determine by multivariate discriminant analysis if the clinical manifestations associated with NMTSS could be differentiated from those observed in MTSS. Their studies revealed that NMTSS was indeed clinically distinct from MTSS in several ways. Patients with NMTSS had an earlier onset of fever and rash with respect to the other clinical features observed in TSS, had less frequent musculoskeletal involvement, more severe anemia, and more frequent central nervous system complications than patients with MTSS. Thus clinically, distinctions between MTSS and NMTSS can be made.

A strict case definition for TSS as outlined by the CDC in the United States has been

established (Table 1). Due to the variations in the clinical manifestations that may occur among patients, a diagnosis of probable TSS can be made if one of the six criteria outlined in Table 1 is absent. Since a specific diagnostic test for TSS is lacking, a diagnosis must be made based upon the exclusion of other clinical entities that present similiarly. Attempts are made to rule out the presence of *Streptococcus pyogenes*, which is the causative agent of scarlet fever, and more recently, has been associated with mediating Streptococcal toxic shock syndrome (Strep TSS)(Cone *et al.*, 1987). In addition, attempts are made to exclude Staphylococcal scalded skin syndrome, Kawasaki's disease, Rocky mountain spotted fever, and the possibilty of drug reactions. The isolation of a TSST-1-producing *S. aureus* from an infected site and the observation that a patient's sera do not have antibody to the toxin, in conjunction with the hematologic and biochemical abnormalities associated with TSS, provides strong support for a diagnosis of TSS.

1.1.4. Treatment of Toxic Shock Syndrome

In periods of acute illness, management of TSS requires immediate rapid fluid replacement to counteract the massive leakage of intravascular fluid into the interstitial space, and to maintain adequate tissue perfusion (Chow, 1995). Therapy is aimed at eliminating *S. aureus* strains from the infected site by removal of the infected foreign body (for example a tampon or intravenous catheter) followed by antibiotic treatment. Most *S. aureus* strains exhibit resistance to penicillin and ampicillin, so ß-lactamase resistant anti-staphylococcal agents such as cloxacillin may be used (Chow, 1995). In penicillin-allergic patients, vancomycin or clindamycin may be preferred alternatives (Chow, 1995). Since TSS is toxin-mediated,

Table 1 **Case Definition for Toxic Shock Syndrome***

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1. Fever (temperature ≥ 38.9°C)

2. Rash (diffuse macular erythrodema)

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 creatine phosphokinase level ≥ 2 X ULN[a])
   - C. Mucous membrane (vaginal, oropharyngeal, or conjunctival hyperemia)
   - D. Renal (BUN[b] or Cr[c] ≥ 2 X ULN or ≥ 5 white blood cells per high power field - in the absence of a urinary tract infection)
   - E. Hepatic (total bilirubin, SCOT[d] or SGPT[e] ≥ 2 X ULN)
   - F. Hematologic (platelets ≤ 100,000/mm^3)
   - G. Central nervous system (disorientation or alterations in consciousness without focal 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

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[a]Twice upper limit of normal for laboratory
[b]Blood urea nitrogen level
[c]Creatine level
[d]Serum glutamic oxaloacetic transaminase level
[e]Serum glutamic pyruvic transaminase level

*(Chesney *et al.*, 1984)

irrigation of the infected site may be warranted to remove any pre-formed toxin. In addition, treatment with pooled intravenous immune globulin (IVIG) preparations, which contain high levels of anti-TSST-1 antibody, have been successful in eliciting protection in animal models of TSS (Melish *et al.*, 1987), and may be warranted in critically ill TSS patients.

1.1.5. Risk Factors Associated with Menstrual and Nonmenstrual Toxic Shock Syndrome

Different risk factors associated with menstrual TSS and nonmenstrual TSS have been noted. In concordance with the observed frequency of TSST-1-producing *S. aureus* strains from TSS patients, women colonized with TSST-1^+ strains are at a higher risk for developing MTSS than their uncolonized counterparts. Age also appears to be a factor, in that women less than 30 years of age appear to be at a higher risk for developing MTSS than women greater than

30 years of age (Davis *et al.*, 1980). This may be accounted for in part by the fact that in a healthy population, the presence of antibodies against TSST-1 correlates closely with age. The majority of individuals develop TSST-1 antibody by the age of 20 (Vergeront *et al.*, 1983). This is consistent with the finding that an increased incidence of MTSS is found in the age group 15-19 years (Broome, 1989). No age-related differences have been found with respect to rate of vaginal *S. aureus* carriage (Davis *et al.*, 1980), although one study did show a trend toward decreased genital colonization in relation to increasing age (Linnemann *et al.*, 1982). Whether age-related or not, women who lack antibody to TSST-1 have a much higher risk of developing MTSS than those with antibody. This is based on studies which have shown that sera from patients with TSS are significantly more likely to lack

antibody to TSST-1 in comparison to healthy controls (Bergdoll *et al.*, 1981; Bergdoll *et al.*, 1982; Notermans *et al.*, 1983; Bonventre *et al.*, 1984). This lack of antibody to TSST-1 is even observed in patients upon convalescence (Bonventre *et al.*, 1984; Stolz *et al.*, 1985), and may explain why recurrences are frequent. The importance of the presence of TSST-1 antibody has been shown in a rabbit model of TSS, in which TSST-1 specific monoclonal antibodies were found to confer protection to rabbits after infection with a nonenterotoxigenic strain of *S. aureus* carrying a plasmid encoding TSST-1 (Best *et al.*, 1988).

A racial predisposition for MTSS has also been suggested, in that the majority of MTSS cases occur in white females (Gaventa *et al.*, 1989; Broome, 1989). This racial distinction does not appear to be due to differences in colonization rates, since black women have been found to be genitally colonized with *S. aureus*, including TSST-1$^+$ *S. aureus,* as frequently, or more frequently, than white women (Linnemann *et al.*, 1982). The fact that less MTSS is noted among this group may result from a failure to note the erythematous rash in women with dark skin, or may be due to some undefined host characteristic (Linnemann *et al.*, 1982).

Women who have vaginal carriage of *Escherichia coli* or other facultative lactose-fermenting gram-negative bacilli in addition to *S. aureus* may also have a greater risk of developing MTSS (Chow and Bartlett, 1982). This association may be consistent with observations that endotoxin can synergize with staphylococcal exoproteins to cause disease *in vivo* (Sugiyama *et al.*, 1964; de Azavedo and Arbuthnott, 1984).

Lastly, tampon usage during menstruation is the best documented risk factor for the development of MTSS. As previously mentioned, the use of one brand of tampon, the Rely^R

brand, has been more frequently associated with MTSS cases than other brands. The association of one particular brand with disease may be related to the chemical composition of the tampon, which has been shown to play an important factor dictating relative risk potential since this aids in determining both the absorbency and Mg^{2+} binding ability (Berkley *et al.*, 1987; Kass and Parsonnet, 1987; Kass, 1989). The higher the absorbency of the tampon, the higher is the relative risk for developing the disease (Berkley *et al.*, 1987; Reingold *et al.*, 1989). Before 1977, tampons were made of rayon, or a blend of rayon and cotton. After 1977, tampons have been made with more absorbent synthetic materials such as polyacrylate fibres and polyester foam (Shands *et al.*, 1980), and this may have led to an increased incidence of the disease. The ability of certain tampon fibres to bind more Mg^{2+} than others is also believed to be important since Mg^{2+} concentration has been shown to regulate TSST-1 production (Mills *et al.*, 1985). Additional factors linking tampon usage with the development of TSS in susceptible individuals have been proposed. The presence of blood on a tampon may provide an important nutrient source that allows increased growth of the organism and/or increased toxin production. In support of this, Lee *et al.* (1987) provided data to suggest that the presence of blood in the culture medium leads to an increased production of TSST-1. The tampon may also act to provide a larger surface area for the organism to colonize. Tampons may also increase the risk of developing MTSS through their ability to cause microulcerations in the vagina (Friedrich and Siegesmund, 1980), which may subsequently lead to an increased absorption of toxin. Also, aerobic conditions are known to favour TSST-1 production more than anaerobic conditions (Todd *et al.*, 1987), and since tampon insertion into the vagina increases the pO_2 of the

microenvironment (Wagner *et al.*, 1984), more toxin may be produced *in vivo* and disease may ensue. Tampon usage during menstruation has also been found to cause shifts in the vaginal microflora when compared to the vaginal microflora of the same women using napkins (Chow and Bartlett, 1989). What role this alteration of microflora may play in TSS, if any, is presently unknown.

Despite all of the above mentioned methods of how tampon usage may significantly increase the risk of developing MTSS, one of the primary roles of the tampon may be to mediate transport of the organism into the vagina. The tampon may act as an intermediary in the transfer of the organism from the hands or perineum of the woman into the vagina. Evidence that possible contamination of the tampon at the site of manufacture is not to blame is based on a study in which 0 of 264 unused tampons tested harboured *S. aureus*, despite the fact that tampons were not sterilized in the U.S.A. at this time (Shands *et al.*, 1980). Consistent with the postulate that transmission of the organism from the hand to the vagina via the tampon may be important is suggested by the observation that use of a non-applicator type of tampon increases the risk for vaginal *S. aureus* carriage (Guinan *et al.*, 1982).

Studies addressing risk factors for NMTSS have also been carried out. NMTSS can result from *S. aureus* infection at any body site, but most commonly in surgical or cutaneous wounds. Unlike that of MTSS, NMTSS can occur in men, women and children of any age who lack protective antibody. Different racial groups appear to be equally susceptible to NMTSS (Reingold *et al.*, 1982). In comparison to MTSS, NMTSS cases occur more significantly in association with a period of hospitalization (Kain *et al.*, 1993), and after a history of previous antimicrobial therapy (Kreiswirth *et al.*, 1986; Kain *et al.*, 1993). Use of

barrier contraceptives has also been shown to increase the risk of NMTSS (Schwartz *et al.*, 1989). Infection with *S. aureus* strains that produce toxins other than TSST-1 are also associated more often with NMTSS cases (Garbe *et al.*, 1985; Schlievert, 1986).

1.2. Toxin Involvement in the Pathogenesis of Toxic Shock Syndrome

Toxic shock syndrome is a toxin mediated disease. This is based on the observation that despite the frequent isolation of *S. aureus* from TSS patients and the dysfunction that is observed in multiple organ systems, bacteremia is rarely noted (Chow, 1983). In rabbit models of TSS, animals that succumb to lethal infection by toxigenic strains of *S. aureus* also exhibit "negative" blood cultures (Rasheed *et al.*, 1985; Melish *et al.*, 1989). The observation that the majority of *S. aureus* strains isolated from TSS patients are toxigenic (Crass and Bergdoll, 1986a) further strengthens this premise. In addition, convalescent sera from patients with TSS more frequently seroconvert to various staphylococcal exoproteins than patients with non-TSS-associated *S. aureus* infections (Whiting *et al.*, 1989). Clinically, symptoms such as the abrupt onset of vomiting and diarrhea and the erythema of skin and mucousal surfaces observed in TSS resemble that of a toxin-induced pathology (Chow, 1983).

1.2.1. Evidence implicating Toxic Shock Syndrome Toxin-1 (TSST-1) in the Pathogenesis of Toxic Shock Syndrome

In 1981, Bergdoll *et al.* (1981) reported the identification of a marker protein for TSS that was produced in over 90% of TSS-associated *S. aureus* isolates. After determining that the protein could induce vomiting in baboons, it was called Staphylococcal enterotoxin F (SEF). This name was subsequently changed to Toxic Shock Toxin (TST) when the emetic

action of the protein could not be reproduced (Reiser *et al.*, 1983). At the same time, Schlievert *et al.* (1981) reported the purification and characterization of a toxin produced by a strain of *S. aureus* that was a potent pyrogen, had nonspecific lymphocyte mitogenic activity, and could enhance host susceptibility to the effects of endotoxin. This protein was referred to as Staphylococcal Pyrogenic Exotoxin C (SPEC). When SEF and SPEC were found to be identical (Bonventre *et al.*, 1983; Bergdoll and Schlievert, 1984), the name was changed to Toxic Shock Syndrome Toxin-1, or TSST-1 (Bergdoll and Schlievert, 1984).

Strong evidence exists to implicate TSST-1 as a causal agent in TSS. First, a large majority of strains isolated from TSS patients produce TSST-1. Second, the sera of some individuals with TSS upon convalescence were shown to seroconvert to TSST-1 (Stolz *et al.*, 1985; Whiting *et al.*, 1989). Third, one group of investigators found that *S. aureus* strains from TSS patients produced significantly more TSST-1 *in vitro* than toxigenic control strains (Rosten *et al.*, 1987). In addition to the above indirect evidence, a role for TSST-1 in pathogenesis has been more directly assessed with the use of animal models. Rabbits are the species of choice for animal models since they can reproduce the majority of the symptoms observed in TSS upon administration of purified TSST-1 or TSS-associated *S. aureus* strains (de Azavedo, 1989). Monoclonal antibodies against TSST-1 have protected rabbits from TSST-1 administered by constant infusion (Bonventre *et al.*, 1988), and from challenge by a nonenterotoxigenic strain of *S. aureus* containing a plasmid encoding TSST-1 introduced into subcutaneously implanted polyethylene chambers (Best *et al.*, 1988). Genetically engineered isogenic strains of *S. aureus* differing in the ability to produce TSST-1 have also been utilized in rabbit studies. Using the subcutaneous infusion model, Rasheed *et al.* (1985)

were among the first investigators to show the importance of TSST-1 in mediating a TSS-like illness *in vivo* using genetically constructed *S. aureus* strains. The construct was made by transducing the TSST-1 marker from a clinical TSST-1^+ isolate into a TSST-1^- strain. Although the TSST-1^+ transductant caused more illness and lethality in rabbits in comparison to the TSST-1^- control strain, of concern is what other DNA sequences besides the TSST-1^+ marker may have been transduced into the construct. In addition, although the subcutaneous infusion model allows a controlled, localized depot of infection, it does not closely resemble the host situation observed in menstrual TSS. Shortly thereafter, de Azavedo *et al.* (1985) constructed a pair of isogenic mutants in TSST-1 production by introducing a plasmid-borne TSST-1 structural gene (*tst)* into a naturally occurring TSST-1^- strain. The isogenic strains were introduced into an uterine chamber that maintained the organisms in a localized region while allowing bacterial exoproteins to be released into the circulation. Using this model, the strain that carried the *tst*-plasmid induced a TSS-like illness in rabbits whereas the control strain that carried the plasmid with a deletion in *tst* had no effect. Criticisms for this experimental design include the fact that the uterine model, although having a genital focus, does not resemble menstrual TSS in which organisms become colonized in the vagina. Also in the experimental design, the rabbits had to be injected daily with erythromycin to maintain the *tst*-carrying plasmid. This requirement may have important implications with regard to the observed clinical outcome, since antibiotic administration can affect the sensitivity of rabbits to TSST-1 (Schlievert, 1983). In a subsequent study, Sloane *et al.* (1991) created TSST-1 isogenic mutants by the method of allele replacement and introduced the strains into the uterine chamber model. Again it was shown that the TSST-1^+ strain was more virulent

than the TSST-1$^-$ isogenic mutant. The method of allele replacement mutagenesis by these investigators represents an ideal method for the creation of isogenic mutants. If this study had made use of a clinical isolate that more closely resembled a TSS strain in phenotypic characteristics in a more clinically relevant animal model, the results would have definitively addressed the role of TSST-1 in menstrual TSS.

A vaginal tampon model described by Melish *et al.* (1989) was the first relevant animal model reported that reproduced the human situation in menstrual TSS. Although TSST-1$^+$ staphylococci could lead to TSS in this model, the role of TSST-1 in menstrual TSS was not demonstrated specifically since isogenic strains were not used. To date, no study has been performed with isogenic TSST-1 mutants in a clinically relevant animal model of TSS.

1.2.2. Evidence implicating Staphylococcal Enterotoxins in the Pathogenesis of Toxic Shock Syndrome

In addition to TSST-1, other staphylococcal exoproteins, in particular the enterotoxins, may play a role in TSS either on their own, or in conjunction with TSST-1. In support, 60% of TSST-1$^+$ strains have been found to co-produce one of the enterotoxins (Crass and Bergdoll, 1986a). Staphylococcal enterotoxin production has also been found in TSS isolates that were TSST-1$^-$, indirectly implicating them as primary causal agents (Bergdoll *et al.*, 1982; Garbe *et al.*, 1985). Moreover, introduction of TSST-1$^-$/enterotoxin$^+$ TSS-associated *S. aureus* strains into the rabbit subcutaneous model of toxic shock have caused a TSS-like illness to develop (Garbe *et al.*, 1985), suggesting that the enterotoxins can induce similiar symptoms as TSST-1 *in vivo*. In TSS patients infected with TSST-1$^-$ *S. aureus* strains who could develop an antibody response, seroconversion to at least one of the enterotoxins was

found to occur more frequently than in TSS patients infected with TSST-1$^+$ *S. aureus* (Whiting *et al.*, 1989).

A. Evidence Suggesting that SEA and TSST-1 Co-Production may be Important in the Pathogenesis of Menstrual Toxic Shock Syndrome

As indicated, the production of SEA by *S. aureus* may also partially contribute to TSS. Upon analysis of 30 NMTSS strains for toxin production, Garbe *et al.* (1985) identified one isolate that produced only SEA. Parsonnet *et al.* (1986) also identified 1 of 20 NMTSS-associated strains that produced SEA alone. In 1990, McCollister *et al.* (1990) isolated a SEA$^+$/TSST-1$^-$ strain of *S. aureus* (D4508) from a patient with NMTSS. Concentrated culture filtrates of the organism administered subcutaneously via osmotic pumps to rabbits induced TSS-like symptoms, whereas culture filtrates pretreated with antibodies against SEA failed to induce the same symptoms. Further evidence that SEA can induce TSS-like symptoms *in vivo* was shown in several studies in which purified SEA introduced into rabbits or monkeys could cause symptoms similiar to that observed in humans with TSS, including death (Bergdoll, 1983; Parsonnet *et al.*, 1986; McCollister *et al.*, 1990).

Instances in which SEA appears to be the sole agent responsible for causing TSS however are limited. Very few TSS-associated strains have been identified that produce SEA alone. Furthermore, Crass and Bergdoll (1986a) found that of 380 TSS-associated strains examined for TSST-1 and enterotoxin production, none were found to produce SEA alone. Although SEA production on its own may not be that important clinically, striking evidence exists to suggest that SEA production in conjunction with TSST-1 by a *S. aureus* strain may be responsible for causing the majority of menstrual TSS cases. As indicated in Table 2, the

combination of TSST-1 and SEA production occurs more frequently among MTSS isolates than NMTSS isolates (Crass and Bergdoll, 1986a; Chang *et al.*, 1991; Kain *et al.*, 1993). In addition, among MTSS isolates, this toxin combination is observed more frequently than any other (Crass and Bergdoll, 1986a). Analyses by multilocus enzyme electrophoresis (Chang *et al.*, 1991) and phage typing (Marples and Wieneke, 1993) suggest that most MTSS cases are caused by a single clone of *S. aureus* which produce both TSST-1 and SEA.

B. Evidence Suggesting a Putative Role for SEB in the Pathogenesis of Toxic Shock Syndrome in TSST-1$^-$ *S. aureus* Isolates

A putative role for SEB in the pathogenesis of TSS is indicated from the results of several studies in which phenotypic characterizations of TSS-associated strains have been performed. Schlievert (1986) found that SEB production was significantly associated with TSS or probable TSS in isolates that did not produce TSST-1. Garbe *et al.* (1985) noted that TSST-1$^-$ TSS-associated *S. aureus* strains were more likely to produce SEB than TSST-1$^+$ strains. Crass and Bergdoll (1986b) found that 8 of 9 TSST-1$^-$ NMTSS isolates produced only SEB, implicating it as the major etiologic agent causing disease in these cases. Finally, Lee *et al.* (1992) reported that SEB production among TSST-1$^-$ isolates was significantly more prevalent in TSS-associated infections than non-TSS associated infections. As with SEA, injection of SEB into monkeys can reproduce many of the symptoms that are observed in TSS, including shock (Beisel 1972; Liu *et al.*, 1978; Bergdoll 1983). In addition, Van Miert *et al.* (1984) found that TSST-1 and SEB could induce similiar clinical hematological and blood biochemical changes when administered intraveneously to goats. Collectively, these results suggest that SEB may play a role in causing TSS.

Table 2. TSST-1 and SEA Production among *S. aureus* isolates

Patient Group (no. of isolates)	TSST-1$^+$ (%)	SEA$^+$ (%)	TSST-1$^+$/SEA$^+$ (%)
MTSS (24)	83	75	71[a]
NMTSS (41)	59	32	24
Non-TSS (146)	25	25	12
Carrier (139)	28	20	13

MTSS, Menstrual toxic shock syndrome; NMTSS, Nonmenstrual toxic shock syndrome; Non-TSS, Non-toxic shock syndrome-associated *S. aureus* infection; Carrier, No infection; TSST-1$^+$, producing toxic shock syndrome; SEA$^+$, producing staphylococcal enterotoxin A

[a]Significantly different from NMTSS, Non-TSS or Carrier strains ($P < 0.001$, Chi square, 2-tailed)

Data compiled from Chang *et al.* (1991).

Of interest is the observation that SEB appears to be only associated with TSS strains that are TSST-1$^-$. In fact, TSST-1 and SEB co-production by the same *S. aureus* strain is extremely rare (Garbe *et al.*, 1985; Crass and Bergdoll, 1986a; Crass and Bergdoll, 1986b; Bohach *et al.*, 1990) and has only been described in a few instances (Ewan *et al.*, 1989; Johnson *et al.*, 1991; Lee *et al.*, 1992). Genotypic studies also suggest that the presence of both the TSST-1 (*tst*) and the SEB (*seb*) genes within the same isolate is rare. Investigations using oligonucleotide probes in conjunction with hybridzation failed to identify the presence of *seb* in TSST-1$^+$ isolates (Bohach *et al.*, 1989; Neill *et al.*, 1990). However Johnston *et al.* (1991) found the TSST-1 and SEB structural genes in 11% (3/28) of their clinical isolates using PCR analysis.

1.2.3. Evidence Implicating Toxins other than TSST-1 and the Enterotoxins in the Pathogenesis of Toxic Shock Syndrome

Although TSST-1 and the staphylococcal enterotoxins are produced in the majority of TSS-related isolates, there still remains some that do not express TSST-1 or any of the enterotoxins. Crass and Bergdoll (1986a) found that 9 of 380 (2.4%) *S. aureus* strains isolated from patients with both menstrual and nonmenstrual TSS did not produce TSST-1, SEA, SEB, SEC, SED, or SEE. In addition, Garbe *et al.* (1985) identified 3 of 10 NMTSS TSST-1$^-$ isolates that did not produce enterotoxin. These results suggest that other toxin(s) besides TSST-1 and the enterotoxins may be responsible for causing TSS in some cases. *S. aureus* produces more than thirty different extracellular proteins, and a detailed examination of the frequency of production of every protein among TSS-associated *S. aureus* isolates has not been carried out. In 1982, elaboration of an unknown epidermal toxin among TSS-

associated strains was described (Kapral, 1982), but evidence to suggest whether it plays a causative role in TSS is lacking. Recently a new toxin, Staphylococcal enterotoxin H (SEH), produced by a TSST-1$^-$ nonmenstrual strain of *S. aureus* was identified (Ren *et al.*, 1994). This new toxin induced an acute TSS-like illness when injected intravenously into rabbits. The percentage of TSS-associated strains producing this toxin has not yet been investigated. Whether SEH may play a role in inducing TSS in TSST-1$^-$, nonenterotoxigenic isolates is currently unknown.

1.2.4. Functional Consequences of TSST-1, SEA and SEB Production by *S. aureus*

A. TSST-1, SEA and SEB as 'SUPERANTIGENS'

TSST-1, SEA and SEB belong to a family of proteins designated as 'superantigens' (White *et al.*, 1989). Two main groups of superantigens - exogenous and endogenous, have been noted. The exogenous group of superantigens are proteins produced by microbes, and in addition to the staphylococcal enterotoxins and TSST-1, include the staphylococcal exfoliating toxins, streptococcal pyrogenic exotoxins (SPE) A, B and C, the M protein molecule and a soluble product secreted by *Mycoplasma arthritidis* called MAM. The endogenous superantigens are comprised of a group of products encoded by unlinked genetic loci in mice, the best known being the minor lymphocyte stimulating antigens (Mls). These type of antigens have now been found to be encoded by mammary tumor viruses (Frankel *et al.*, 1991).

Superantigen is used to describe these unique proteins since they can stimulate a large number of T cells at a lower concentration than that required by a conventional antigen. Moreover, these antigens stimulate only T cells bearing specific V_β components of the T cell

receptor. Each superantigen has its own unique V_β profile (Schlievert, 1993). TSST-1 stimulates human $V_\beta 2$ T cells (Choi *et al.*, 1990), SEB V_β 3,12,14,15,17,20 T cells (Choi *et al.*, 1989), and SEA V_β 6.3,6.9,7.3,7.4 T cells (Zumla 1992). This unique feature of superantigens enables them to interact with more T cells than a conventional antigen since the actual region contacted by the superantigen on the V_β chain of the T cell receptor is relatively invariant within each V_β gene family (Drake and Kotzin, 1992). In addition, superantigens differ from conventional antigens in other aspects: Superantigens do not require processing by the antigen presenting cell before binding to the MHC Class II molecule. Instead, superantigens interact with the MHC Class II molecule outside of the conventional peptide binding groove, and thus superantigen presentation by T cells is not MHC-restricted.

Superantigens are unique molecules capable of triggering profound disturbances within the immune system. In addition to causing massive stimulation of T cells, superantigens have been found to induce T cell nonresponsiveness, either through functional inactivation (anergy) (Rellahan *et al.*, 1990; O'Hehrir *et al.*, 1990) or clonal deletion (MacDonald *et al.*, 1991; Kawabe and Ochi, 1991). Superantigens also mediate staphylococcal enterotoxin-dependent cell-mediated cytotoxicity, or SDCC, in which rapid killing of antigen presenting cells, irrelevant of their nominal specificity, is evoked (Kalland *et al.*, 1991). The cytotoxic activities can be mediated by both $CD4^+$ and $CD8^+$ T cells (Dohlsten *et al.*, 1990) and T cells expressing the gamma-delta T cell receptor (Rust *et al.*, 1990). This latter effect also has the potential for functional inactivations of T-cell mediated responses. It may be speculated that the production of these toxins by *S. aureus* may represent a method by which the organism avoids recognition by T cells (Kalland *et al.*, 1991). Indeed, SDCC might play

a role in inhibiting immunoglobulin production by B cells, since Class II^+ B cells would also be destroyed in such a cytotoxic response. As proposed by Mourad *et al.* (1993), down-regulation of the humoral response by these superantigens may provide a putative explanation for why TSS patients do not generate TSS specific-antibodies and why recurrences of this disease are frequent.

B. The MHC Class II Molecule is the Receptor for TSST-1, SEA and SEB

Although TSST-1 is non-cytotoxic for a variety of cell lines (Drumm *et al.*, 1989), specific binding to cervical epithelial (Kushnaryov *et al.*, 1984a; Kushnaryov *et al.*, 1984b), and vascular endothelial (Kushnaryov *et al.*, 1989) cells has been observed. Binding of TSST-1 to epithelial cells leads to internalization via receptor-mediated endocytosis (Kushnaryov *et al.*, 1984b). Once transported to the inside by epithelial cells, TSST-1 may interact with endothelial cells to become translocated from the extravascular space across the vessel wall into the lumen, where interaction with peripheral blood mononuclear cells may occur.

Many researchers have provided evidence to suggest that the MHC Class II molecule on accessory cells such as antigen presenting cells (APC) is the receptor for TSST-1, SEA and SEB (Fischer *et al.*, 1989; Herrmann *et al.*, 1989; Mollick *et al.*, 1989; Scholl *et al.*, 1989a; See *et al.*, 1992a). Although all three toxins bind to the same receptor, unique binding epitopes have been observed. Scholl *et al.* (1989b) showed by cross-competition binding studies that SEB and TSST-1 bind to distinct sites on both HLA-DR and HLA-DQ transfected cell lines. Two distinct binding sites for TSST-1 and SEB on HLA-DR were also shown by Chintagumpala *et al.* (1991). Consistent with this, See *et al.* (1990) were unable to inhibit labelled TSST-1 binding to human peripheral blood mononuclear cells with excess

unlabelled SEB. In contrast, the same investigators were able to show that excess unlabelled SEA could inhibit labelled TSST-1 binding to human peripheral blood mononuclear cells, suggesting that TSST-1 and SEA may bind to overlapping epitopes. Similiarly SEA was shown by Fraser (1989) to compete with SEB for binding to HLA-DR. It was thus postulated that SEA may bind to HLA-DR at a site that overlaps the binding epitopes for TSST-1 and SEB (See *et al.*, 1992a).

Isotypic and allelic polymorphisms of the MHC molecule have been shown to influence the binding of staphylococcal toxins. TSST-1, SEA, and SEB bind preferentially to the MHC isotypes in the order HLA-DR>DQ>DP (Scholl *et al.*, 1989a; Herman *et al.*, 1990; Uchiyama *et al.*, 1990; Imanishi *et al.*, 1992). Preferential binding of these toxins to specific HLA-DR alleles expressed on L-cells has also been demonstrated (Herman *et al.*, 1990; Scholl *et al.*, 1990).

In addition to the MHC Class II molecule, a second toxin binding site for superantigens on murine macrophages may be present. In a recent report by Beharka *et al.* (1994), macrophages obtained from Class II deficient transgenic mice were found to bind various staphylococcal exotoxins and induce cellular activation. Other investigations have also indicated that staphylococcal enterotoxins might bind to sites other than the MHC Class II molecule (Dohlsten *et al.*, 1991; Herrmann *et al.*, 1991). These putative non-MHC Class II toxin binding sites still remain to be defined.

C. Activation of Monocytes by Staphylococcal Superantigens

Interaction of staphylococcal superantigens with the MHC Class II molecule on monocytes leads to transcription of genes for monocyte-derived cytokines such as IL-1ß and TNFα

(Trede *et al.*, 1991). It has been found that direct contact between monocytes and T cells is required for purified TSST-1 to stimulate TNFα and IL-1ß secretion (See *et al.* 1992c). Furthermore, studies by Gjorloff *et al.* (1991), and Fisher *et al.* (1990) showed that SEA induction of IL-1 and TNFα from monocytes required the participation of T cells.

The binding of superantigens to MHC Class II molecules on monocytes is a prerequisite for cytokine induction, as evidenced by the fact that antibodies to Class II molecules in the presence of staphylococcal superantigens can diminish TNFα and IL-1ß production (Grossman *et al.*, 1990; Matsuyama *et al.*, 1993). Superantigens can induce TNFα release from monocytes independent of T cell mitogenesis since a SEA mutant toxin that was nonmitogenic for T cells was still able to bind to Class II molecules and induce TNFα release (Grossman *et al.*, 1992). In addition Fisher *et al.* (1990) used an intracytoplasmic staining technique to show that intracellular TNFα production did occur in monocytes in the presence of T cells after stimulation with SEA.

D. Activation of T Cells by Staphylococcal Superantigens

Interaction of the superantigen/MHC Class II complex with specific $V_ß$ components of the T cell receptor results in the activation of T cells. Upon activation, T cells proliferate and secrete cytokines such as TNF, IL-2 and IFN-δ. The dependency on the MHC Class II molecule for mediating superantigen-induced T cell activation was suggested by the observation that monoclonal antibodies against the HLA-DR molecule inhibited both TSST-1 and SEA induced T cell proliferation (Fleischer and Schrezenmeier, 1988; Fischer *et al.*, 1989; See *et al.*, 1992a). The secretion of a T cell specific cytokine, TNFß, has also been found to be dependent on the MHC Class II molecule, since T cells from DR4-negative

donors, stimulated with TSST-1, were found to secrete TNFß only in the presence of L cells transfected with DR4 genes and not control L cells (Akatsuka *et al.*, 1994). Moreover this response could be abrogated by the addition of an anti-HLA-DR monoclonal antibody. Although T cell activation by superantigens is dependent on Class II MHC, evidence suggests that superantigens can interact with T cells in the absence of the antigen presenting cell to induce intracellular signals through the T cell receptor. Fleischer and Schrezenmeier (1988) showed that staphylococcal enterotoxins incubated with purified resting T cells caused a rise in intracellular Ca^{2+} concentrations. Lagoo *et al.* (1994) also showed that superantigens induced cytokine gene expression but not cytokine secretion in highly purified blood T cells in the absence of Class II^+ cells. It therefore appears that although signals are induced to the T cell upon interaction with superantigens, additional accessory signals mediated by crosslinking of the T cell receptor, Class II molecule, and adhesion molecules are required for complete T cell activation.

Both $CD4^+$ and $CD8^+$ T cells can be activated to proliferate and secrete cytokines upon superantigen exposure in the presence of APC (Calvano *et al.*, 1984; Fischer *et al.*, 1990). Activation of $CD8^+$ cells by superantigens has been associated with an inhibition of immunoglobulin production (Poindexter and Schlievert, 1987). Class I molecules do not appear to be necessary for the $CD8^+$ T cell response since Carlsson *et al.* (1988) found that Daudi cells, which lack Class I molecules, are able to support $CD8^+$ T cell activation. Likewise, although $CD4^+$ cells are activated, the presence of CD4 on T cells does not appear to be absolutely necessary for the Class II mediated effects since transfection of $CD4^-$ murine T cell hybridomas with CD4 cDNA did not affect the proliferative response of the majority

of the hybridomas to various superantigens (Sekaly *et al.*, 1991). These data suggest that the V_β segment on a $CD8^+$ cell is also capable of being cross-linked to MHC Class II molecules by superantigens.

E. Evidence Implicating a Central Role for T Cell Mitogenesis and Cytokine Secretion in the Pathogenesis of Superantigen-Mediated Shock

Since the staphylococcal toxins believed to be responsible for causing TSS belong to the superantigen family of proteins, it is reasonable to hypothesize that these unique superantigen-related activities might contribute to the pathogenesis of TSS. The initial immunological response induced by these toxins upon interaction with MHC Class II molecules and T cells is the expansion of T cell clones bearing specific V_β molecules. In addition to proliferation, T cells are activated to release cytokines such as TNFα, IL-1ß, IL-2 and IFN-τ. The postulate that T cell mitogenesis followed by excessive secretion of cytokines, particularly TNFα, plays a critical role leading to lethal shock upon superantigen stimulation is based on collected evidence from numerous studies. An indirect role for T cells in TSS was suggested by Choi *et al.* (1990), who found that during TSS, T cell stimulation occurs on a scale that is not observed in responses to conventional antigens. Direct evidence for a primary role of T cells in the toxicity induced by SEB was shown by Marrack *et al.* (1990). These investigators found that both T cell deficient nude mice, and mice genetically bred to express a low number of SEB reactive V_β-T cells, experienced less weight loss than control mice upon injection of SEB. These investigators concluded that SEB-induced weight loss in mice was mediated by T cells. Experiments confirming these observations were performed by Miethke *et al.* (1992) using D-Galactosamine (D-gal)

sensitized mice. D-galactosamine is a hepatotoxic agent that sensitizes mice to the biologic effects of superantigens as well as endotoxin by unknown mechanisms. D-Gal sensitized SCID mice, deficient in T and B cells, did not succumb to lethal shock upon injection with SEB. Upon reconstitution with T cells, however, the mice died. The same investigators performed the identical experiments with TSST-1, and also demonstrated a requirement for T cells in TSST-1-mediated lethal shock (Meithke *et al.*, 1993). These experiments provide the most direct evidence to support a primary role for T cells in mediating lethal shock. In a complementary investigation, Bonventre *et al.* (1993) identified a critical residue in the TSST-1 molecule that was required for mediating the mitogenic action on T cells. Substitution of the histidine residue at position 135 to an alanine by site-directed mutagenesis yielded a TSST-1 mutant (H135) that retained MHC Class II binding ability, but lost the ability to induce T cell activation (as measured by mitogenesis and cytokine assays) (Bonventre *et al.*, 1993; Cullen *et al.*, 1995). The mutant toxin failed to induce death in a rabbit model of TSS (Bonventre *et al.*, 1993) or a murine model of lethal shock (Bonventre *et al.*, 1995) whereas the wild-type toxin was lethal.

TNF has been implicated as the central mediator in the pathogenesis of TSS. TNF introduced into experimental animals can induce most of the symptoms that are observed in TSS patients, including fever (Dinarello *et al.*, 1986), hypotension, and shock (Ikejima *et al.*, 1988; Dinarello *et al.*, 1989). Many studies have shown that anti-TNF monoclonal antibodies can confer protection against the toxic effects of both *S. aureus* and its purified toxins. For example, antibody against TNF was found to protect rabbits against a lethal infusion of purified TSST-1 (Parsonnet *et al.*, 1988); an anti-TNF antibody was found to

prevent death in baboons infused with live *S. aureus* (Hinshaw *et al.*, 1992); an anti-TNF monoclonal antibody was found to provide protection to mice in a dose-dependent manner against the lethal effects of TSST-1 (Miethke *et al.*, 1993); and anti-TNF antibody was found to protect mice against the lethal effects of SEB (Miethke *et al.*, 1992). Evidence that T cells might be the source of the cytokine(s) mediating toxicity comes from the observation that cyclosporin A (an agent that suppresses lymphokine secretion by T cells) treatment of D-Gal sensitized mice conferred protection against the lethal effects of SEB (Miethke *et al.*, 1992) and TSST-1 (Miethke *et al.*, 1993). Whether the T cell requirement for toxicity is due to the direct release of TNF from T cells, or is due to another cytokine released from T cells that enhance TNF production from monocytes and/or T cells is not clear.

Although evidence exists to suggest that TNF is the primary mediator leading to TSS, other cytokines, such as IL-1, may also be important. IL-1 and TNF have been found to act synergistically to induce a shock-like state in rabbits (Ikejima *et al.*, 1988; Okusawa *et al.*, 1988; Dinarello *et al.*, 1989), and each can induce the production of the other. In addition, TNFα and IL-1ß can induce the *in vivo* production of IL-6 (Shalaby *et al.*, 1989). IL-6 may in fact be an important mediator of TNFα and IL-1ß functions *in vivo* since injection of both cytokines in mice correlated with a greater increase in serum IL-6 and mortality compared to animals injected with either cytokine alone (Shalaby *et al.*, 1989).

1.2.5. Genetic Control of TSST-1, SEA and SEB Production in *S. aureus*

A. Regulatory Loci Involved in Controlling Toxin Production

Most staphylococcal exoproteins including TSST-1 and SEB, are produced maximally during the post-exponential phase of growth *in vitro*. Other exoproteins are produced during

the exponential phase of growth, including coagulase, Protein A and SEA (Czop and Bergdoll, 1974; Borst and Betley, 1993). During the transition from exponential to post-exponential growth, regulatory systems alter the expression of accessory traits. One regulatory locus in *S. aureus* that appears to be involved in gene activation during this transition is the 'accessory gene regulator', or *agr* (Recsei *et al.*, 1986) [previously referred to as *exp* (Morfeldt *et al.*, 1988)]. This locus regulates a number of *S. aureus* exoprotein genes, in that it can be both stimulatory and inhibitory for various gene products. For example, while mutations in *agr* led to the undetectable production of proteins such as α-hemolysin (Recsei *et al.*, 1986), TSST-1 (Recsei *et al.*, 1986) and SEB (Gaskill and Khan, 1988), others such as coagulase and Protein A were increased (Recsei *et al.*, 1986).

Due to the differential effects on various proteins, it seems probable that *agr* may act through a number of other intermediate regulatory steps to mediate its effect on gene expression. In this regard, three other related regulatory systems in *S. aureus* have been identified. Smeltzer *et al.* (1993) identified a locus called *xpr* that was also found to be responsible for regulating the production of a number of extracellular staphylococcal proteins. Transposon insertion mutagenesis into *xpr* led to a decrease in the expression of extracellular proteins that were similiar to the proteins found to be decreased in *agr* mutants. It was proposed that *xpr* and *agr* may interact to regulate genes of pathogenic importance. A second locus called *sar*, for staphylococcal accessory regulator, was also identified. *Sar* appears to be involved in regulating the expression of extracellular and cell-wall associated proteins (Cheung *et al.*, 1992). This locus appeared distinct from agr, since *sar* mutants produced more α-hemolysin and protease than the wild-type, in direct contrast to the *agr*

mutants. The *sar* locus may therefore act as a counter-regulatory system to that of *agr* (Cheung *et al.*, 1992). Evidence for a joint role for both loci in the regulation of exoproteins was suggested by Cheung and Projan (1994), who found that optimal transcription of RNAIII (a transcript of *agr* that is believed to be the *agr*-specific regulator of exoprotein synthesis) was dependent on an intact *sar* locus. Finally, a third locus designated *sae* for *S. aureus* exoprotein expression, has been identified (Giraudo *et al.*, 1994). Mutations in this locus result in diminished or null levels of α- and ß-hemolysins, DNAase, coagulase and Protein A. Production of lipase, staphylokinase, SEA and proteases however were not affected. As a result of the novel phenotype of these mutants, this locus appears to be distinct from that of *agr, xpr* or *sar.*

TSST-1 is under control of the *agr* locus. *Agr* exerts its effect on TSST-1 production at the transcriptional level, since *agr* mutants have less TSST-1 mRNA in comparison to wild-type cells (Rescei *et al.*, 1986). SEB is also under control of the *agr*, and regulation appears to be at the transcriptional level (Gaskill and Khan, 1988). In addition to *agr*, the *xpr* locus may also play a role in regulating SEB production, since *xpr* mutants were found to produce undetectable levels of SEB (Smeltzer *et al.*, 1993). In contrast to TSST-1 and SEB, SEA is not under control of the *agr* (Tremaine *et al.*, 1993).

B. Genetic Analysis of TSST-1 Production

TSST-1 is a 22 kD exoprotein produced by some strains of *S. aureus*. Initial purification and biochemical analyses of the toxin were carried out by Bergdoll *et al.* (1981), Schlievert *et al.* (1981), and Blomster-Hautamaa *et al.* (1986b). The TSST-1 structural gene was cloned into *E. Coli* on a 10.6 kb chromosomal fragment in the plasmid pRN6100 from an

alpha-hemolysin negative derivative of the *S. aureus* strain 3-14 (Kreiswirth *et al.*, 1983). The nucleotide and partial amino acid sequence of TSST-1 was determined by Blomster-Hautamaa *et al.* (1986a). The TSST-1 structural gene (*tst*) consists of 708 nucleotides. The toxin has a 40 amino acid signal peptide that upon cleavage yields a 194 amino acid mature toxin molecule.

Variability in TSST-1 production observed among *S. aureus* strains is determined by the presence or absence of *tst*, since the concordance between TSST-1 production and detection of the chromosomal gene with oligonucleotide probes is >99% (Bonventre *et al.*, 1989). The TSST-1 determinant is not plasmid linked (Kreiswirth *et al.*, 1984). Initial studies found that *S. aureus* strains isolated from patients with TSS were more frequently lysogenized by temperate bacteriophage than non-TSS-associated *S. aureus* strains. This observation led to the suggestion that the TSST-1 determinant may be carried by a bacteriophage (Schutzer *et al.*, 1983). This theory was refuted by Kreiswirth *et al.* (1983), when transfer of the TSST-1 determinant by lysogenic conversion could not be demonstrated. DNA from TSST-1^+ isolates hybridized to probes derived from sequences flanking the TSST-1 gene, whereas DNA from TSST-1^- isolates did not. This suggested that the TSST-1 gene was located within a larger segment of DNA present only among TSST-1^+ isolates. Subsequently, Kreiswirth *et al.* (1989) reported that the TSST-1 gene is located on a unique genetic element of ~4-7 kb in size. Digesting whole cell DNA with *Cla*I (a restriction enzyme that does not cleave within the TSST-1 genetic element) followed by hybridization with a TSST-1 specific gene probe revealed *Cla*I fragments of various sizes. This indicated that the TSST-1 genetic element may be mobile. Consistent with this was the observation that protoplast fusion and

transformation mapping had placed the TSST-1 gene in at least two places - very close to the tryptophan (*trp*) operon in some strains, and very close to the tyrosine B (*tyrB*) locus in another strain (Chu *et al.*, 1988). The *trp* operon appeared to be the preferred location for the TSST-1 genetic element, since the vast majority of TSST-1$^+$ *S. aureus* strains were tryptophan auxotrophs (Chu *et al.*, 1985; Kreiswirth *et al.*, 1989). The observation that the majority of *trp*$^-$ isolates (51 out of 58) shared common phenotypic characteristics and a common pattern by Southern hybridization with a *tst* probe suggest that these *trp*$^-$ isolates are clonal in nature (Kreiswirth *et al.*, 1989). Multilocus enzyme electrophoresis (MLEE) analyses indicated that a single clone of *S. aureus* (ET41) was responsible for the majority of female urogenital TSS cases (Musser *et al.*, 1990). MLEE analysis also suggests that since the TSST-1 gene occurred in a number of various clonal lineages representing diverse species of *S. aureus*, the gene is evolutionarily old, and hence, has not been recently evolved or acquired by *S. aureus* (Musser *et al.*, 1990).

To date, the TSST-1 genetic element is referred to as Heterologous Insertion 555 (Chu *et al.*, 1988). This term is used to indicate the presence of an extra segment of chromosomal DNA in an organism associated with a specific trait (Kreiswirth *et al.*, 1984). The observation that the TSST-1 determinant was not able to be transferred via transduction, in conjunction with 4-7 kb being the estimated size of the TSST-1 genetic element (a size that is smaller than the genome of any staphylococcal phage) provides evidence to support that the TSST-1 determinant is not mediated by phage. In 1989, the original investigators who studied the molecular biology of the TSST-1 determinant proposed that the TSST-1 genetic element is a staphylococcal transposon (Kreiswirth *et al.*, 1989). To date however, no

experiments to demonstrate that the TSST-1 genetic element can undergo successive transposition to nonhomologous targets have been reported. The exact nature of the TSST-1 genetic element thus remains unclear.

C. Genetic Analysis of SEA Production

Staphylococcal enterotoxin A is the toxin that is most frequently associated with staphylococcal food poisoning outbreaks caused by the ingestion of preformed toxin in contaminated food sources (Minor and Marth, 1971). *In vitro*, *S. aureus* produces very small amounts of SEA, usually not exceeding 5 - 10 μg/ml of medium (Iandolo and Dyer, 1981). In 1978, the SEA structural gene (*sea*) was proposed to reside on the chromosome since SEA production was not associated with detectable plasmid DNA in a number of isolates (Shafer and Iandolo, 1978b). Chromosomal mapping experiments revealed a preferred location for *sea*, since 24 of 29 strains had *sea* located on the chromosome between the *pur*-110 and *ilv*-129 markers (Pattee and Glatz, 1980; Mallonee *et al.*, 1982). Subsequently, *sea* was cloned on a 2.5 kb *Hin*dIII fragment and expressed in *E. coli* (Betley *et al.*, 1984). Further mapping studies by Betley *et al.* (1984) verified that although *sea* was located at the *pur-ilv* site in some strains, other chromosomal locations could be found. The SEA determinant was thus proposed to be mobile, and analysis by Southern blotting found it to be associated with a DNA segment of 8-12 kb in size. Shortly thereafter, Betley and Mekalanos (1985) reported that *sea* was carried by a family of related phages that differed by restriction fragment length polymorphisms (RFLPs) surrounding the gene. The variability in the sequences of the phages led to differences in the upstream promoter regions of *sea*, which played a role in dictating the amount of SEA produced by each strain (Borst and

Betley, 1994). The phages that carry a functional SEA gene may be either viable or defective (Betley and Mekalanos, 1985). In addition, related phages that do not carry *sea* have been found (Betley *et al.*, 1984; Betley and Mekalanos, 1985). There was also a report of a phage that carried a SEA-like gene, called *sezA*$^+$ (Soltis *et al.*, 1990). Although *sezA*$^+$ had a high degree of nucleotide identity with *sea*, there was no translation into an enterotoxin-like protein due to the lack of a translation initiation codon. Recently, *sea* has been found associated with triple converting phages (Coleman *et al.*, 1989). These novel serotype F staphylococcal phages mediated the simultaneous triple lysogenic conversion for ß-lysin, staphylokinase and enterotoxin A. The structural genes for staphylokinase and *sea* were found closely linked in the phage genome. Insertional inactivation of the ß-lysin determinant occurs upon lysogenization of the triple converting phage, yielding a ß-lysin$^-$, staphylokinase$^+$, enterotoxin A$^+$ strain.

In 1988, the nucleotide sequence of *sea* was reported (Betley and Mekalanos, 1988). The gene is 771 bp and encodes a SEA precursor of 257 amino acids, that upon processing yields the mature form of SEA with a molecular mass of 27,100 kD.

D. Genetic Analysis of SEB Production

Similiar to TSST-1 and SEA, SEB is another example of a variable genetic trait in *S. aureus*. SEB is produced more abundantly *in vitro* than SEA, usually exceeding 100 µg/ml of medium (Iandolo, 1990). There is wide variation with respect to the amount of SEB produced by various *S. aureus* strains. The deduced nucleotide sequences of *seb* from a high SEB producer and from a low SEB producer were similiar, negating the possiblity that nucleotide sequence differences in the gene or promoter region were responsible for the

observed variability (Compagnone-Post *et al.*, 1991). One possibility is that variations in SEB levels between strains arise from variations in cellular proteins involved in transcriptional activation. In this regard, the cloned SEB gene from a high and low producer, when introduced on a plasmid into the same strain, were found to produce comparable amounts of both SEB and SEB mRNA (Compagnone-Post *et al.*, 1991). Thus unknown host factors may play a role in regulating SEB production. One such host factor may be the delta-lysin RNA. High SEB producers were shown to have in general more delta-lysin mRNA than low SEB producers (Compagnone-Post *et al.*, 1991).

Initial studies attempting to analyse the SEB determinant in *S. aureus* focussed on the methicillin resistant strain DU4916. Studies with DU4916 led to the suggestion that SEB production was plasmid mediated in this strain (Dornbusch and Hollander, 1973). Subsequently Shafer and Iandolo (1979) found that *seb* was chromosomal in two methicillin resistant plasmid-negative strains. It was then concluded that *seb* could be located either on a plasmid or the chromosome. Further studies by Dyer and Iandolo (1981) led to the suggestion that the plasmid believed to be associated with SEB production (called pSN2) may not contain *seb*, but may be involved in the regulation of SEB synthesis. Shortly thereafter, Khan and Novick (1982) refuted a regulatory role for pSN2 in SEB production. Their results were based on experiments in which cured derivatives of DU4916 were still found to produce SEB. DNA-DNA hybridization experiments verified that pSN2 had not integrated into the chromosome of the derivatives. Next, the nucleotide sequence of pSN2 was determined, and found to encode a polypeptide of ~20 kD. This was different in size than the 28 kD protein that corresponds to SEB. Through their studies, Khan and Novick (1982)

thus tentatively labelled the SEB gene in DU4916 as chromosomal.

The most recent report to examine the genetics of SEB production appeared in 1988. Johns and Khan (1988) provided evidence to suggest that *seb* is associated with a genetic element of at least 26.8 kb in size. SEB^+ isolates were shown to have identical sized restriction fragments with homology to a probe putatively identified as encompassing the right hand junction of the SEB genetic element and common chromosomal DNA, whereas SEB^- isolates had heterogenous sized fragments to the probe. This prompted the investigators to conclude that the 26.8 kb region surrounding *seb* (termed the SEB genetic element) is found in one chromosomal location in these strains. As observed for the genetics of SEA production, some SEB^- strains, although lacking *seb*, had sequences that were homologous to the SEB genetic element. Whether this represented an incomplete or related element was not established. Johns and Khan (1988) theorized that the SEB genetic element could be a bacteriophage. The possibility that it was a common transposon was ruled out since staphylococcal transposons as large as 26.8 kb have not been described. The size of the SEB genetic element is similiar to that of other phages, including the phage that carries *sea*. Furthermore, the location of *seb* at approximately 1.5 kb upstream of one end of the element is consistent with the location of other toxin genes carried by other phages (Betley and Mekalanos, 1985; Johnson *et al.*, 1986). Attempts to induce putative phage carrying the SEB structural gene however have been unsuccessful. This does not exclude the possibility that SEB may be phage-mediated, since defective phage that are unable to produce infectious particles upon induction may be present.

Alternatively Johns and Khan (1988) hypothesized that *seb* could be part of a large plasmid

that had integrated into the chromosome. They noted that Altboum *et al.* (1985) had described a 56.2 kb plasmid, called pZA10, that frequently integrated and excised from the chromosome with concomitant rearrangements in DNA sequence. Upon transfer of pZA10 into SEB^- strains, several transformants became SEB^+. Further support for plasmid location of *seb* came from the observation that the G-C content of *seb* is lower than the majority of *S. aureus* chromosomal genes, but similiar to that of other *S. aureus* plasmid genes (Jones and Khan, 1986). To date, no further work has been reported in this regard, and thus the exact nature of the SEB genetic element and its chromosomal location still remain elusive.

1.3. Focus of Thesis Proposal

Staphylococcus aureus is a complex pathogen that produces many extracellular proteins including TSST-1, SEA and SEB, all of which have been implicated in causing TSS. TSST-1 is believed to be the primary toxin involved in causing the disease, since much evidence is present to support an etiologic role. Unfortunately however, key experiments addressing its biological role in menstrual TSS have not yet been performed. These experiments consist of using isogenic mutants in TSST-1 production in a relevant animal model. The primary objective of this research was to determine if a role for TSST-1 in menstrual TSS could be established by performing the above experiment. Upon establishment of a role for TSST-1 in menstrual TSS, interaction of TSST-1 with other staphylococcal exoproteins, specifically SEA and SEB, on both a functional and molecular level was warranted. The interaction of TSST-1 with these specific toxins are believed to have important clinical significance due to two very important observations that have been made among TSS-associated *S. aureus* isolates. First is the observation that MTSS isolates of *S. aureus* co-produce TSST-1 and

SEA more frequently than NMTSS, non-TSS or carrier isolates (Chang *et al.*, 1991). Second is the observation that production of TSST-1 and SEB by *S. aureus* is mutually exclusive or that co-production of these toxins is extremely rare (Garbe *et al.*, 1985; Crass and Bergdoll, 1986a; Crass and Bergdoll, 1986b; Ewan *et al.*, 1989; Bohach *et al.*, 1990; Johnson *et al.*, 1991; Lee *et al.*, 1992).

Co-production of TSST-1 and SEA by *S. aureus* is postulated to have important functional consequences *in vivo* due to the designation of both of these toxins as superantigens. The co-expression of two such proteins by a *S. aureus* strain might lead to a more virulent strain than production of a single 'superantigenic' toxin. If true, this might provide a putative explanation for why a TSST-1^+/SEA^+ clone of *S. aureus* is frequently associated with MTSS. What role SEA may play in conjunction with TSST-1 specifically in MTSS however is unclear.

In addition to TSST-1, SEB has also been suggested to play a role in TSS. Therefore, the phenotypic observation that TSST-1 and SEB production among *S. aureus* strains appears mutually exclusive has important clinical implications, and a novel regulatory system involved in mediating the control of TSST-1 and SEB expression may exist. Although it is generally accepted that this toxin combination rarely exists at the phenotypic level, controversy still exists at the genotypic level over whether *tst* and *seb* can be found within the same isolate. In light of this controversy, an examination of *S. aureus* isolates for the presence of the TSST-1 and SEB genes and determination of the molecular mechanism(s) involved in regulating this toxin phenotype, was warranted.

In view of the above, the following **Questions** provided the focus for the thesis proposal:

1. Is there conclusive evidence that TSST-1 plays a causative role in the pathogenesis of Menstrual TSS?

2. Does co-production of TSST-1 and SEA by a *S. aureus* strain mediate increased virulence?

3. Is the "mutually exclusive" production of TSST-1 and SEB due to the absence of structural genes in the chromosome?

4. What are some possible mechanisms that may explain the "mutual exclusivity" of TSST-1 and SEB co-production?

Chapter 2. Materials and Methods

2.1. Bacterial growth media and antibiotics

Escherichia coli strains were propogated on Luria-Bertani (LB) media (Sambrook *et al.*, 1989) containing antibiotics where appropriate (ampicillin 50 μg/ml; ampicillin + methicillin 80 and 20 μg/ml respectively; erythromycin [Em] 100 μg/ml). *Staphylococcus aureus* strains were propogated on Tryptic Soy Agar (TSA), Tryptic Soy Broth (TSB) or Brain Heart Infusion media (BHI) (Difco, Detroit, MI) containing antibiotics where appropriate (Em 10 μg/ml; chloramphenicol [Cm] 10 μg/ml).

2.2. Bacterial strains and plasmids

The *Staphylococcus aureus* control or reference strains and clinical isolates used in this study are listed in Tables 3 and 4 respectively. Plasmids used in this study are listed in Table 5.

2.3. Phenotypic Analyses of *S. aureus*

2.3.1. Toxin Production

A. Immunoblotting

S. aureus isolates were grown overnight in either BHI or TSB containing antibiotics where appropriate. The post-exponential phase cultures were centrifuged (1,200 x *g*; 10 min) and the supernatant was filter-sterilized. Proteins in the culture supernatants were concentrated by either lyophilization or ultrafiltration through a Centricon-10 or Microcon-3 concentrator (10,000 and 3,000 molecular weight cut-offs respectively; Amicon, Beverly, MA).

Table 3 *Staphylococcus aureus* Reference and Control Strains Used in this Study

Strain	Phentoypic Results TSST-1+	SEB+	Genotypic Results *tst*+	*seb*+	Clinical and other features	Reference or Source
RN450	-	-	-	-	NCTC 8325	Murphy *et al.*, 1981
RN4220	-	-	-	-	restriction deficient	Kresiwirth *et al.*, 1983
RN3984	+	-	+	+	TSSM/SEA+ *trp*+	Kreiswirth *et al.*, 1989 Chu *et al.*, 1988
RN7043	-	-	-	+	isogenic mutant of RN3984; TSST-1+/SEA+	This study
INT#1	+	-	+	+	isogenic mutant of RN3984; TSST-1+/SEA-	This study
FR11169	+	-	+	+	TSSM, *trp*-	Kreiswirth *et al.*, 1989 Chu *et al.*, 1988
0507	-	+	-	+	Non-TSS SEB+	ATCC 13566
8527	-	-	-	-	Non-TSS SEA+	ATCC 13565
8528	-	+	-	+	Non-TSS SEB+	ATCC 14458
8530	-	+	-	+	Non-TSS SEC+	ATCC 19095
8531	-	-	-	nd	Non-TSS SEE+	ATCC 27664

Abbreviations: TSST-1+, producing toxic shock syndrome toxin-1; SEA+, producing staphylococcal enterotoxin A; SEB+, producing staphylococcal enterotoxin B; SEC+, producing staphylococcal enterotoxin C; SEE+, producing staphylococcal enterotoxin E; *tst*$^+$, containing TSST-1 structural gene; *seb*$^+$, containing SEB structural gene; *trp*+, requiring tryptophan for growth; *trp*-, not requiring tryptophan for growth; TSSM, menstrual toxic shock syndrome; Non-TSS, non-toxic shock syndrome-associated *S. aureus* infection; nd, not determined

Table 4 *Staphylococcus aureus* Clinical Isolates Used in this Study

Cinical Isolates	Phenotypic Results TSST-1+	SEB+	Genotypic Results *tst*+	*seb*+	Electrophoretic Type (ET)	Clinical Characteristics
7051	+	-	+	+	5	Carrier
7054	-	+	-	+	6	Carrier
7128	-	+	nd	+	6	Carrier
7140	+	-	+	+	21	Carrier
7142	-	+	-	+	6	Carrier
7145	-	+	-	+	6	Carrier
7163	-	+	-	+	6	Carrier
7294	+	-	+	+	21	TSSNM
7333	-	+	-	+	5	TSSNM
7335	-	+	nd	+	22	TSSNM
7370	-	+	-	+	6	Carrier
7371	-	+	-	+	6	Carrier
7372	-	+	-	+	6	Carrier
7385	-	+	-	+	6	TSSNM
7402	-	+	-	+	6	Non-TSS
7405	-	+	-	+	6	Non-TSS
7407	-	+	-	+	5	Non-TSS
7423	-	+	-	+	6	Non-TSS
7429	-	+	-	+	6	Non-TSS
7431	-	+	-	+	6	Non-TSS
7513	-	+	-	+	6	TSSNM
7599	-	+	nd	+	6	TSSNM
7616	+	-	+	+	21	Carrier
7660	-	-	-	nd	10	Carrer
7690	-	-	nd	-	49	Non-TSS
7692	-	+	-	+	6	Non-TSS
7694	-	+	-	+	50	Non-TSS
7695	-	+	-	+	51	Non-TSS
7785	-	+	-	+	48	TSSM
7835	-	+	-	+	5	Non-TSS
7836	-	+	-	+	50	TSSNM
8254	-	+	-	+	59	Carrier
8270	+	-	+	+	21	Carrier
8292	-	+	-	+	50	TSSNM
8297	-	-	-	nd	8	Carrier
8315	-	+	-	+	6	Carrier
8385	-	+	-	+	62	Non-TSS
8395	-	+	-	+	63	Non-TSS
8426	-	+	-	+	6	Non-TSS
VI-5	+	-	+	+	nd	TSSNM

Abbreviations: TSST-1+, producing toxic shock syndrome toxin-1; SEB+, producing staphylococcal enterotoxin B; *tst*$^+$, containing TSST-1 structural gene; *seb*$^+$, containing SEB structural gene; TSSNM, nonmenstrual toxic shock syndrome, TSSM, menstrual toxic shock syndrome; Non-TSS, non-toxic shock syndrome-associated *S. aureus* infection; Carrier, no infection; nd, not determined. ET analysis was performed by J. M. Musser in the laboratory of R. K. Selander (Pennsylvania State University)

Table 5 Plasmids Used in This Study

Plasmid	Host	Marker	Relative Properties	Reference
pUC19	*E. coli*	Ap^r	Cloning Vector	Yanisch-Perron *et al.*, 1985
pRN6550	*E. coli*	Ap^r	pBR322 carrying *tst*	Kreiswirth *et al.*, 1987
pSK155	*E. coli*	Ap^r	pBR322 carrying *seb*	Ranelli *et al.*, 1985
pMJB38	*E. coli*	Ap^r	pBR322 carrying *sea*	Betley and Mekalanos, 1988
pMLD6872	*E. coli*	Ap^r	pUC19 with a 624 bp *Bam*HI-*Hin*dIII fragment from pMJB38 containing *sea* sequences	This study
pRN6441/pRN6442	*S. aureus*	Em^r	t^s derivatives of pE194 carrying an Em^r marker on a 1.4 kb TaqI fragment; pUC18 polylinker at the PstI site is located in opposite orientations in these plasmids	Horinouchi and Weisblum, 1982; Villafane *et al.*, 1987; Sambrook *et al.*, 1989
pMLD6873	*S. aureus*	Em^r	pMLD6872 with a 1.4 kb *Taq*I fragment (encoding Em^r) from pRN6442	This study
pMLD6873C-1	*S. aureus* *E. coli*	Em^r Apr	pMLD6873 ligated to pRN6441; t^s origin of replication	This study
pJW1	*S. aureus* *E. coli*	Em^r Cm^r/Ap^r	pHPS9 containing *tst*	Haima *et al.*, 1990; This study
pMLD6876-1	*S. aureus* *E. coli*	Em^r Ap^r	pUC19 containing *seb* ligated to pRN6441	This study

Ap^r, ampicillin resistant; Em^r, erythromycin resistant; Cm^r, chloramphenicol resistant, t^s, temperature sensitive origin of replication

Lyophilized samples were suspended in distilled water. Equal amounts of total protein (Bio-Rad Protein Assay; Bio-Rad Laboratories Ltd., Missisauga, Ontario) were loaded onto a 14% SDS-polyacrylamide gel (SDS-PAGE) prepared by standard procedures (Sambrook *et al.*, 1989). After electrophoretic separation of the samples, proteins were transferred to a nitrocellulose membrane with the Semi-Dry Electroblotter A (Ancos Dimension Laboratories Inc., Mississauga, Ontario, Canada; 30-45 min, 150 mA, room temperature) using transblot buffer (25 mM TRIS-HCl, 192 mM glycine, 20% methanol; pH 8.3). The membrane was incubated overnight in a 1:100 dilution of polyclonal rabbit anti-serum raised against purified TSST-1 from *S. aureus* MN8 (Rosten et al., 1989) or 1 μg/ml of rabbit anti-serum to SEB (Toxin Technology, Sarasota, FLA) in 5% skim milk (Difco) in a volume sufficient to cover the membrane (30-50 ml). Following three 2-min washes with TRIS-buffered saline (1.21 g/l TRIS-HCl, 9 g/l NaCl; pH 7.4) containing 1% Tween-20 (TBS-T) filters were incubated with a 1:1,000 dilution of biotinylated goat-anti-rabbit immunoglobulin G (Gibco/BRL) in TRIS-buffered saline containing 0.5% bovine serum albumin (TBS-BSA) for approximately 2 h. After washing with TBS-T, the filter was incubated with a 1:1,000 dilution of streptavidin-horseradish peroxidase conjugate (Gibco/BRL) in TBS-BSA for 30 min. Following a final wash step with TBS-T, colour development was achieved with 4-chloronaphthol as described by the manufacturer (Gibco/BRL).

B. Immunoassay

Production of TSST-1 by *S. aureus* isolates was quantitated by a non-competitive enzyme-linked immunosorbent assay (ELISA) procedure similiar to the method previously described (Rosten *et al.*, 1987) with one modification. Biotinylated-TSST-1 was used as the capture

antibody in place of anti-TSST-1 conjugated to alkaline phosphatase. Biotinylation of TSST-1 was accomplished using the Protein Biotinylation System (Gibco/BRL) following the manufacturer's instructions. After addition of biotinylated anti-TSST-1 [diluted 1:2,000 in PBS containing 0.05% Tween-20 (PBS-T)] to the wells (0.1 ml), the ELISA plate was incubated for 90 min at 37°C. This was followed by three 2-min washes with PBS-T. Streptavidin-alkaline phosphatase (Gibco/BRL; diluted 1:2,000 in PBS-T) was added (0.1 ml/well) and plates were incubated for 20 min at 37°C. Detection was performed as described previously (Rosten et al., 1987) using *p*-nitophenyl phosphate as the substrate (Sigma Chemical Co., St. Louis, MO). The sensitivity limit of the ELISA was 0.5 ng/ml of TSST-1.

SEA production by *S. aureus* was determined by a non-competitive ELISA procedure. Affinity purified anti-SEA (Toxin Technology) (2 μg/ml) in 0.05 M bicarbonate-carbonate buffer (pH 9.6), was coated onto microtiter plates (0.1 ml/well) (ImmulonI, Dynatech Laboratories, Chantilly, VA) overnight at room temperature. Unbound antibodies were removed by three 2-min washes with PBS-T. A SEA standard (Toxin Technology), serially diluted from 128 to 0.5 ng/ml in PBS-T or BHI (when appropriate) or culture filtrates pretreated with normal rabbit serum (10% [vol/vol] final concentration) to eliminate protein A (Rosten et al., 1987), were added in 0.1 ml volumes to the wells. After incubation at 37°C for 1.5 h, wells were washed with PBS-T, and 0.1 ml of alkaline phosphatase conjugated anti-SEA diluted 1/250 in PBST (prepared by the method of Nakamura *et al.*, 1986) was added to each well followed by incubation for 1.5 h at 37°C. Finally wells were washed with PBS-T and incubated with 0.1 ml of a 1 mg/ml solution of *p*-nitrophenyl

phosphate (Sigma) followed by measurement of the O.D. at 410 nm using a Dynatech MR5000 microplate reader (Dynatech Laboratories). The sensitivity limit of the immunoassay was 1 ng/ml of SEA.

SEB production by *S. aureus* isolates was determined by a non-competitive ELISA procedure as described by Lee *et al.* (1992). The sensitivity limit of the SEB immunoassay was 0.5 ng/ml of SEB.

The total amount of protein found in the culture filtrates of strains was quantitated using the Bio-Rad Protein Assay (Bio-Rad Laboratories).

2.4. Molecular Biology Techniques

2.4.1. DNA Manipulation

DNA modifying enzymes (restriction enzymes, ligase, Klenow, alkaline phosphatase) were purchased from New England Biolabs (Mississauga, Ontario, Canada), Gibco/BRL Life Technologies Inc. (Gaithersburg, MD) or Boehringer Mannheim (Laval, Quebec, Canada) and used according to the manufacturer's instructions.

2.4.2. Isolation of Plasmid DNA

Plasmid DNA was isolated from *Escherichia coli* via lysis by alkali using standard procedures (Sambrook *et al.*, 1989). Isolation of plasmid DNA from *Staphylococcus aureus* was performed by the alkali lysis procedure modified by the use of lysostaphin as described by Novick (1991).

2.4.3. Transfer of DNA

Plasmids were transformed into competent $CaCl_2$-treated cells of *E. coli* JM109 (Yanisch-Perron *et al.*, 1985) by standard procedures as described by Sambrook *et al.* (1989) or into

cells of XL-1 Blue (Bullock *et al.*, 1987) following the protocol of Stratagene (1992). Introduction of plasmid DNA into *S. aureus* RN4220 (restriction-deficient mutant capable of accepting shuttle plasmids propogated in *E. coli*) (Kreiswirth *et al.*, 1983) was accomplished by electroporation (Bio-Rad Laboratories Ltd.) following the method of Augustin and Gotz (1990). Introduction of plasmid DNA into *S. aureus* RN3984 was performed via transduction with phage 80α (Novick, 1963). Phage 80α was maintained by propagation on *S. aureus* RN450.

2.4.4. Transduction

Phage 80α transducing lysates were prepared on donor strains using the agar layer method described by Swanstrom and Adams (1951). A swab of an overnight culture of donor cells was suspended into 0.2 ml of phage buffer (1mM $MgSO_4$, 4mM $CaCl_2$, 50 mM TRIS-HCl (pH 7.8), 5.9 g/l NaCl, 1.0 g/l gelatin) (Novick, 1991) and inoculated with $\sim 10^7$ PFU of phage 80α. Cultures were gently mixed and the phage was allowed to absorb for 5 min at room temperature. Tryptic Soy Broth Top Agar (TSBTA; TSB containing 0.7% agar) (2.5 ml) was added and the mixture poured onto TSA plates containing 5mM $CaCl_2$. After overnight incubation, 2.5-5.0 ml of phage buffer was added to each plate and the agar layer scraped into centrifuge tubes. Tubes were shaken vigorously for several minutes and centrifuged at 10,000 X *g* for 10 min at 4°C. Supernatants were filter-sterilized. Transduction was performed essentially as described by Kasatiya and Baldwin (1967) with the modification of a 20 min incubation at 37°C before addition of 0.02M sodium citrate as described by Schroeder and Pattee (1984). Transductants were selected on BHI agar containing 10 μg/ml erythromycin and 5 μg/ml sodium citrate.

2.4.5. **Preparation of chromosomal DNA**

DNA was prepared from overnight cultures of *S. aureus* grown at 37°C. The cells were washed by centrifugation in TEE buffer (1 M TRIS-HCl (pH 7.6) containing 0.1 M EDTA and 10 mM EGTA), resuspended in TEE buffer (450 μl/ml of culture) containing lysostaphin (10 units/ml; Sigma) and RNaseA (50 μg/ml; Boehringer Manheim), and incubated at 37°C for 1 h. Cells were lysed by the addition of 30 μl of 10% SDS and 0.1 mg/ml proteinase K (Boehringer Mannheim), followed by incubation at 65°C for 1 h. Sodium chloride was added to 500 mM (final concentration) followed by 80 μl per ml of volume of a 10% solution of CTAB (hexadecyl trimethylammonium bromide; Sigma) in 0.7 M NaCl. The sample was incubated at 65°C for 10 min and then extracted with phenol-chloroform (Sambrook *et al.*, 1989). The nucleic acids were precipitated with ethanol as previously described (Sambrook *et al.*, 1989). The DNA was dissolved in TE buffer (10 mM TRIS-HCl (pH 7.6), containing 1 mM EDTA) and stored at 4°C until use.

2.4.6. **Preparation of DNA probes**

pRN6550 carrying *tst* (Kreiswirth *et al.*, 1987) (provided by R. P. Novick and B. N. Kreiswirth, Public Health Research Institute, New York), pSK155 carrying *seb* (Ranelli *et al.*, 1985) (provided by S. A. Khan, University of Pittsburg), pMJB38 carrying *sea* (Betley and Mekalanos, 1988) (provided by R. P. Novick) and pRN6441 (Villafane *et al.*, 1987) (provided by R. P. Novick) were digested with the appropriate restriction endonucleases. The DNA fragments to be used as probes were isolated from a low-melting agarose gel (Sigma) with the use of Gelase (Epicentre Technologies, Madison, WI), following the manufacturer's instructions. The DNA fragments were labelled by the random primed

incorporation of digoxigenin-labeled dUTP (Boehringer Mannheim) as outlined by the supplier.

2.4.7. Southern hybridization analysis

Chromosomal DNA from the bacterial isolates was digested with restriction endonucleases *Cla*I, *Hin*dIII or *Kpn*I under conditions recommended by the supplier. DNA was electrophoresed through a 1% agarose gel in TRIS-acetate-EDTA buffer (40 mM TRIS-acetate, 2 mM EDTA). Digoxigenin-labelled molecular size standards (Boehringer Mannheim) were used as the size controls.

DNA fragments were transferred to nylon membranes (Boehringer Mannheim) under alkaline conditions (0.4 M NaOH) as described by Sambrook *et al.* (1989). The hybridization conditions were: 20-50 ng/ml digoxigenin-labelled probe in a solution of 50% formamide, 5X SSC (1X SSC= 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 2% blocking reagent (Boehringer Mannheim), 0.1% N-lauroylsarcosine (wt/vol), and 0.02% SDS (wt/vol) at 42°C overnight. Washing (0.1X SSC, 0.1% SDS [wt/vol]) was done at 68°C. Detection by luminescence was accomplished by the addition of a high-affinity alkaline phosphatase-conjugated anti-digoxigenin antibody, followed by the substrate AMPPD (disodium salt) under conditions described by Boehringer Mannheim. Probes were removed from the nylon blots with two 20-min washes at 37°C in 0.2 M NaOH containing 0.1% SDS (wt/vol), followed by two 5-min washes in 2X SSC at room temperature. Removal of the probe was verified by lack of a hybridization signal after the wash step.

2.4.8. Polymerase chain reaction (PCR) analysis

Sequences for the oligonucleotide primers designated SEB1 and SEB2 targeting sequences

located at positions 310-329 and 634-657 respectively of the SEB structural gene were prepared at the Nucleic Acid and Protein Service Unit (University of British Columbia). These primers were designed by Johnson *et al.* (1991) and give an amplification product of 478 bp in SEB^+ isolates. PCR was done essentially as described by Johnson *et al.* (1991), except that 60 ng of nucleic acid was used as template for the reaction.

Sequences for the oligonucleotide primers designated MLD-1 and MLD-2 were chosen with the use of 'Primer Detective' software. The oligonucleotide sequence for MLD-1 was 5'- CTAAACCAGATGAGTTGCACAAATCG -3', and the sequence for MLD-2 was 5'-AATTCCCTAACTTAGTGTCC -3'. These primers target SEB structural gene sequences located at positions 17-39 and 162-181 respectively, with an expected PCR product of 165 bp in SEB^+ isolates. The PCR was performed in a 50 μl reaction mixture using 60 ng of nucleic acid as template and 0.2 μM of each primer under the following conditions: denaturation for 30 sec at 95°C, annealing of primers for 30 sec at 55°C, primer extension for 1 min at 72°C. This was repeated for 40 cycles. PCR products were separated on a 2.5% agarose gel in TRIS-acetate-EDTA buffer.

2.5. Construction of Isogenic Mutants

2.5.1. *S. aureus* RN3984 - Wildtype isolate used for isogenic mutant construction

RN3984 is a TSST-1-producing strain of *S. aureus* isolated from the vagina of a patient with menstrual TSS (Barbour, 1981). It has been also referred to as the Harrisburg strain (Schlievert *et al.*, 1979). It is beta-hemolytic, tryptophan-negative, coagulase positive, and belongs to phage group 1 (major types 29/52A/79) (Barbour, 1981; Schlievert *et al.*, 1979;

Kreiswirth *et al.*, 1989). It produces staphylococcal enterotoxin A but not staphylococcal enterotoxins B, C, D or E as tested by immunoblot analysis described previously in this section with the addition of 1 μg/ml of rabbit anti-serum to SEA, SEB, SEC_{1-3}, SED or SEE respectively (Toxin Technology).

2.5.2. Inactivation of *tst* in *S. aureus* RN3984

The TSST-1 structural gene of RN3984 was inactivated by allele replacement by R. P. Novick and B. N. Kreiswirth as previously described (Sloane *et al.*, 1991). In brief, the *tst* shuttle plasmid pRN6684 [*tst*::Tc^r; temperature sensitive origin of replication (t^s)] was introduced into RN3984 at the permissive temperature for replication. Dilutions of a broth culture were plated onto TSB containing Tc and incubated at 43°C. After integration of the plasmid into *tst* by a single crossover event, transduction of the co-integrate into wild-type RN3984 followed by Tc^r screening of the transductants was performed. Transductants were scored for Em^s to identify recombinants where plasmid excision had occurred and caused allele replacement of *tst* by *tst*::Tc^r. The Tc^r fragment was ~2.9 kb. One transductant, RN7043, was kept for further characterization.

2.5.3. Inactivation of *sea* in *S. aureus* RN3984

A. Construction of plasmids

A 624 bp *Bam*HI-*Hin*dIII fragment (containing *sea*) was excised from pMJB38 and ligated to *Bam*HI-*Hin*dIII digested pUC19 (2.7 kb) to create pMLD6872 (3.3 kb) containing a unique *Cla*I site within *sea*. Inactivation of *sea* in pMLD6872 was accomplished by inserting a 1.4 kb *Taq*I fragment encoding the erythromycin resistance determinant (Em^r) of the *S. aureus* plasmid pRN6442 (Villafane *et al.*, 1987) into the unique *Cla*I site creating

pMLD6873 (4.7 kb). All attempts to introduce pMLD6873 as a suicide plasmid into RN3984 by electroporation were unsuccessful. Next *Eco*RI digested pMLD6873 was ligated to *Eco*RI digested pRN6441 (~3.8 kb) (Villafane *et al.*, 1987) to create pMLD6873C-1 (~8.5 kb) (a shuttle plasmid that can replicate in both *S. aureus* and *E. coli* containing a temperature sensitive origin of *S. aureus* replication). Refer to Figure 1 for an overview of plasmid construction.

B. Introduction of plasmid pMLD6873C-1 into RN3984 and isolation of SEA$^-$ transductants

pMLD6873C-1 was introduced into *S. aureus* RN4220 by electroporation and transformants selected for resistance to Em. Next, pMLD6873C-1 was transferred from RN4220 to RN3984 (TSST-1$^+$/SEA$^+$) by transduction with phage 80α. After incubation at 30-32°C, (the permissive temperature for plasmid replication) transductants were selected by resistance to Em. Several Em^r transductants of RN3984 were selected and after 8 h growth at 32°C in TSB containing Em, the transductants were grown overnight at 43°C (the non-permissive temperature for plasmid replication). Overnight cultures were plated (0.1 ml) onto solid media (TSA with Em) and grown overnight at 43°C. The passage of cells from solid to liquid media at 43°C was performed for three consecutive cycles. Em^r colonies were analysed for SEA production by immunoassay as previously described in section 2.3.1. At this stage, the genomic DNA of several SEA$^-$ transductants were digested with *Hin*dIII and analysed by Southern hybridization analysis with a 624 bp *Bam*HI-*Hin*dIII *sea* probe (Betley and Mekalanos, 1988). The results indicated that in these transductants, pMLD6873C-1 had integrated into the *sea* chromosomal locus since the expected *Hin*dIII fragment that exceeded

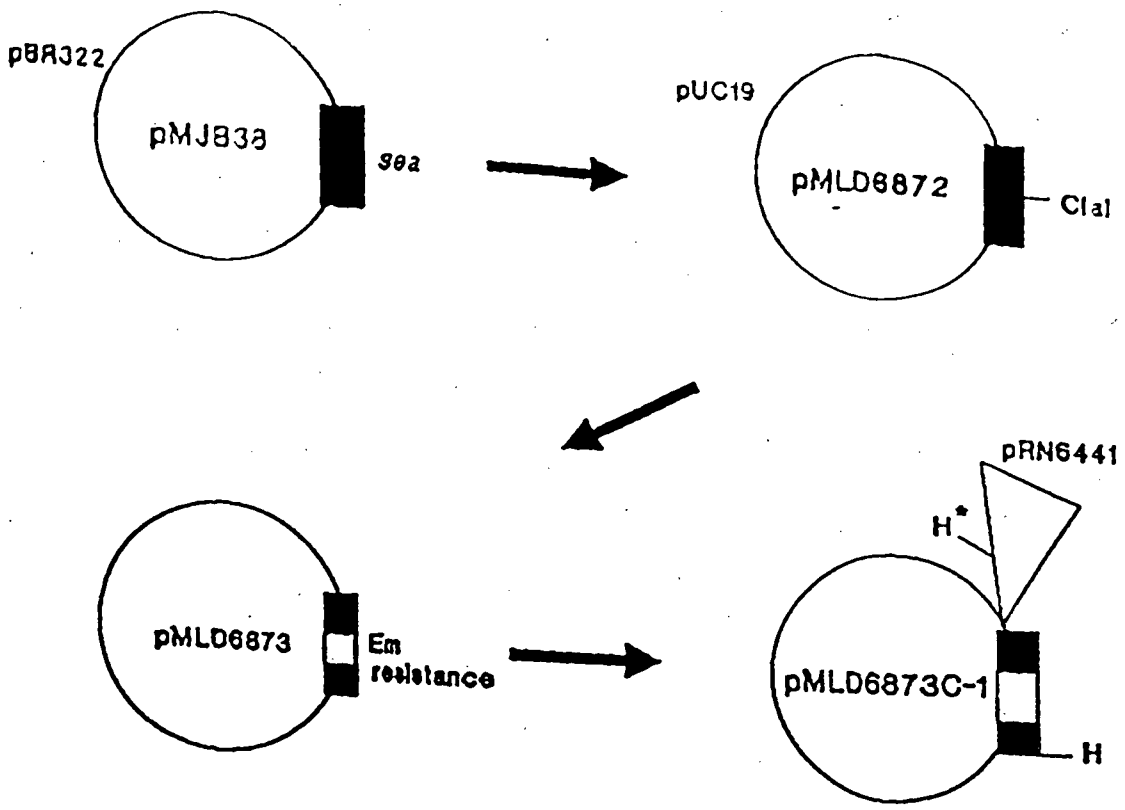


Figure 1. Plasmid constructs generated to assist in the inactivation of *sea* in RN3984 by the method of plasmid integration.
Em, erythromcyin; H, *Hin*dIII;, H*, *Hin*dIII site not definitely known

the *Hin*dIII fragment observed with RN3984 by 1.4 kb was not observed (the size of the Em^r marker used to inactivate *sea* in pMLD6873). Instead, three *Hin*dIII fragments that hybridized to the probe were found in the SEA$^-$ transductants. Since pMLD6873C-1 contains two *Hin*dIII sites (see Figure 1) this would be consistent with recombination of the plasmid into the *sea* locus via a single crossover event. To increase the likelihood of obtaining a SEA$^-$ mutant derived by allele replacement in which only the inactivated *sea* and not the plasmid backbone had recombined into the chromosome, one SEA$^+$/Em^r transductant was chosen and the Em^r marker again transduced into wild-type RN3984 with phage 80α. This facilitates plasmid excision and allele replacement at a high frequency (McDevitt *et al.*, 1992). Sixty of sixty-four Em^r transductants screened after this step were SEA$^-$. Several transductants analysed by Southern hybridization analysis using the *sea* probe revealed results that were again consistent with integration of pMLD6873C-1 into *sea*. Attempts to remove the plasmid backbone of one transductant by growing without antibiotic selection at the non-permissive temperature for a number of generations as previously described (Foster *et al.*, 1990) were unsuccessful. One SEA$^-$/Em^r mutant, designated INT#1, was chosen for further characterization.

C. Verification of the stability of the SEA$^-$ phenotype of INT#1 after *in vivo* growth

Thirty BALB/c mice were inoculated intraperitoneally (i.p.) with $\sim 10^6$-10^7 CFU of washed RN3984 (TSST-1$^+$/SEA$^+$) or INT#1 (TSST-1$^+$/SEA$^-$) suspended in sterile phosphate-buffered saline (PBS) (15 per group). For the following 5 days, bacteria were collected from three mice in each group by injection of 5 ml of sterile PBS i.p., followed by reaspiration.

The collected aspirate was centrifuged at 1,200 X *g* for 10 min and the pellet was resuspended in 0.5 ml of sterile PBS. Samples of the recovered bacteria were plated onto Mannitol salt agar (Difco) and incubated overnight at 37°C. Cells from several isolated colonies on mannitol plates (chosen by the presence of a yellow halo surrounding the colony) were grown overnight in TSB and the culture supernatant was analysed for SEA by immunoassay.

2.6. Creation of a TSST-1 and SEB Producing Strain of *S. aureus*

2.6.1. Construction of plasmids

A. pJW1 (*tst* containing plasmid)

Construction of pJW1 was performed by Julian Wood (University of Victoria, Co-op Student). In brief, the cloned TSST-1 structural gene resided in the pBR322-based plasmid pRN6550 (Kreiswirth *et al.*, 1987). A 1.6 kb *Bgl*I fragment containing *tst* was excised from pRN6550 and ligated to *Hinc*II digested pBluescript (Short *et al.*, 1988). Next, *tst* was excised on a 1.62 kb *Hin*dIII-*Xho*I fragment and ligated to the *Sma*I digested *B. subtilis* plasmid pHPS9 (Haima *et al.*, 1990) to create pJW1. pJW1 is a shuttle vector that can replicate in both *S. aureus* and *E. coli*, and contains antibiotic resistant genes for chloramphenicol and erythromycin.

B. pMLD6876-1 (*seb* containing plasmid)

The cloned SEB structural gene resided in the plasmid pSK155 (Ranelli *et al.*, 1985). *seb* was excised on a 1.8 kb *Kpn*I-*Cla*I fragment and ligated to *Hinc*II digested pUC19 to create pMLD6876. A shuttle vector derivative of pMLD6876 was next constructed by ligating pMLD6876 with pRN6441 (Villafane *et al.*, 1987) to create pMLD6876-1. pMLD6876-1

contains a gene encoding resistance to erythromycin.

2.6.2. Introduction of pJW1 and pMLD6876-1 into *S. aureus*

pMLD6876-1 was introduced into *S. aureus* RN4220 by electroporation. Colonies were selected for resistance to Em. SEB production was verified by immunoblot analysis in the Em^r transformants. Next, pJW1 was introduced into RN4220(pMLD6876-1) by electroporation. Colonies were selected for resistance to Em and Cm. Production of TSST-1 and SEB by Em^r/Cm^r transformants was determined by immunoblot analysis.

2.7. *In Vitro* Effects of TSST-1, SEA and the Culture Filtrates of Isogenic Strains on Human Peripheral Blood Mononuclear Cells and Rabbit Splenocytes

2.7.1. Purification of TSST-1 and SEA

TSST-1 was purified from culture supernatants of *S. aureus* MN8 by Winnie Kum (University of British Columbia) by preparative isoelectric focusing and chromatofocusing as previously described (Kum *et al.*, 1993). This procedure results in a pure TSST-1 preparation, as verified by silver staining after SDS-PAGE, by immunoblotting with polyclonal rabbit antiserum raised against the crude culture supernatant of *S. aureus* MN8, and by autoradiography after iodination and SDS-PAGE (Kum *et al.*, 1993).

SEA was purchased from Toxin Technology and further purified by Winnie Kum by chromatofocusing using a pH 6 to 8 gradient polybuffer exchanger (PBE 94; Pharmacia Fine Chemicals). SEA (2 mg) was reconstituted in 5 ml of TRIS-acetate buffer (25 mM, pH 8.3) and applied to a column (K 15.30, 1.5 by 30 cm; Pharmacia) containing 80 ml of PBE 94 equilibrated with TRIS-acetate buffer. Elution of SEA was accomplished with polybuffer 96-acetate (pH 6.0; Pharmacia) at a flow rate of 36 ml/h. Fractions were assayed for SEA by

immunoassay, and those containing SEA were pooled, dialysed against several changes of deionized water for 72 h at 4°C, and lyophilized. Purity of the SEA preparation was verified by the observation of a single band at ~28 kD after separation on SDS-PAGE followed by silver staining.

2.7.2. **Purification of Human Peripheral Blood Mononuclear Cells**

Fresh human peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers by the method previously described (See *et al.*, 1992c). In brief, cells were obtained by centrifugation of platelet-pheresis buffy coats over Histopaque 1.077 (Sigma). Mononuclear cells at the interface were washed with Hank's balanced salt solution before being separated into T- and non-T cell populations by rosetting with sheep erythrocytes as described previously (Madsen and Johnson, 1979). Non-rosetted cells were separated into monocyte and B lymphocyte fractions by density gradient centrifugation over Percoll (Pharmacia Fine Chemicals, Dormal Quebec, Canada). RPMI 1640 and 10% heat inactivated (56°C, 30 min) fetal calf serum (FCS) (Gibco/BRL) (~16 ml) was added to the non-T cell fraction and mixed with Percoll to give a final specific gravity of 1.062 g/ml. One ml of RPMI plus 10% FCS was layered on the top of each suspension and centrifuged at 800 X *g* for 15 min. The monocyte-containing interface was collected and cells were washed three times with Hank's. This procedure results in a ≥90% pure monocyte preparation as assessed by nonspecific esterase staining of cytospin preparations (See *et al.*, 1992c). For isolation of purified human T lymphocytes, E-rosetted cells were treated with ammonium chloride to remove sheep erythrocytes, and washed three times with Hank's. At this stage, T cells are >80% $CD2^+$ by flow cytometric analysis as determined previously in our

laboratory by Raymond See.

2.7.3. Preparation of Rabbit Splenocytes

Rabbit spleens from female New Zealand White rabbits were removed asceptically, and single cell suspensions were obtained by pressing the spleen through a stainless steel grid into RPMI 1640 medium. Red blood cells were removed from the cell suspension by lysis with 0.85% NH_4Cl_2 similiar to the method previously described (Mishell *et. al.*, 1980). Cells were washed in RPMI 1640 and resuspended to the appropriate concentration in RPMI 1640 supplemented with 10% heat inactivated FCS, 2mM L-glutamine, 25 mM Hepes buffer (pH 7.2) and 10 μg/ml polymyxin B sulfate.

2.7.4. Preparation of Culture Filtrates from Isogenic Strains

Culture filtrates from isogenic strains were prepared by growing as indicated for the *in vivo* model inocula preparation (BHI, post-exponential phase) (see Section 2.8.2) followed by centrifugation at 2,500 X *g* for 10 min. Culture filtrates were filter-sterilized and stored at -20°C.

2.7.5. Immunoassay Detection of Cytokine Production by Human PBMC or Rabbit Splenocytes Stimulated with Toxin or Culture Filtrate

T cells and monocytes (1:1 ratio; 2 X 10^6 cells/well) in supplemented RPMI 1640 (described in section 2.7.3) were cultured in 1.0 ml volumes in conjunction with various concentrations of TSST-1, SEA or TSST-1 plus SEA in 24-well culture plates (Becton-Dickinson). After 19.5 h at 37°C, 5% CO_2, supernatants were harvested and contaminating cells removed by centrifugation at 800 X *g* for 5 min. Supernatants were stored at -70°C.

A. Human TNFα

TNFα present in the culture supernatants was assayed by ELISA as previously described (See *et al.*, 1992c). The sensitivity limit of the TNFα ELISA was 250 pg/ml.

B. Human IL-1ß

IL-1ß in the culture supernatants was assayed by ELISA as previously described (See and Chow, 1992). The sensitivity limit of the IL-1ß ELISA was 500 pg/ml.

C. Human IL-6

Levels of IL-6 in culture supernatants was determined by ELISA. Goat anti-human IL-6 (R + D Systems, Minneapolis, MN; 0.08 ml at 2 μg/ml in 0.05 M bicarbonate-carbonate buffer, pH 9.6) was used to coat flat-bottomed 96-well microtiter plates (ImmulonI, Dynatech Laboratories, Inc.) overnight at room temperature. Unbound antibodies were removed by three 2-min washes with PBS containing 0.1% Tween 20 (PBS-TW). A human IL-6 standard (R + D Systems) serially diluted from 2500 to 0.2 pg/ml in PBS containing 3% BSA (PBS-BSA), or test sample, was added in duplicate 0.08 ml volumes to the wells and plates were incubated for 90 min at 37°C. After washing with PBS-TW, 0.08 ml of biotinylated goat anti-human IL-6 (R + D Systems) diluted 1/8,000 in PBS-BSA was added to wells and incubated for 1.5 h. Plates were then washed and wells incubated with 0.08 ml of streptavidin-alkaline phosphatase (Gibco/BRL) diluted 1/8,000 in PBS-BSA for 20 min at 37°C. Wells were washed five times with 0.2 ml of 50 mM TRIS-buffered saline (pH 7.5) and an ELISA amplification system (Gibco/BRL) was used as previously described (See *et al.*, 1992c) to increase the sensitivity of the assay. Optical density at 490 nm was measured in a Titertek Multiscan spectrophotometer (Flow Laboratories, Mississauga, Ontario,

Canada). The sensitivity limit of the IL-6 assay was 625 pg/ml. In each case the background values (PBMC in media alone) were subtracted from the observed values.

D. Rabbit TNFα

Rabbit splenocytes (2 X 10^6 cells/0.5 ml) were incubated with 0.3 ml supplemented RPMI 1640 plus 0.2 ml of 10-fold serially diluted culture filtrates of RN3984, RN7043 or INT#1 in 24-well tissue culture plates (Becton Dickinson). Addition of BHI (0.2 ml undiluted + 0.3 ml supplemented RPMI 1640) served as the media control. After incubation at 37°C, 5% CO_2 for 24 h, the media was collected and contaminating cells present were removed by centrifugation at 800 X *g* for 5 min. Supernatants were stored at -70°C. TNFα induced from rabbit splenocytes was quantitated by a rabbit TNFα ELISA. In brief, goat-anti-rabbit TNFα (Pharminogen, San Diego, CA) (4.0 μg/ml) in 0.05 M bicarbonate-carbonate buffer (pH 9.6), was used to coat microtiter plates (Dynatech Laboratories) overnight at room temperature (0.08 ml/well). Unbound antibodies were removed by three 2-min washes with PBS-TW. Rabbit TNFα conditioned medium (Pharminogen), serially diluted from 5 ng/ml to 0.15 ng/ml in PBS-BSA were added in duplicate 0.08 ml volumes to the wells. Test samples (harvested supernatants of 24 h stimulated rabbit splenocyte preparations) were added in triplicate 0.08 ml volumes. BHI incubated with rabbit splenocytes alone served as the background control. After incubation at 37°C for 1.5 h, wells were washed with PBS-TW and 0.08 ml of biotinylated goat-anti-rabbit TNFα (Pharminogen) (2 μg/ml) in PBS-BSA was added. Following incubation at 37°C for 1.5 h and washing, plates were incubated with streptavidin-alkaline phosphatase (Gibco/BRL) diluted 1/1,000 in PBS-BSA for 20 min at 37°C. Finally, plates were washed and incubated for 1.5 h at 37°C with *p*-nitrophenyl

phosphate (Sigma) as the substrate. The O.D. at 410 nm was measured using a Dynatech MR5000 microplate reader (Dynatech Laboratories). The sensitiviy limit of the TNFα assay was 150 pg/ml.

2.7.6. Proliferative Response of Human PBMC or Rabbit Splenocytes Upon Stimulation with Toxin or Culture Filtrate

Mitogenesis of human PBMC was assessed by quantitation of [^{3}H]thymidine incorporation into cellular DNA after exposure to TSST-1, SEA or TSST-1 plus SEA. A 1:1 ratio of T cells and monocytes (3 X 10^5 cells/well) suspended in supplemented RPMI 1640 were cultured in 0.2 ml volumes with various concentrations of TSST-1, SEA or TSST-1 plus SEA in 96-well U-bottom tissue culture plates (Falcon Labware, Becton-Dickinson) for 3 days at 37°C, 5% CO_2. Cells were labelled with 1 μCi of [^{3}H]thymidine (ICN Flow Laboratories, Irvine CA; 6.7 Ci/mmol) 18 hr before completion of incubation and harvested onto glass fiber filter paper with an automatic harvester (Skatron, Sterling, VA). Radioactivity was detected in a liquid scintillation counter (Beckman LS1800).

The proliferative response of rabbit splenocytes to the culture filtrates of isogenic strains was measured as described above. Rabbit splenocytes (3 X 10^5 cells/0.1 ml) were cultured in the presence of 0.05 ml of supplemented RPMI 1640 plus 0.05 ml of 10-fold serially diluted culture filtrates in supplemented RPMI 1640. BHI alone served as the media control. For experiments involving TSST-1 or SEA complementation to RN7043 or INT#1 culture filtrates respectively, 0.05 ml of purified TSST-1 or SEA diluted in supplemented RPMI 1640 was added to the appropriate well to equal the known concentration of TSST-1 or SEA present in the RN3984 culture filtrate sample as quantitated by immunoassay.

The proliferative response of rabbit splenocytes to purified TSST-1 and SEA was determined as described above by incubating rabbit splenocytes (3 X 10^5 cells/0.1 ml) with 0.05 ml supplemented RPMI 1640 and various concentrations of TSST-1, SEA, or TSST-1 plus SEA in a total volume of 0.2 ml. For the generation of TSST-1 and SEA induced mitogenic dose response curves, a pooled rabbit splenocyte preparation from 5 rabbits was used in an attempt to minimize the variation in proliferative responses that have been observed among rabbits.

2.8. Animal Model Studies

2.8.1. Animals

Female New Zealand White (NZW) rabbits were purchased from R and R Rabbitry (Washington, USA). They were housed separately and fed water and rabbit chow *ad libitum*.

Eight week old female BALB/c mice were purchased from Charles River Canada (Quebec). They were housed 5 per cage.

2.8.2. Preparation of Isogenic Mutant Inocula for *In Vivo* Studies

RN3984 (TSST-1^+/SEA$^+$), RN7043 (TSST-1^-/SEA$^+$) and INT#1 (TSST-1^+/SEA$^-$) were streaked onto BHI and two isolated colonies were inoculated into 20 ml volumes of dialysed BHI broth (Rosten *et al.*, 1989). BHI media and all glassware used for bacterial culture preparation were maintained in pyrogen-free conditions to prevent endotoxin contamination (Rosten *et al.*, 1989). Cultures were incubated with shaking at 150 rpm, 37°C overnight in a controlled environment incubator-shaker (New Brunswick Scientific, Edison, NJ). The post-exponential phase *S. aureus* strains were centrifuged at 550 X *g* for 10 min at 4°C. After two washes in sterile PBS, cultures were resuspended in BHI. The number of organisms in

the staphylococcal suspensions were determined on BHI agar plates.

2.8.3. Introduction of isogenic mutants into two animal models of lethal shock

A. Tampon-associated vaginal infection model of TSS in the New Zealand White rabbit

NZW rabbits weighing 3.3-4.5 kg were anesthetized with ketamine (30 mg/kg), acepromazine (0.8 mg/kg) and xylaxine (3 mg/kg) and maintained on 4% oxygen and 1.5-3.5% halothane during the surgical procedure. A suprapubic abdominal incision was made to expose the length of the vagina. A preweighed, sterile tampon (rayon/cotton, Tampax Super Plus, Tambrands Canada Inc., 0.5-0.55 g) placed in an uncuffed endotracheal tube applicator (Tip-to-Tip, 7 mm internal diameter; Mallinckroft, Argyle, NY) was inserted through the urogenital sinus into the vagina and dislodged against the cervix using a sterile wooden dowelling. A loose ligature was placed around the distal end of the vagina with a silk 2-0 sterile nonabsorbable suture to prevent spontaneous extrusion of the tampon. A 2 ml inoculum containing $\sim 10^{10}$ CFU *S. aureus* in BHI, mixed with 0.1% of rabbit blood (as simulated menses, obtained from the same rabbit) was injected into the tampon through the vaginal wall with a 25 guage needle (Becton Dickinson, Cockeysville, MD). The abdominal incision was closed with synthetic suture and after administration of 0.02-0.05 mg/kg of buprenephrine, rabbits were allowed to recover for observation. Rabbits were monitored for a period of up to 8 days with respect to mortality, and incidence of lipaemia and diarrhea. Weight and rectal temperature measurements were taken daily. These clinical parameters are indirect indicators of *in vivo* cytokine secretion, since TNF and IL-1 have been shown to suppress lipoprotein lipase production (Beutler *et al.*, 1985; Price *et al.*, 1986), induce fever

(Dinarello *et al.*, 1986), and cause weight loss (Moldawer *et al.*, 1988).

B. D-Galactosamine sensitized mouse model of lethal shock

BALB/c mice were injected i.p. with 20 mg of D(+)-galactosamine-hydrochloride (Sigma) dissolved in sterile saline 2 h prior to bacterial challenge. The mice (30 in total, 15 per group) were inoculated i.p. with $\sim 10^8$ CFU of RN3984 or INT#1. In a preliminary experiment with RN3984, this dose corresponded to the i.p. LD_{80} after 48 h. Infection with BHI media alone in 2 mice served as the control. Deaths were recorded up to 72 h after inoculation.

Chapter 3. Determination of whether TSST-1 plays a pivotal role in the pathogenesis of MTSS by the introduction of isogenic mutants in TSST-1 production in a Tampon-associated Vaginal Infection Model

3.1. Introduction

Although *S. aureus* produces many different extracellular products, the observation that TSST-1 was produced by the majority of TSS isolates caused it to be implicated in TSS early in the investigation of this disease. Nothwithstanding the numerous studies that have been performed that strongly point to TSST-1 as a causative agent in this syndrome, definitive evidence proving a role for TSST-1 in menstrual TSS was lacking. This may have resulted from the lack of an appropriate animal model and/or unavailability of appropriate test strains in earlier studies. These models and constructs are currently available however. A vaginal tampon model developed in the NZW rabbit has been described by Melish *et al.* (1989). In addition, the construction of various isogenic mutants in TSST-1 production have been reported (Rasheed *et al.*, 1985; de Azavedo *et al.*, 1985; Sloane *et al.*, 1991). Before this thesis work began, no one had made use of an appropriate animal model that resembled menstrual TSS in conjunction with isogenic *S. aureus* mutants in TSST-1 production to verify TSST-1 as an etiologic agent. In light of this, the first objective of this research was to introduce isogenic mutants in TSST-1 production into a vaginal infection model of TSS to

determine if the TSST-1$^+$ strain caused more mortality or morbidity than the TSST-1$^-$ construct. In addition, the effects of culture filtrates from isogenic strains on rabbit splenocytes with respect to T cell proliferation and TNFα secretion - two biologic activities which are proposed to play an important role in the pathogenesis of superantigen-mediated shock, were examined.

3.2. Results

3.2.1. Characterization of the TSST-1$^-$ Mutant RN7043

Loss of TSST-1 production by RN7043 was verified by immunoblot analysis (Figure 2) and immunoassay (Table 6) as described in Materials and Methods. As can be seen in Figure 2, a band with a molecular mass of 22 kD (molecular mass of TSST-1) having immunological reactivity to the anti-TSST-1 antibody is missing in the culture filtrate of RN7043 (Lane 1) compared to the culture filtrate of RN3984 (Lane 2). This indicates that no TSST-1 is stably secreted into the medium by RN7043.

The size of the *Cla*I fragment containing *tst* was examined by Southern hybridization analysis of *Cla*I digested genomic DNA of RN7043 with a 297 bp *Hinc*II-*Bam*HI *tst* specific gene probe (Kreiswirth *et al.*, 1989). As shown in Figure 3, the *Cla*I fragment that hybridized to the *tst* gene probe in RN7043 was larger than the *Cla*I fragment that hybridized to the probe in the TSST-1$^+$ wildtype strain RN3984. The larger *Cla*I fragment observed to hybridize with RN7043 DNA as compared to RN3984 DNA was consistent with the expected increase in size (approximately 2.9 kb) if the Tc^r fragment inserted into *tst* of RN7043. This observation provided support for the supposition that *tst* in RN7043 had been inactivated by allele replacement.

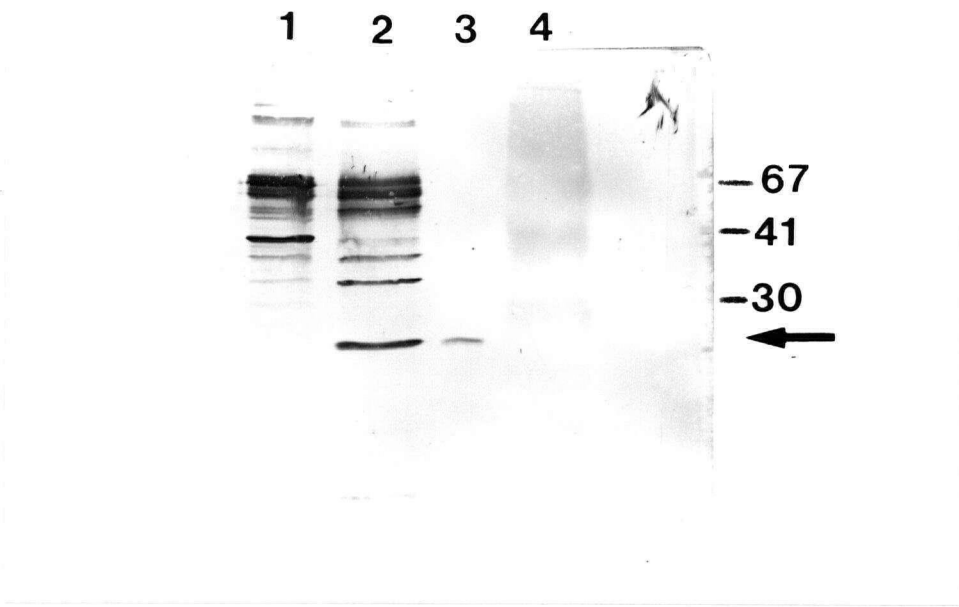


Figure 2. Immunoblot analysis of culture filtrates from TSST-1⁻ mutants separated by SDS-PAGE and blotted with a polyclonal anti-TSST-1 antibody.

Lane# 1. RN7043 culture filtrate
2. RN3984 culture filtrate
3. 8 ng of purified TSST-1
4. Bio-Rad Kaleidoscope Pre-Stained Molecular Mass Standards (kD)

Arrow indicates the position of the TSST-1 standard.

Table 6. Detection of TSST-1 in the Culture Filtrates of RN3984 and RN7043.

Strain	[TSST-1] in Culture Filtrate (ng/ml)
RN3984	107.2 ± 4.2
RN7043	nd

TSST-1 levels in culture filtrates were quantitated by immunoassay as described in Materials and Methods. Two single colonies of each strain were inoculated into 20 ml BHI and incubated at 37°C for 10 h. Culture filtrates were obtained by centrifugation and filter-sterilization. Values represent the mean ± SEM of 5 culture filtrates prepared from each strain tested in at least duplicate wells by immunoassay. The sensitivity limit of the immunoasaay was 0.5 ng/ml of TSST-1. nd, not detected

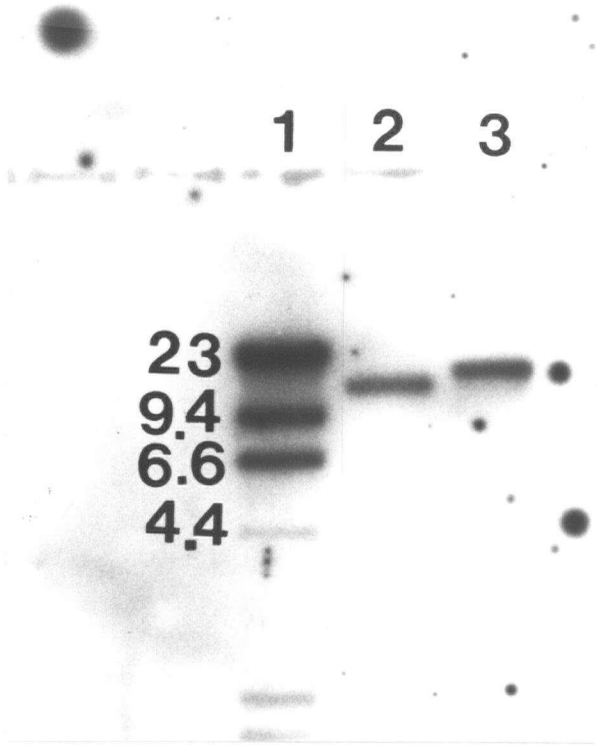


Figure 3. Southern hybridization analysis of TSST-1⁻ mutant genomic DNA digested with *Cla*I and hybridized with a 297 bp *Hinc*II-*Bam*HI *tst*-specific gene probe (Kreiswirth *et al.*, 1989) as described in Materials and Methods.

Lane# 1. Digoxigenin-labelled DNA Molecular Mass Standards (kb)
2. RN3984
3. RN7043

SEA is the only staphylococcal enterotoxin produced by RN3984. Since SEA is in the superantigen family of proteins and could be a causal agent in MTSS, a comparison of the amount of SEA produced by the TSST-1$^-$ mutants was performed. For some unexplained reason, in relation to the total amount of protein (pg of SEA/μg of protein), RN7043 was found to produce significantly less SEA than RN3984 (9.7 ± 2.4 vs 2.6 ± 0.3; $P = 0.03$, Mann-Whitney test, 2-tailed, see Figure 4). This could be important if SEA plays a contributory role in MTSS, since any observed differences between the pathogenicity of the TSST-1$^-$ mutants *in vivo* could be attributed to the higher amount of SEA produced by RN3984.

3.2.2. Virulence of RN3984 and RN7043 in a tampon-associated vaginal infection model of toxic shock syndrome

A. Mortality

RN3984 was more lethal in the tampon-associated vaginal infection model than RN7043. 4 of 13 (31%) rabbits infected with RN3984 died as compared to 0 of 13 (0%) rabbits infected with RN7043 ($P < 0.05$, Fisher's test, one-tailed). Three of the four deaths occurred within the first three days of experimental infection. Our definition of mortality included symptoms of 'severe clinical distress' necessitating immediate sacrifice as mandated by the University of British Columbia Animal Care Committee. Conditions which warranted immediate sacrifice for humane reasons in this model was defined as the presence of any two of the following three objective criteria:

i) weight loss greater than 20% of that prior to challenge

ii) a recumbency position of the rabbit

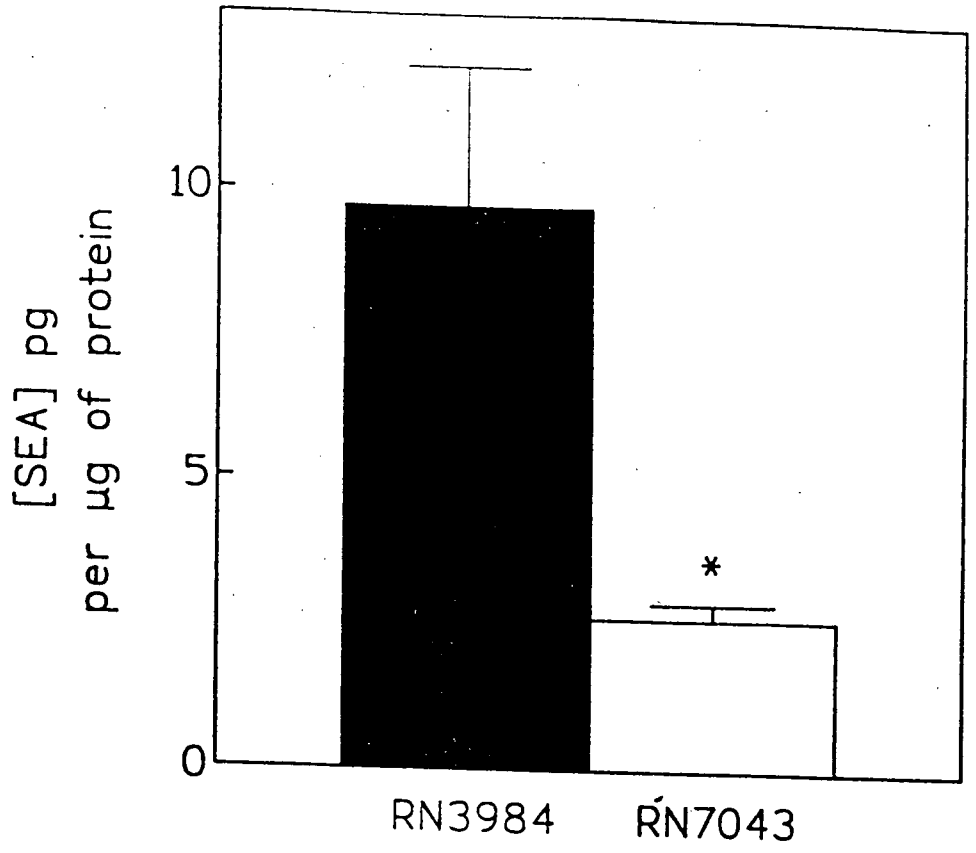


Figure 4. Comparison of SEA levels with respect to total protein in the culture filtrates of the TSST-1⁻ mutants. Cultures were prepared by inoculating two single colonies into 20 ml of BHI followed by incubation at 37°C for 10 h. Bars represent the mean SEA level (detected by immunoasay) ± SEM per µg of protein in 5 prepared culture filtrate samples. The difference between the values was significant (P = 0.03, Mann-Whitney test; 2-tailed).

iii) no response (ie. failure to get up) to various stimuli, including sound, pain or touch

B. Temperature change

Rabbits infected with RN3984 exhibited a significantly higher mean average rectal temperature over a 6 day observation period than rabbits infected with the TSST-1$^-$ mutant RN7043. The mean highest temperature (°C) ± SEM of the survivors infected with RN3984 was 40.2 ± 0.1 vs 39.7 ± 0.1°C for rabbits infected with RN7043 (P = 0.0003, student's t-test, one-tailed).

C. Weight loss

Although it appeared that rabbits infected with RN3984 lost more weight as compared to pre-infection baseline values (Day 0) than rabbits infected with RN7043 (Figure 5), differences in weight loss between the infected groups over the observation period was not statistically significant. Both groups of rabbits lost weight up until Day 3 following infection, however rabbits infected with RN3984 continually lost weight thereafter, whereas rabbits infected with RN7043 exhibited a gradual increase in weight over the same time period.

3.2.3. Effect of culture filtrates of isogenic strains on rabbit splenocytes

A. T cell proliferation

A mitogenic effect as measured by [^{3}H]thymidine incorporation into cellular DNA (described in Materials and Methods) was observed when diluted culture filtrate preparations were added to rabbit splenocytes (Figure 6). RN3984 culture filtrate induced a significantly greater mean effect (± SEM) in T cell proliferation than the RN7043 culture filtrate at dilutions of 1/1,000 (842,527 ± 178,477 vs 510,372 ± 120,297 cpm; P = 0.01, paired t-test, one-tailed) and 1/10,000 (385,567 ± 96,984 vs 98,529 ± 16,303 cpm; P = 0.01,

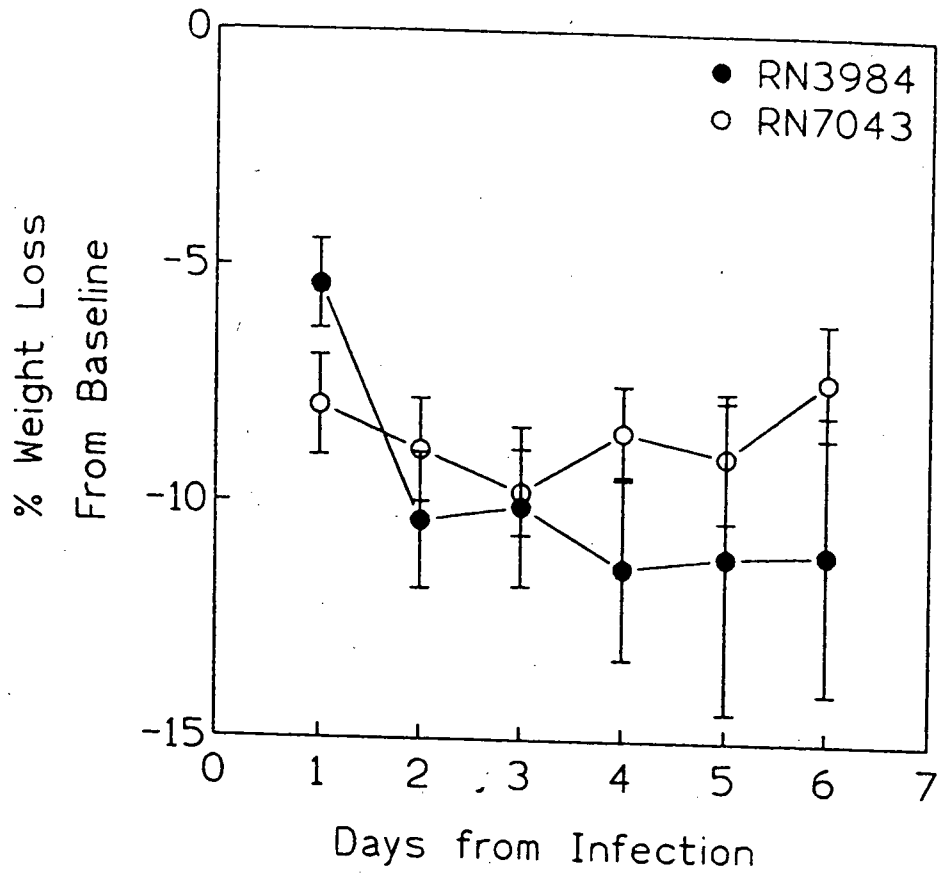


Figure 5. Weight loss in rabbits infected with RN3984 and RN7043. Values represent the mean ± SEM in all rabbits (including those that subsequently succumbed to the infection) in which weight values were obtained.

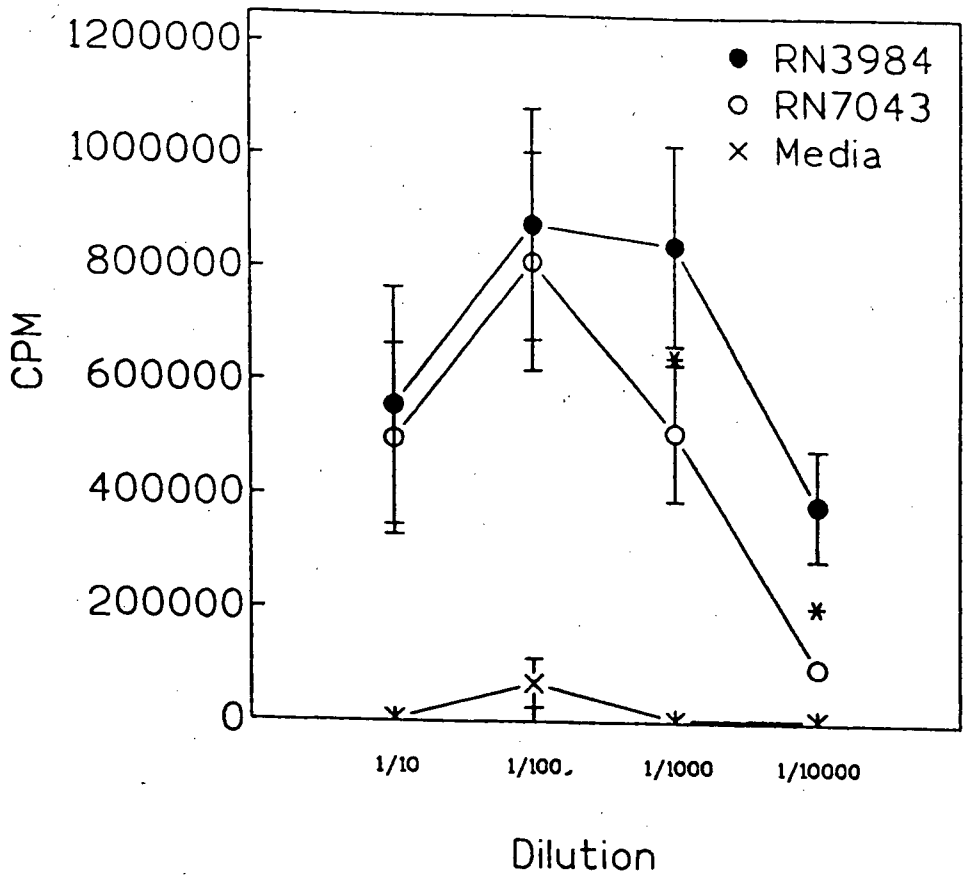


Figure 6. Mitogenic response induced by RN3984 and RN7043 culture filtrates on rabbit splenocytes. Values are expressed as the mean ± SEM measured by [^{3}H]thymidine incorporation into cellular DNA in at least 7 rabbit splenocyte samples, tested in a minimum of triplicate determinations. BHI served as media control. * indicates a significant difference in proliferation compared to RN3984 (P = 0.01, paired t-test, one-tailed)

paired t-test, one-tailed). The decreased level of T cell proliferation caused by RN7043 culture filtrate in comparison to RN3984 culture filtrate could be explained by the lack of TSST-1. If this were true, adding TSST-1 to the RN7043 culture filtrate would be expected to increase the level of T cell proliferation back to the level observed with the RN3984 culture filtrate. To test this, purified TSST-1 was added with a 1/10,000 dilution of RN7043 culture filtrate (at a concentration that equalled the known amount found in the diluted RN3984 culture filtrate as determined by immunoassay) to rabbit splenocytes and the amount of T cell proliferation was measured by [^{3}H]thymidine incorporation into cellular DNA. As observed in Figure 7, purified TSST-1 added to the culture filtrate of RN7043 restored the amount of cell proliferation back to that observed with RN3984.

B. **TNFα Secretion**

Culture filtrates of RN3984 and RN7043 stimulated rabbit splenocytes to secrete TNFα as measured by a rabbit TNFα specific immunoassay described in Materials and Methods (Figure 8). Incubation of rabbit splenocytes with undiluted RN3984 culture filtrate induced significantly more TNFα (mean $\pm$ SEM) than incubation with undiluted RN7043 culture filtrate (25.74 $\pm$ 1.19 vs 21.71 $\pm$ 1.47 ng/ml; P = 0.03, paired t-test, one-tailed). Similiarly, a significant increase in TNFα secretion from rabbit splenocytes was observed upon incubation with a 1/10 dilution of RN3984 culture filtrate as compared to a 1/10 dilution of RN7043 culture filtrate (25.57 $\pm$ 1.7 vs 22.54 $\pm$ 1.76 ng/ml; P = 0.04, paired t-test, one-tailed).

3.3. Discussion

These studies support the hypothesis that TSST-1 is an important virulence determinant in

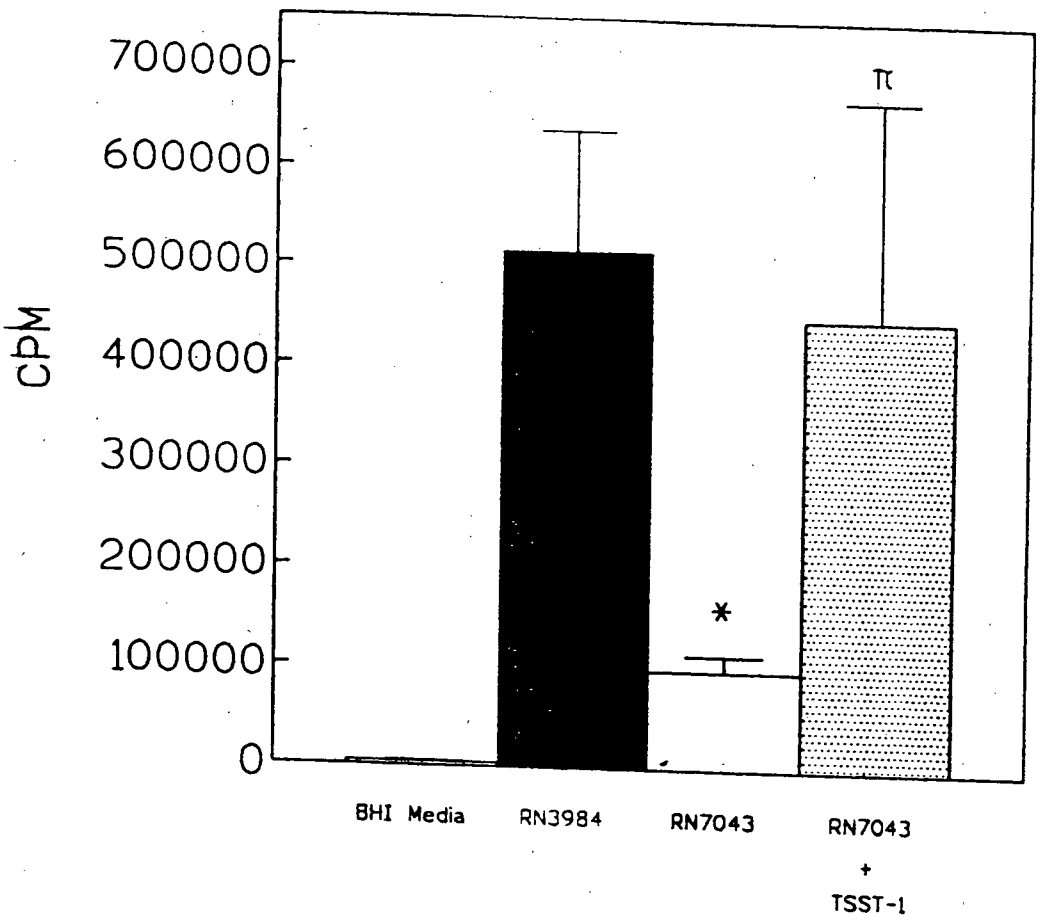


Figure 7. Increase in the proliferative response of rabbit splenocytes to RN3984 culture filtrate is consistent with the presence of TSST-1. A 1/10,000 dilution of RN7043 culture filtrate was added with purified TSST-1 (at a concentration that equalled the known amount found in the diluted RN3984 culture filtrate as determined by immunoassay) to rabbit splenocytes and the amount of T cell proliferation was assessed by incorporation of [^{3}H]thymidine into cellular DNA. Results are the mean ± SEM in 5 rabbit splenocyte samples each tested in a minimum of triplicate cultures. * indicates a significant difference in proliferation compared to RN3984 ($P < 0.05$, paired t-test, one-tailed); ¶ indicates no significant difference compared to RN3984 ($P > 0.05$, paired t-test, one-tailed)

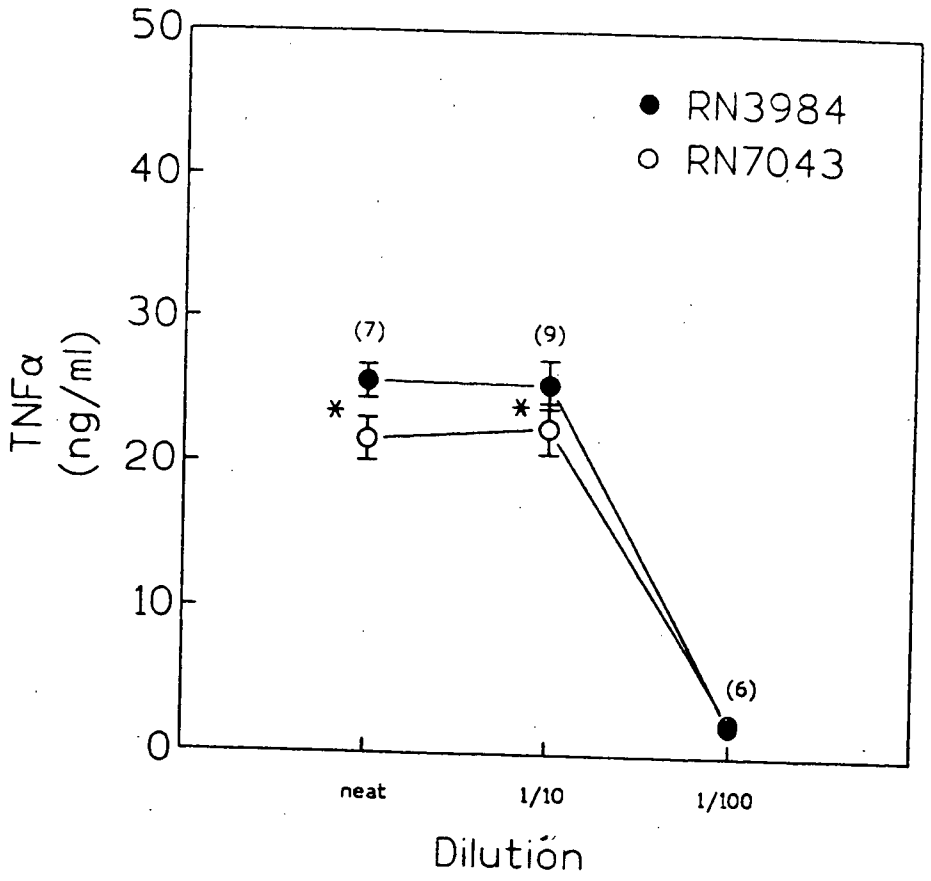


Figure 8. TNFa secretion induced by RN3984 and RN7043 culture filtrates from rabbit splenocytes. Values represent the mean ± SEM tested in a minimum of duplicate cultures. BHI served as the media control and gave background values of <0.04 ng/ml. Numbers in brackets indicate the number of rabbits tested at each dilution. * indicates a significant difference in proliferation compared to RN3984 ($P < 0.05$, paired t-test, one-tailed)

the pathogenesis of menstrual toxic shock syndrome. The TSST-1$^+$ *S. aureus* strain was able to induce more lethality and fever than its TSST-1$^-$ isogenic derivative in the tampon-associated vaginal infection model. This is the first study to test a role for TSST-1 in menstrual TSS using isogenic mutants in a relevant animal model. In addition, using mitogenesis and TNFα secretion as indicators of toxicity potential, the *in vitro* observation that the RN3984 culture filtrate induced an increased amount of T cell proliferation and TNFα secretion from rabbit splenocytes than RN7043, further supports the *in vivo* data.

RN3984 was initially isolated from a patient with menstrual TSS (MTSS) and phenotypically resembles the majority of MTSS isolates since it produces both TSST-1 and SEA (Kain et al., 1993), and requires tryptophan for growth [Chu et al., 1988]. No other enterotoxins besides SEA are produced by RN3984. Similiar to TSST-1, SEA also belongs to the superantigen family of proteins, and as such, can cause profound immunological disturbances. It is puzzling why SEA is produced in lower amounts by RN7043 as compared to RN3984 when grown *in vitro*. Since growth in a test-tube does not mimic growth *in vivo,* partly because different environmental signals may exist to regulate the expression of various virulence determinants, it is not known whether RN7043 produces a lower amount of SEA than RN3984 *in vivo*. It is possible that TSST-1 influences the expression of SEA in some manner, such that inactivation of *tst* in RN7043 resulted in the reduction of SEA production. Alternatively it is possible that during construction of RN7043, insertion of the Tc^r cassette into *tst* resulted in polar effects to occur in downstream genes which may have been involved in regulating the expression of several staphylococcal proteins.

In terms of superantigen expression, these strains differ in both TSST-1 and SEA

production. Whether SEA is an important mediator in menstrual TSS is not known at present. These results suggest that it may not be (at the concentration produced by RN7043) since there was not any mortality observed in rabbits infected with RN7043 (TSST-1$^-$/SEA$^+$). However since the amount of SEA produced by RN7043 was lower than for RN3984, this result does not provide adequate assessment of its potential role. Due to the lower amount of SEA produced by the TSST-1$^-$ mutant, determination of whether TSST-1 plays a causative role in MTSS remains inconclusive. To determine if SEA production enhances the virulence of a TSST-1$^+$ strain, the pathogenicity of a TSST-1$^+$/SEA$^+$ and a TSST-1$^+$/SEA$^-$ strain were next compared upon introduction into two animal models of lethal shock.

Chapter 4. Effect of TSST-1 and SEA Superantigen Co-Production in the Pathogenesis of Menstrual TSS

4.1. Introduction

In the previous chapter evidence that TSST-1 is an important virulence determinant in the pathogenesis of menstrual toxic shock syndrome remained inconclusive since the TSST-1$^-$ mutants differed in the amount of SEA produced. It is believed that production of enterotoxins in conjunction with TSST-1 may exacerbate the illness. Since enterotoxins belong in the superantigen family of proteins, an enterotoxin$^+$/TSST-1$^+$ strain may cause greater immunological disturbances in an infected host than a strain producing only TSST-1. Co-production of SEA and TSST-1 appears to be clinically important since this toxin combination is produced by menstrual TSS isolates of *S. aureus* more frequently than nonmenstrual, non-TSS or carrier isolates (Chang *et al.*, 1991). Analyses by multilocus enzyme electrophoresis (MLEE) (Chang *et al.*, 1991) and phage typing (Marples and Wieneke, 1993) suggest that a single clone of *S. aureus* producing both TSST-1 and SEA is responsible for the majority of menstrual TSS cases. In light of these observations, it was hypothesized that co-production of SEA and TSST-1 by *S. aureus* may act to increase various superantigen-associated activities which may lead to a more virulent strain than production of TSST-1 alone. To test this hypothesis, TSST-1, SEA, and TSST-1 plus SEA at minimally effective concentrations were incubated with human PBMC and the effects on T cell proliferation and cytokine secretion (TNFα, IL-1ß, IL-6) were examined. Since both

TSST-1 and SEA are known to bind to the MHC Class II molecule on human PBMC to induce these activities (Grossman *et al.*, 1990; Fleischer and Schrezenmeier, 1988; Fischer *et al.*, 1989; See *et al.*, 1992a) an enhancement in these effects was fully expected upon co-incubation at low concentrations. To examine the effects of this toxin combination *in vivo*, the SEA structural gene (*sea*) of a TSST-1^+/SEA^+ strain of *S. aureus* was inactivated to create a TSST-1^+/SEA^- mutant. The TSST-1^+/SEA^+ and TSST-1^+/SEA^- strains were introduced into a rabbit tampon-associated vaginal infection model of TSS and a D-galactosamine mouse model of lethal shock to determine if the TSST-1^+/SEA^+ strain of *S. aureus* causes more mortality and morbidity than the TSST-1^+/SEA^- strain. In addition, the *in vitro* effects (T cell proliferation, TNFα secretion) of culture filtrates from the isogenic pair and purifed TSST-1 and SEA were examined on rabbit splenocytes.

4.2. Results

4.2.1. *In vitro* effect of purified TSST-1 and SEA on human peripheral blood mononuclear cells

A. T cell proliferation

TSST-1 and SEA can induce the proliferation of T cells from human PBMC as observed in Figures 9 and 10 (data by Winnie Kum). The dose response curves observed with these cells are similiar to that observed by other investigators in that TSST-1 and SEA were found to mediate T cell proliferation over a broad dose range (Langford *et al.*, 1978; Calvano *et al.*, 1984; Poindexter and Schlievert, 1985; Kum *et al.*, 1993). Direct comparison between T cell populations is difficult due to variation in the amplitude of the proliferative response (Calvano *et al.*, 1984).

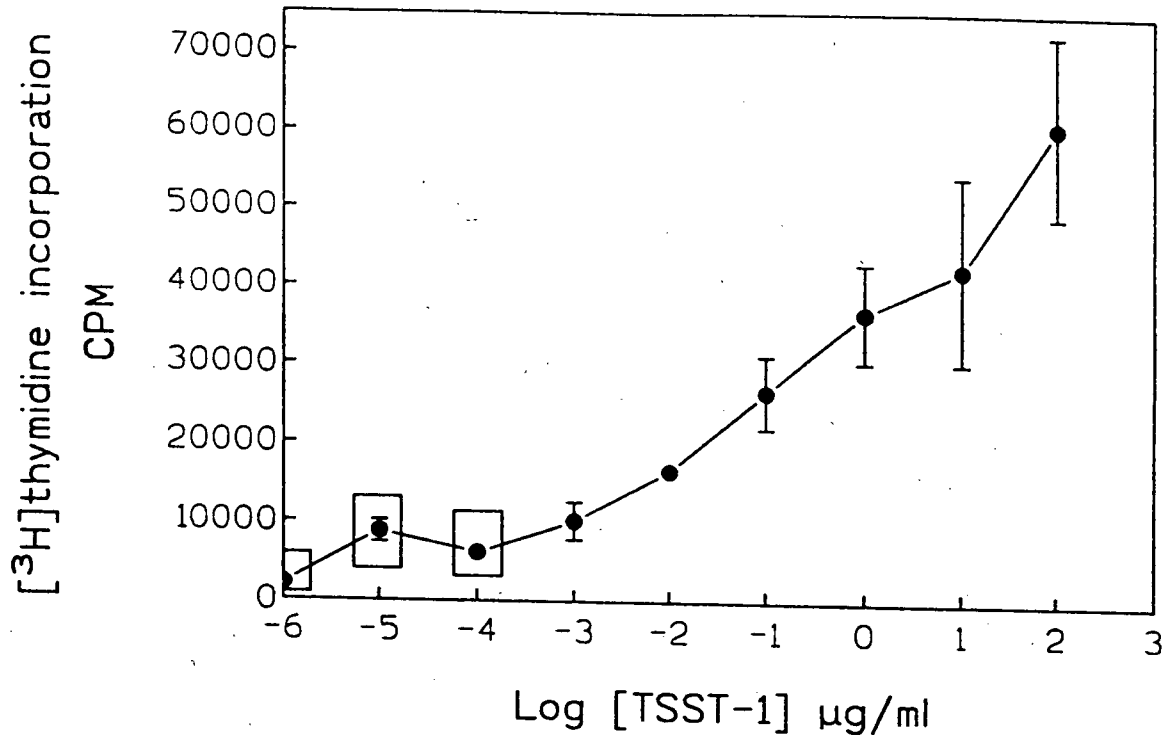


Figure 9. Mitogenic response of human PBMC to TSST-1 as measured by [^{3}H]thymidine incorporation into cellular DNA. Each point represents the mean ± SEM from one human PBMC culture tested in triplicate determinations. Background activity has been subtracted. Concentrations of TSST-1 outlined by the boxes indicate the doses used for the co-incubation studies. (Experimental data provided by Winnie Kum).

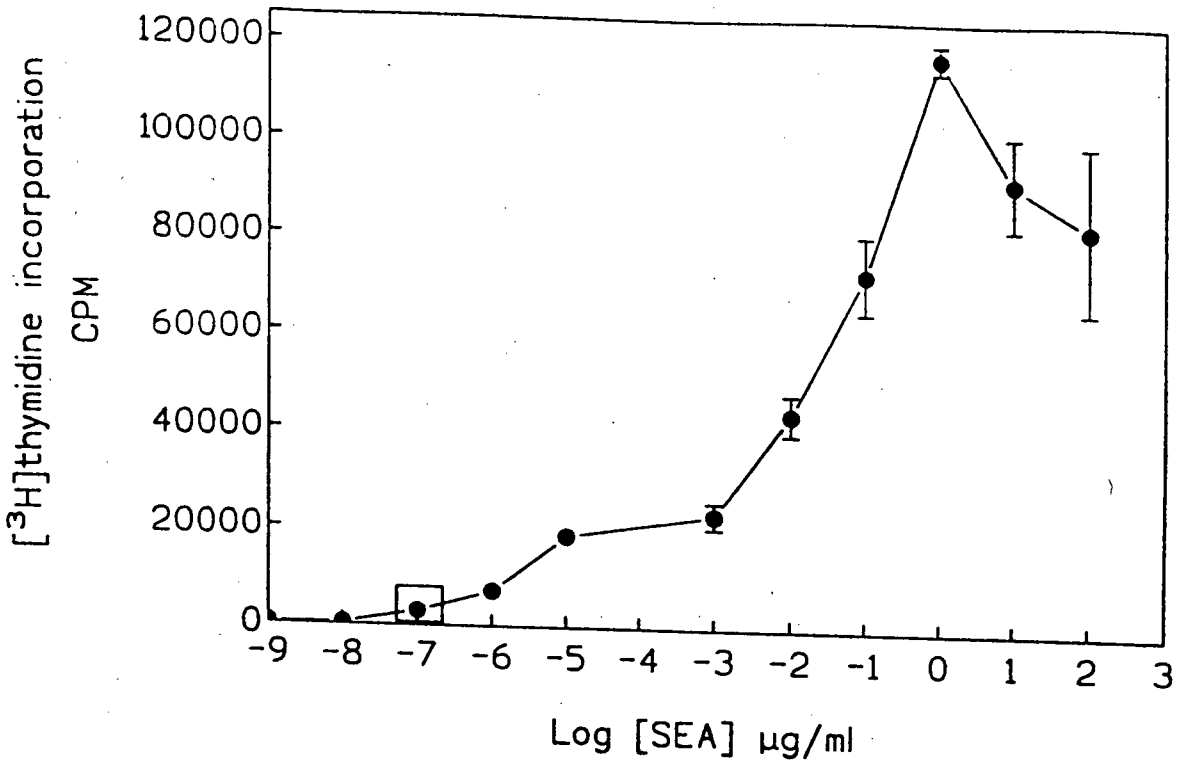


Figure 10. Mitogenic response of human PBMC to SEA as measured by [^{3}H]thymidine incorporation into cellular DNA. Each point represents the mean ± SEM from one human PBMC culture tested in triplicate determinations. Background activity has been subtracted. The concentration of SEA outlined by the box indicates the dose used for the co-incubation studies. (Experimental data provided by Winnie Kum).

To test if co-incubation of TSST-1 and SEA on human PBMC can result in an enhancement in T cell proliferation as compared to incubation with either toxin alone as expected, concentrations of TSST-1 and SEA that caused a minimal amount of T cell proliferation according to the dose response curves were chosen (Figures 9 and 10, indicated by boxes). Three concentrations of TSST-1 (1, 10 and 100 pg/ml) either alone, or in conjunction with SEA (0.1 pg/ml) were added to human PBMC. Three doses of TSST-1 were used in these studies in an attempt to overcome the anticipated variability of responses between human T cell populations. As observed in Figure 11, a significant increase in T cell proliferation (cpm $\pm$ SEM) occurred upon incubation of human PBMC with both TSST-1 (1 pg/ml) and SEA (0.1 pg/ml) (21,224 $\pm$ 6613) as compared to either TSST-1 (12,111 $\pm$ 3909; $P = 0.013$, paired t-test, 2-tailed) or SEA (8,046 $\pm$ 2310; $P = 0.035$, paired t-test, 2-tailed) alone. In addition, 3 cultures of human PBMC tested with increasing concentrations of TSST-1 (10 and 100 pg/ml) in conjunction with SEA (0.1 pg/ml) also exhibited a significant increase in T cell proliferation as compared to incubation with either toxin alone (2 of 3 human PBMC cultures using TSST-1 at 10 pg/ml, and 3 of 3 human PBMC cultures using TSST-1 at 100 pg/ml). The increased effect in mitogenesis appeared to be additive rather than synergistic since the mean proliferative response observed upon co-incubation with TSST-1 and SEA did not significantly exceed the sum of the proliferative response observed upon incubation with TSST-1 or SEA alone.

B. Cytokine secretion (TNFα, IL-1ß, IL-6)

The effect of co-incubation of TSST-1 and SEA on human PBMC with respect to cytokine secretion was next investigated. A preliminary experiment with the human PBMC of one

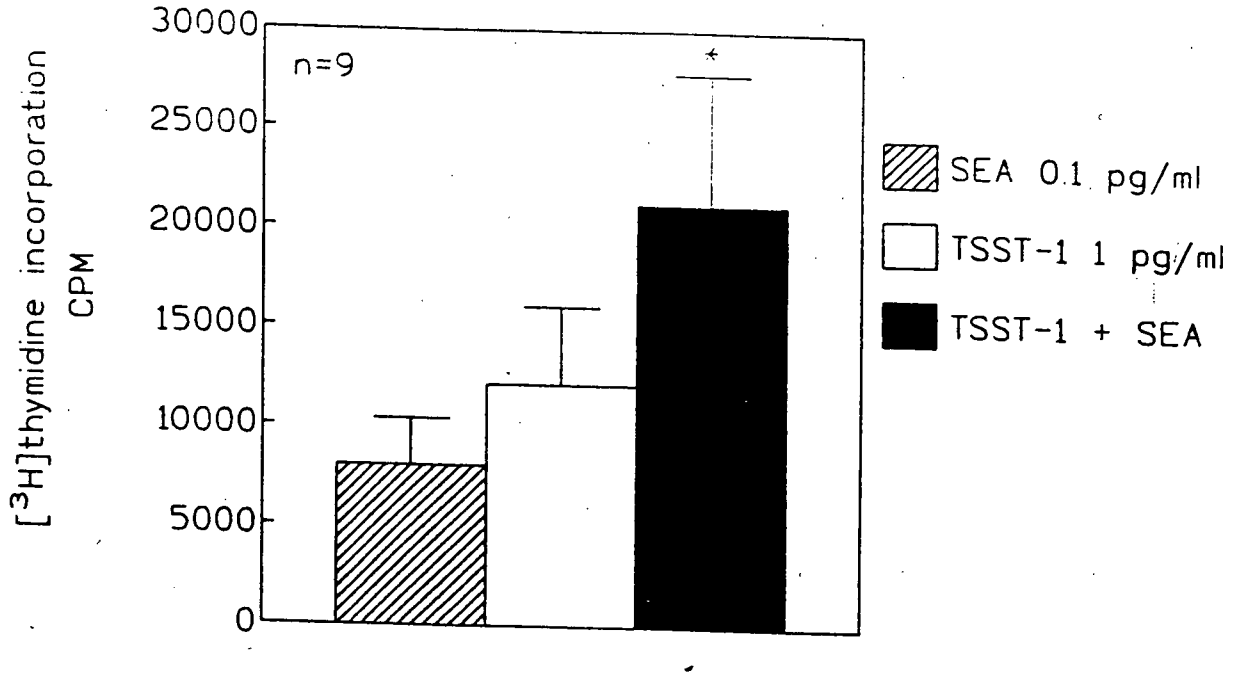


Figure 11. Human PBMC mitogenic response induced upon co-incubation with TSST-1 and SEA as measured by [^{3}H]thymidine incorporation into cellular DNA. The values represent the mean ± SEM in a minimum of quadruplicate determinations performed in 9 human PBMC samples. Background activity has been subtracted.
* significantly different from the mitogenic response observed with TSST-1 or SEA alone ($P < 0.05$, paired t-test, 2-tailed)

donor was performed to generate a dose response curve of TSST-1 and SEA induced TNFα, IL-1ß, and IL-6 production. Concentrations of TSST-1 and SEA ranging from 0.001 pg/ml to 1 μg/ml were used to stimulate human PBMC for 19.5 h. Although dose response effects were observed for toxin induced TNFα and IL-6 production, this particular donor was a low IL-1ß responder to these toxins at the concentrations used. Stimulation with either TSST-1 or SEA at 1 μg/ml (the highest dose used) resulted in the release of IL-1ß into the culture supernatant, but at the low end of the sensitivity limit for the IL-1ß immunoassay. Due to the inherent variabilities among human PBMC samples with respect to cytokine induction (de Azavedo *et al.*, 1988; Endres *et al.*, 1988; Fast *et al.*, 1988; Ikejima *et al.*, 1988; Schindler *et al.*, 1990; Gjörloff *et al.*, 1991; Müller-Alouf *et al.*, 1994; Krakauer 1995), the choice of an appropriate concentration of either TSST-1 or SEA that would be suitable for all donors is extremely difficult. Furthermore different concentrations may be needed to stimulate each cytokine. As a result, based on this preliminary experiment, an arbitrary concentration of 10 pg/ml of TSST-1 and either 1 or 10 pg/ml of SEA was chosen.

To determine if co-incubation of TSST-1 and SEA with human PBMC enhanced TNFα production as compared to incubation with TSST-1 or SEA alone, five human PBMC samples were stimulated with TSST-1 (10 pg/ml) and SEA (1 pg/ml) either alone, or in combination. As shown in Figure 12, a significant increase in the mean TNFα level (ng/ml ± SEM) present in the culture supernatants harvested from the human PBMC was observed upon addition of both TSST-1 and SEA (1.5 ± 0.21) as compared to either TSST-1 (0.85 ± 0.22; $P = 0.034$, paired t-test, 2-tailed) or SEA (0.56 ± 0.35; $P = 0.017$, paired t-test, 2-tailed) alone. A significant increase in the mean TNFα level in culture supernatants was also

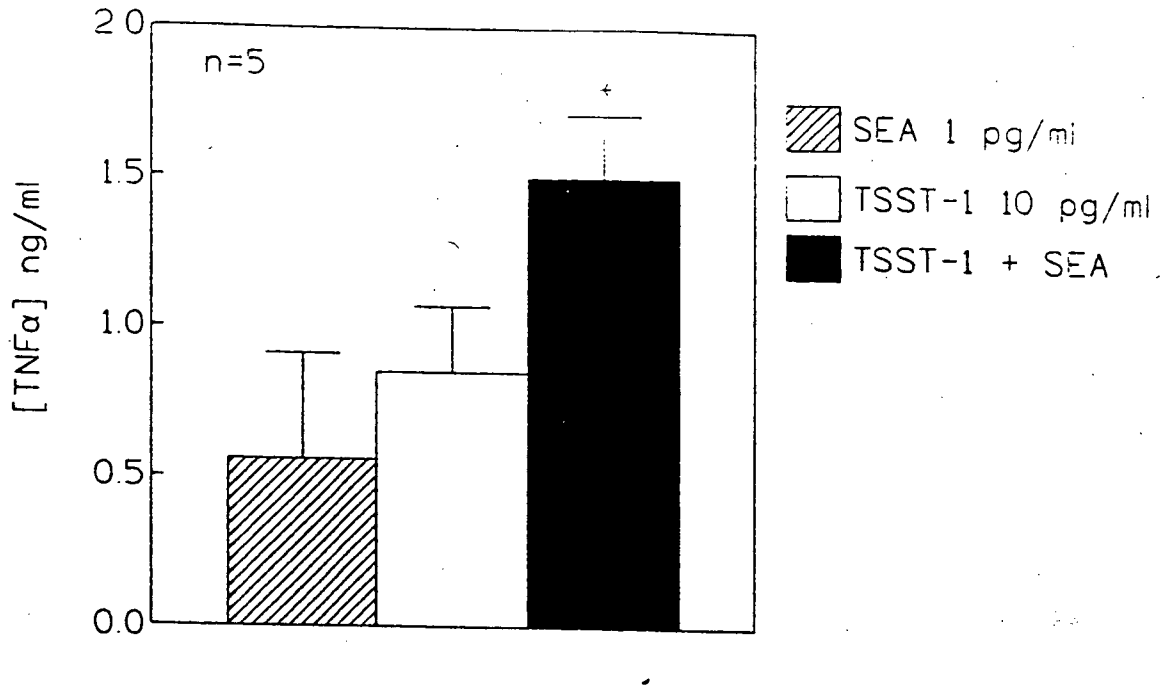


Figure 12. TNFa secretion from human PBMC upon co-incubation with TSST-1 and SEA. Human PBMC were incubated for 19.5 h with TSST-1, SEA or TSST-1 plus SEA and culture supernatants were tested for TNFa by immunoassay. Values represent the mean ± SEM in 5 human PBMC samples each studied in duplicate determinations. Background values (culture media alone) were below the levels of detection of the immunoassay.

* significantly different from the TNFa level observed with TSST-1 or SEA alone ($P < 0.05$, paired t-test, 2-tailed)

observed when TSST-1 was co-incubated with SEA at 10 pg/ml (2.1 ± 0.3) as compared to incubation with TSST-1 (0.85 ± 0.22; P = 0.013, paired t-test, 2-tailed) or SEA (1.4 ± 0.27; P = 0.008, paired t-test, 2-tailed) alone. Similiar to the effect on T cell mitogenesis, incubation of human PBMC with TSST-1 and SEA appeared to cause an increase in TNFα production in an additive rather than synergistic manner, since the concentration of TNFα secreted upon co-incubation with TSST-1 and SEA did not significantly exceed the sum of the response observed with either toxin individually.

Unlike the ability of TSST-1 and SEA to enhance TNFα secretion from human PBMC, IL-1ß or IL-6 levels were not significantly increased upon co-stimulation with both toxins. The mean IL-1ß level (ng/ml ± SEM) in the culture supernatants of eight human PBMC samples upon stimulation with TSST-1 (10 pg/ml) and SEA (1.0 pg/ml) (5.34 ± 2.4) was not significantly greater than incubation with TSST-1 (5.61 ± 2.7; P = 0.84, paired t-test, 2-tailed) or SEA (3.71 ± 2.0; P = 0.16, paired t-test, 2-tailed) alone (Figure 13). Increasing the concentration of SEA to 10 pg/ml did not alter the results. Similiarly, the mean IL-6 concentration (ng/ml ± SEM) measured in the culture supernatants of eight human PBMC samples upon addition of both TSST-1 (10 pg/ml) and SEA (1 pg/ml) (1.19 ± 0.43) was not significantly increased when compared to incubation with TSST-1 alone (0.99 ± 0.41; P = 0.5, paired t-test, 2-tailed), although a significant increase was observed in comparison to SEA alone (0.53 ± 0.28; P = 0.02, paired t-test, 2-tailed) (Figure 14). Increasing the concentration of SEA to 10 pg/ml did not change the results.

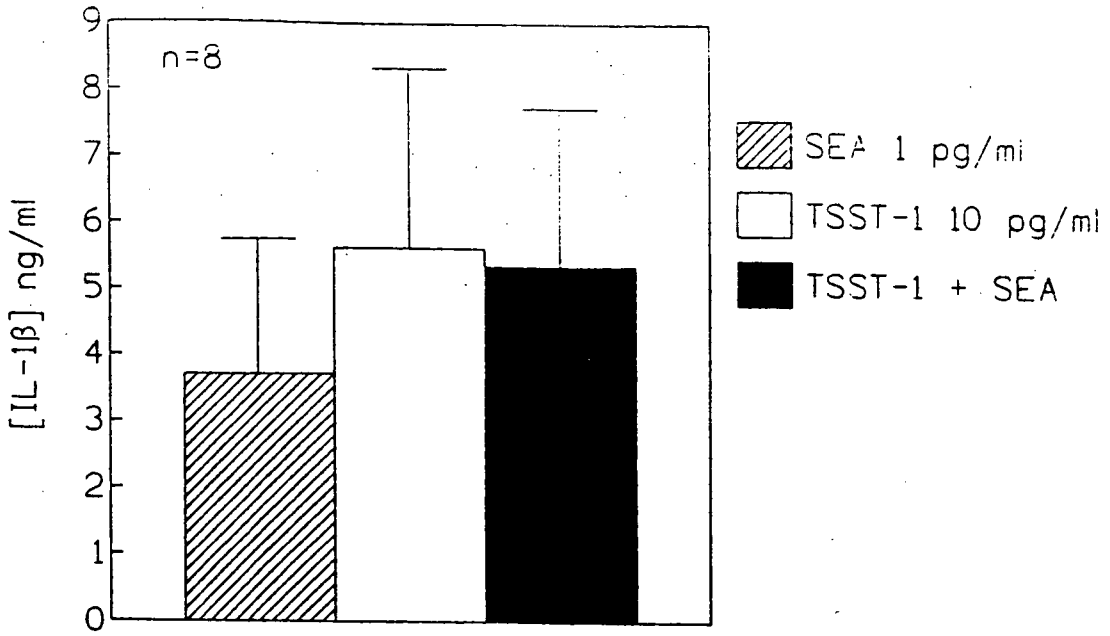


Figure 13. IL-1ß secretion from human PBMC upon co-incubation with TSST-1 and SEA. Human PBMC were incubated for 19.5 h with TSST-1, SEA or TSST-1 plus SEA and culture supernatants were tested for IL-1ß by immunoassay. Values represent the mean ± SEM in 8 human PBMC samples each studied in duplicate determinations. Values observed with culture media alone were subtracted from the reported values.

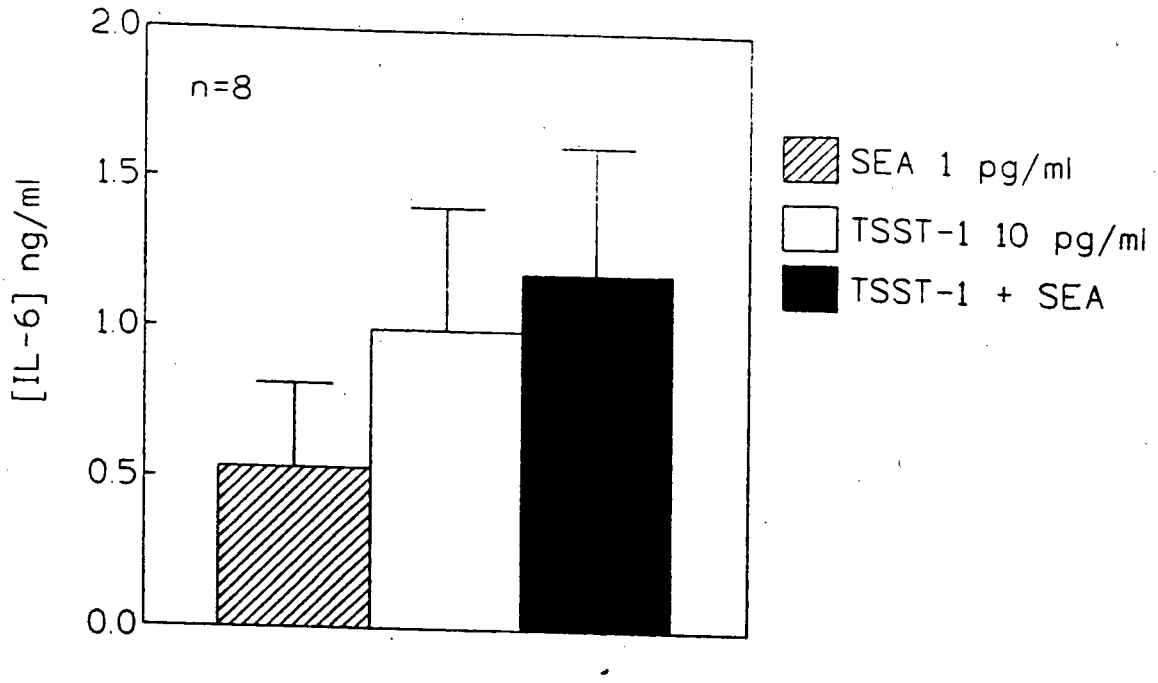


Figure 14. IL-6 secretion from human PBMC upon co-incubation with TSST-1 and SEA. Human PBMC were incubated for 19.5 h with TSST-1, SEA or TSST-1 plus SEA and culture supernatants were tested for IL-6 by immunoassay. Values represent the mean ± SEM in 8 human PBMC samples each studied in duplicate determinations. Background values (culture media alone) were below the levels of detection of the immunoassay.

4.2.2. Characterization of the TSST-1$^+$/SEA$^-$ mutant INT#1

Mutagenesis of *sea* in RN3984, a TSST-1$^+$/SEA$^+$ clinical strain isolated from a patient with menstrual TSS, was accomplished by plasmid integration as described in Materials and Methods. SEA in the culture filtrate of INT#1 was lower than the limit of detection by immunoassay, performed as described in Materials and Methods (Table 7). The amount of TSST-1 detected in the culture filtrate of INT#1 by immunoassay (as described in Materials and Methods) was not significantly different from the amount detected in the culture filtrate of RN3984 (Figure 15). This indicated that inactivation of *sea* by plasmid integration did not interfere with TSST-1 production.

Disruption of the SEA structural gene was verified by performing Southern hybridization analysis on *Hin*dIII digested genomic DNA of INT#1 with a 624 bp *Bam*HI-*Hin*dIII *sea* specific gene probe (Betley and Mekalanos, 1988). As can be seen in Figure 16, the sum of the INT#1 chromosomal *Hin*dIII fragments that hybridized to the probe (~6.4, 5.1 and <2 kb) was greater than the *Hin*dIII fragment that hybridized in RN3984 (~4.0 kb) and was consistent with the size expected if pMLD6873C-1 (~8.4 kb) recombined at the *sea* chromosomal locus. The presence of three *Hin*dIII fragments that hybridized to the *sea* probe in INT#1 is proposed to have arisen via a single crossover event between the chromosomal *sea* of RN3984 and the inactivated *sea* of pMLD6873C-1 near the 5' end of the gene as illustrated in Figure 17. The position of the *Hin*dIII site in pMLD6873C-1 indicated by an asterix is not definitively known since the orientation in which *EcoRI* digested pRN6441 inserted into pMLD6873 to generate pMLD6873C-1 was not determined (refer to Materials and Methods).

Table 7. Detection of SEA levels in the Culture Filtrates of RN3984 and INT#1.

Strain	[SEA] in Culture Filtrate (ng/ml)
RN3984	25.5 ± 3.7
INT#1	nd

SEA levels in culture filtrates were quantitated by immunoassay as described in Materials and Methods. Two single colonies of each strain were inoculated into 20 ml BHI and incubated at 37°C for 10 h. Culture filtrates were obtained by centrifugation and filter-sterilization. Values represent the mean ± SEM of 5 culture filtrates prepared from each strain tested in at least duplicate wells by immunoassay. The sensitivity limit of the immunoassay was 1 ng/ml of SEA. nd, not detected

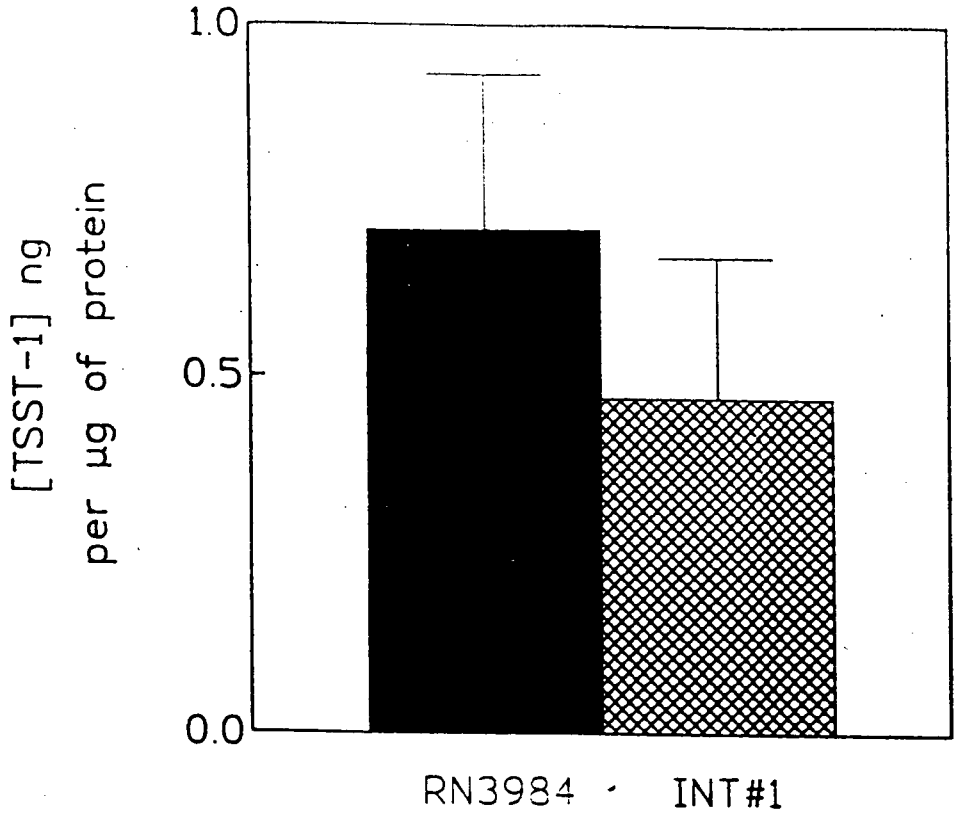


Figure 15. Comparison of TSST-1 levels with respect to total protein in the culture filtrates of RN3984 and INT#1. Cultures were prepared by inoculating two single colonies into 20 ml BHI followed by incubation at 37°C for 10h. Bars represent the mean TSST-1 level (detected by immunoassay) ± SEM per µg of protein in 5 culture filtrates prepared from RN3984 and 4 culture filtrates prepared from INT#1.

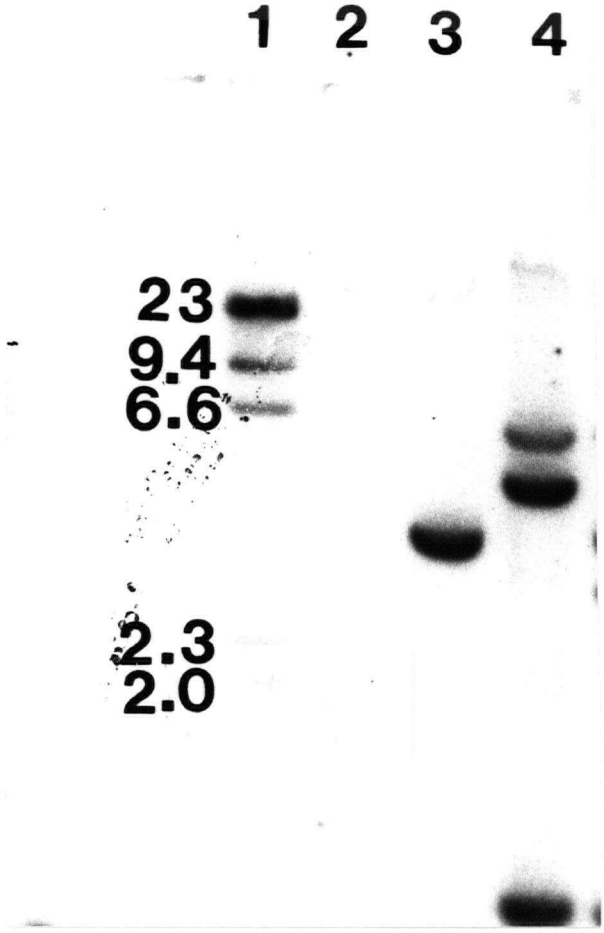


Figure 16. Southern hybridization analysis of SEA$^-$ mutant genomic DNA digested with *Hin*dIII and hybridized with a 624 bp *Bam*HI-*Hin*dIII *sea* specific gene probe.

Lane # 1. Digoxigenin-labelled DNA molecular size standards (kb)
2. RN450 (*sea*$^-$ control isolate)
3. RN3984
4. INT#1

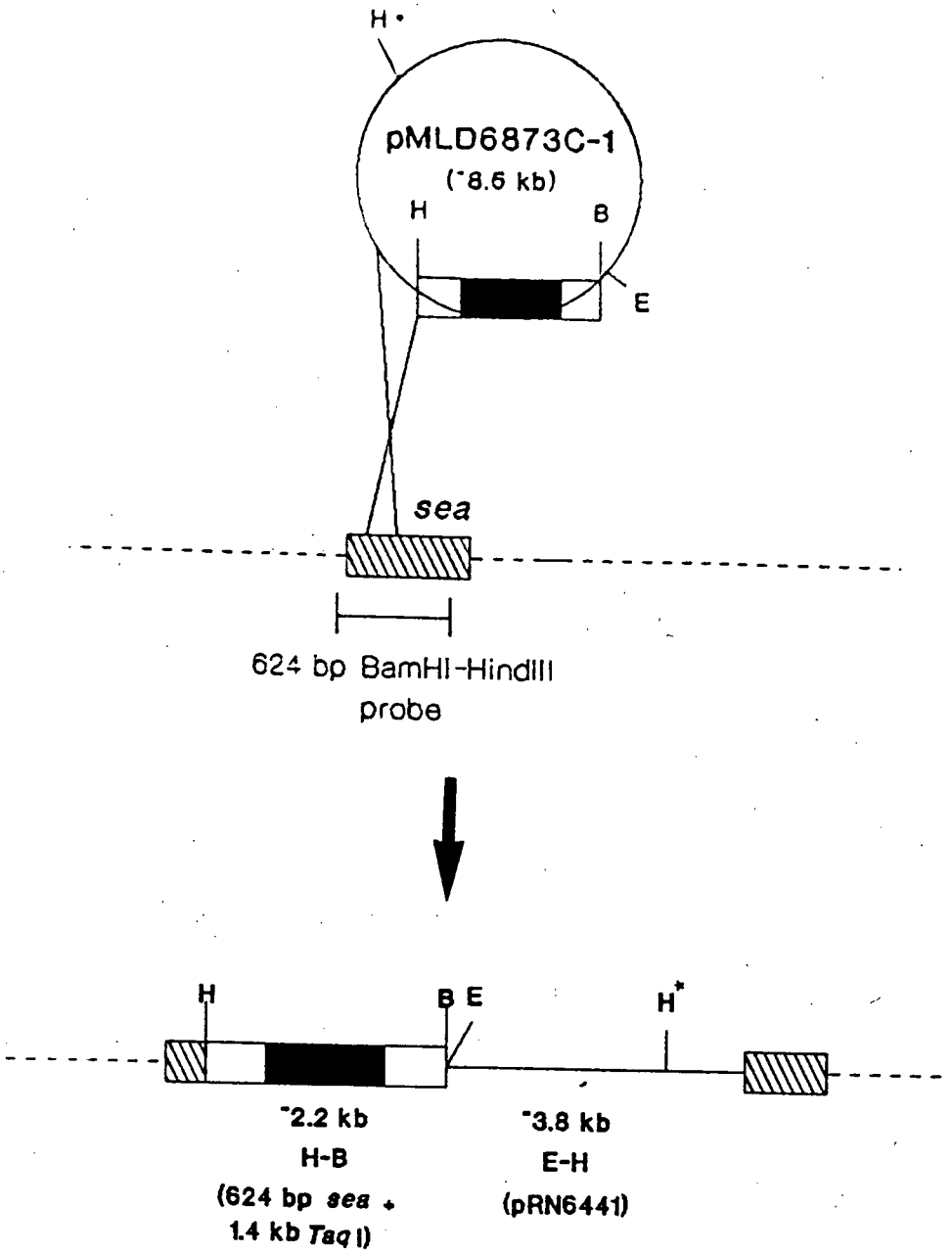


Figure 17. A possible recombination event between pMLD6873C-1 and *sea* in RN3984 to generate the SEA⁻ mutant INT#1. The indicated point of integration of pMLD6873C-1 into *sea* is consistent with the presence of three *Hin*dIII fragments that hybridized to the 624 bp *Bam*HI-*Hin*dIII *sea* probe in Figure 16, one of which was ~6.0 kb (refer to molecular sizes of the constructed plasmids in Materials and Methods). The dotted line represents the chromosomal DNA of RN3984 and the hatched box represent *sea* in RN3984. The unfilled box represents sea in pMLD6873C-1 inactivated by the 1.4 kb *Taq*I fragment of pRN6441 (indicated by solid box) as described in Materials and Methods. H, *Hin*dIII; H*, indicates possible location of *Hin*dIII (see Materials and Methods); B, *Bam*HI; E, *Eco*RI

Digestion of INT#1 with *Cla*I and hybridization with the 1.4 kb *Taq*I probe revealed one fragment with homology (Figure 18). The 1.4 kb *Taq*I fragment encoded Em^r and was used to inactivate *sea* in pMLD6873 (as described in Materials and Methods, section 2.5.3). The faint band observed slightly above the darker band in Figure 18 is believed to have arisen by hybridization with incompletely digested chromosomal DNA. The presence of only one band that hybridized to the probe is consistent with the premise that pMLD6873C-1 inserted only once into the chromosome. It should be noted that for some unexplained reason, one of the *Cla*I sites was destroyed in pMLD6873 upon insertion of the 1.4 kb *Taq*I fragment (see Materials and Methods, section 2.5.3). This explains why a *Cla*I fragment larger than 1.4 kb was observed upon digestion of INT#1 with *Cla*I followed by hybridization with the 1.4 kb *Taq*I probe (Figure 18).

4.2.3. Virulence of RN3984 and INT#1 in two animal models of lethal shock

A. Tampon-associated vaginal infection model

To determine whether a TSST-1^+/SEA^+ strain of *S. aureus* is more virulent than a TSST-1^+/SEA^- strain, monitoring of lethality, rectal temperature, weight loss, incidence of lipaemia and incidence of diarrhea was performed over eight days in 24 NZW rabbits - 10 infected with either RN3984 or INT#1, and 4 infected with culture media alone. Monitoring of rabbits was performed blinded with respect to infection group. As outlined in Table 8, no difference in lethality was observed between rabbits infected with RN3984 or INT#1. Other clinical parameters such as mean rectal temperature (Table 8), percent weight loss (Figure 20), and incidence of lipaemia (Table 8) were also not significantly different between the groups. Although the trends in weight loss were similiar to those observed for RN3984 and

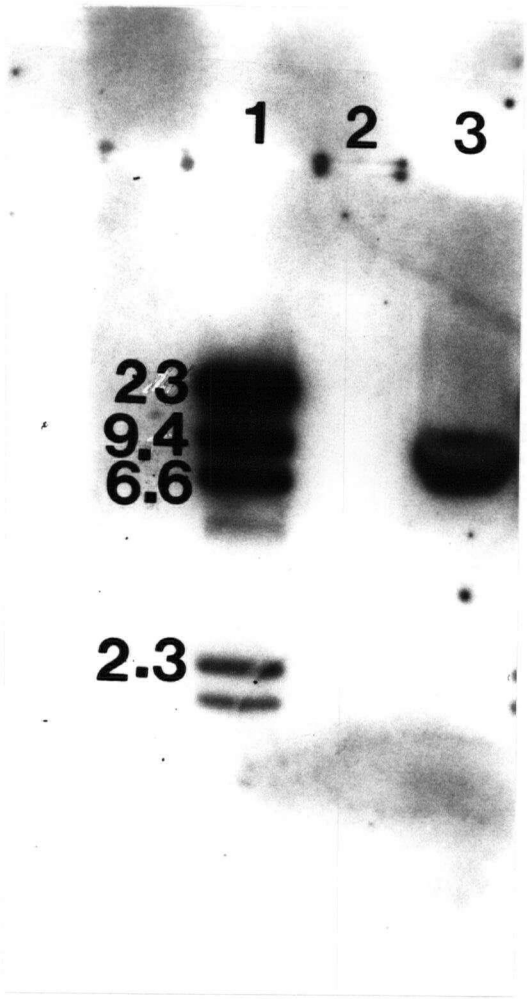


Figure 18. Southern hybridization analysis of SEA⁻ mutant genomic DNA digested with *Cla*I and probed with a 1.4 kb *Taq*I fragment of pRN6441. The presence of only one band that hybridized to the probe indicated that pMLD6873C-1 had integrated only once into the chromosome. The faint band with a higher molecular size observed to hybridize to the probe is believed to be the result of incomplete digestion of chromosomal DNA.

Lane # 1. Digoxigenin-labelled DNA molecular size standards (kb)
2. RN3984
3. INT#1

Table 8. Mortality and Morbidity mediated by RN3984 and INT#1 in a Tampon-associated Vaginal Infection Model of TSS in the NZW Rabbit.

Rabbit Infection Group	Mortality (# died/# infected)	Incidence of Lipaemia (%)	Incidence of Diarrhea (%)	Mean maximum rectal temperature[a] (°C ± SEM)
RN3984	1/10	4/9 (44)	4/9 (44)	40.1 ± 0.1
INT#1	2/10	4/8 (50)	0/8 (0)[b]	40.2 ± 0.2
Media control	0/4	0/4 (0)	0/4 (0)	39.7 ± 0.3

[a]Mean maximum rectal temperature over the 8 day observation period among survivors
[b]Significantly different from RN3984 ($P < 0.05$, Fisher's test, one-tailed)

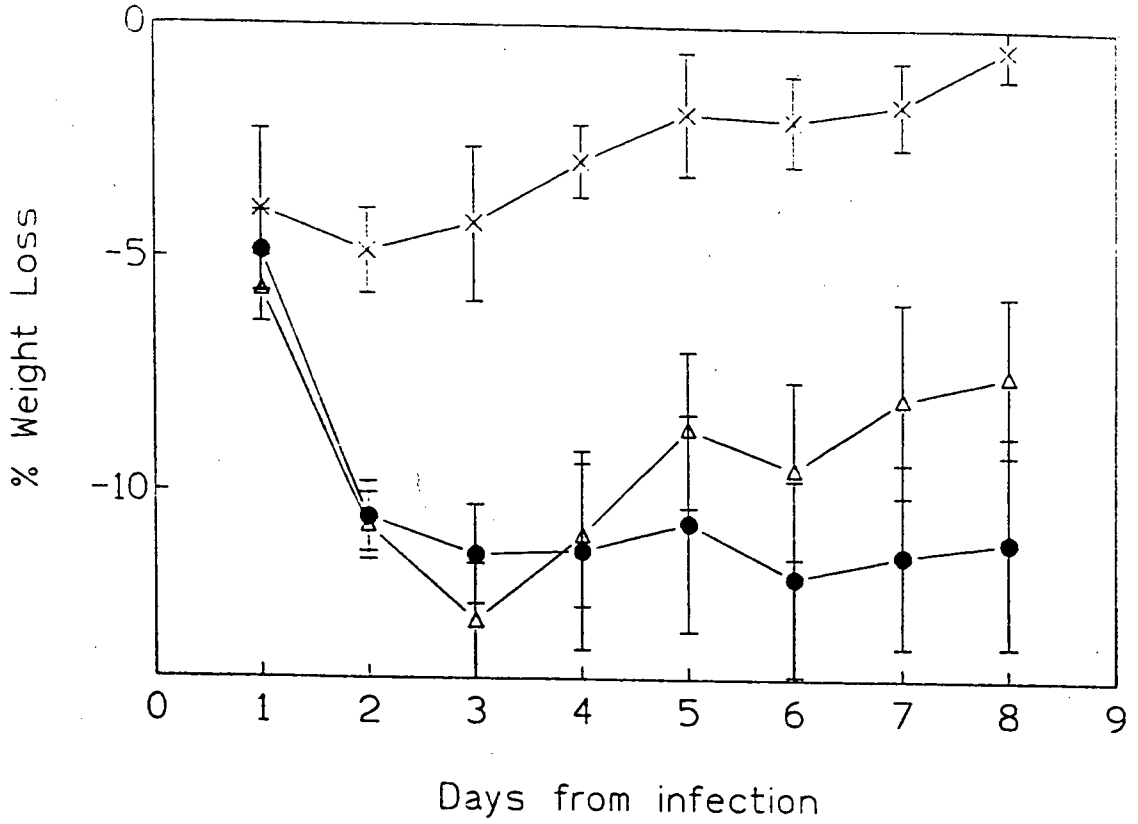


Figure 19. Weight loss in rabbits infected with RN3984 (●), INT#1 (△), or Media alone (X). Values represent the mean ± SEM in all available measurements gathered from rabbits on each day.

RN7043 in that the INT#1 infected group began to gain more weight in comparison to the RN3984 infected group, which may be suggestive of less morbidity upon INT#1 infection, no statistically significant difference was observed between the groups on any observation day. By comparing the weight loss graphs of RN3984 and RN7043 (Figure 5) and RN3984 and INT#1 (Figure 19) it was readily apparent that up until Day 6 there was a greater difference in weight loss between rabbits infected with RN7043 and RN3984 than rabbits infected with INT#1 and RN3984.

Detection of circulating rabbit TNFα by immunoassay (described in Materials and Methods) in the sera of the infected rabbits over the 8 day observation period was also attempted. Unfortunately, only 3 of 23 rabbits examined had detectable levels and thus differences between the groups using this as a parameter was not useful. Assaying for systemic TNF has many potential pitfulls, including the presence of specific binding proteins in the sera such as autoantibodies (Jeffes *et al.*, 1989) and soluble TNF receptors (Chouaib *et al.*, 1991), that may interfere with detection. In addition, due to the rapid clearance of TNF *in vivo*, the timing of blood sampling is a crucial factor (van Deuren, 1994). The collection periods may not have been optimal in this study.

As expected, a significant increase was observed in the number of rabbits that exhibited diarrhea upon RN3984 infection in comparison to INT#1 infection (4/8 vs 0/8; $P < 0.05$, Fisher's test, one-tailed) (Table 8). This indirectly supports SEA production by RN3984 and stability of the SEA$^-$ phenotype in INT#1 *in vivo*, since SEA is a common cause of staphylococcal food poisoning, in which one of the main symptoms is diarrhea.

B. D-galactosamine sensitized mouse model

As another test of the TSST-1$^+$/SEA$^+$ and TSST-1$^+$/SEA$^-$ strains, a second animal model of lethal shock was used to compare the virulence of RN3984 and INT#1. After 72 h, 12 of 15 mice infected with RN3984 died as compared to 8 of 15 mice infected with INT#1 ($P = 0.12$; Fisher's test, one-tailed). 2 of 2 mice infected with culture media alone survived. In order to conclude that INT#1 was less virulent than RN3984, a $\geq 50\%$ decrease in mortality was desired. Using a sample size of 15, this experiment yielded a 20% chance of observing a Type II statistical error.

4.2.4. *In vivo* stability of the SEA$^-$ phenotype of INT#1

Although plasmid integration can be utilized to generate site-specific mutations in *S. aureus* (Foster *et al.*, 1990; Proctor 1992), its use has been limited by concerns that this type of mutation may be unstable after prolonged growth *in vivo* as a result of the presence of direct duplications flanking the insertion (Foster *et al.*, 1990). In light of this, the stability of the SEA$^-$ phenotype of INT#1 was monitored for five days upon inoculation into BALB/c mice. Production of SEA by bacteria isolated from mice was tested by immunoassay as described in Materials and Methods. All of the 97 colonies screened from 15 mice over a five day period were SEA$^-$. Stability of the SEA$^-$ phenotype of INT#1 after *in vivo* growth in rabbits was also examined. While performing the experiments in rabbits, bacteria were recovered from tampons between Days 1 and 8 and analysed for SEA production. All of the cells from 26 colonies recovered from 9 rabbits infected with RN3984 produced SEA, in comparison to 0 of 67 cells from colonies recovered from 7 rabbits infected with INT#1. This suggests that the SEA$^-$ phenotype of INT#1 is stable *in vivo*, and that the lack of

difference in mortality and morbidity observed upon infection with RN3984 and INT#1 in both animal models is not due to reversion of the SEA$^-$ phenotype in INT#1.

4.2.5. *In vitro* effect of culture filtrates from isogenic strains on rabbit splenocytes

A. T cell proliferation

To determine if the culture filtrates of the TSST-1$^+$/SEA$^+$ and TSST-1$^+$/SEA$^-$ strains differed in their ability to induce T cell proliferation, diluted culture filtrate preparations of RN3984 and INT#1 were added to rabbit splenocytes. A mitogenic effect was observed upon incubation of rabbit splenocytes with each of two independently prepared culture filtrate samples from RN3984 and INT#1 (Figure 20). A significantly higher amount of T cell proliferation as assessed by [^{3}H]thymidine incorporation into cellular DNA (described in Materials and Methods) (cpm $\pm$ SEM) was observed in splenocytes incubated with RN3984 culture filtrate as compared to INT#1 culture filtrate at a dilution of 1/10,000 (410,831 $\pm$ 108,121 vs 202,191 $\pm$ 76,968; P = 0.038, paired t-test, 2-tailed). The decreased T cell proliferation caused by INT#1 culture filtrate compared to RN3984 culture filtrate could be explained by the lack of SEA. If this were true, adding SEA to the INT#1 culture filtrate would be expected to increase the level of T cell proliferation back to the level observed with RN3984 culture filtrate. To test this, purified SEA was added with a 1/10,000 dilution of INT#1 culture filtrate (at a concentration that equalled the known amount present in RN3984 culture filtrate as determined by immunoassay) to rabbit splenocytes and the amount of T cell proliferation was measured by [^{3}H]thymidine incorporation into cellular DNA. As observed in Figure 21, addition of SEA to the INT#1 culture filtrate restored the amount of T cell proliferation back to wild-type levels.

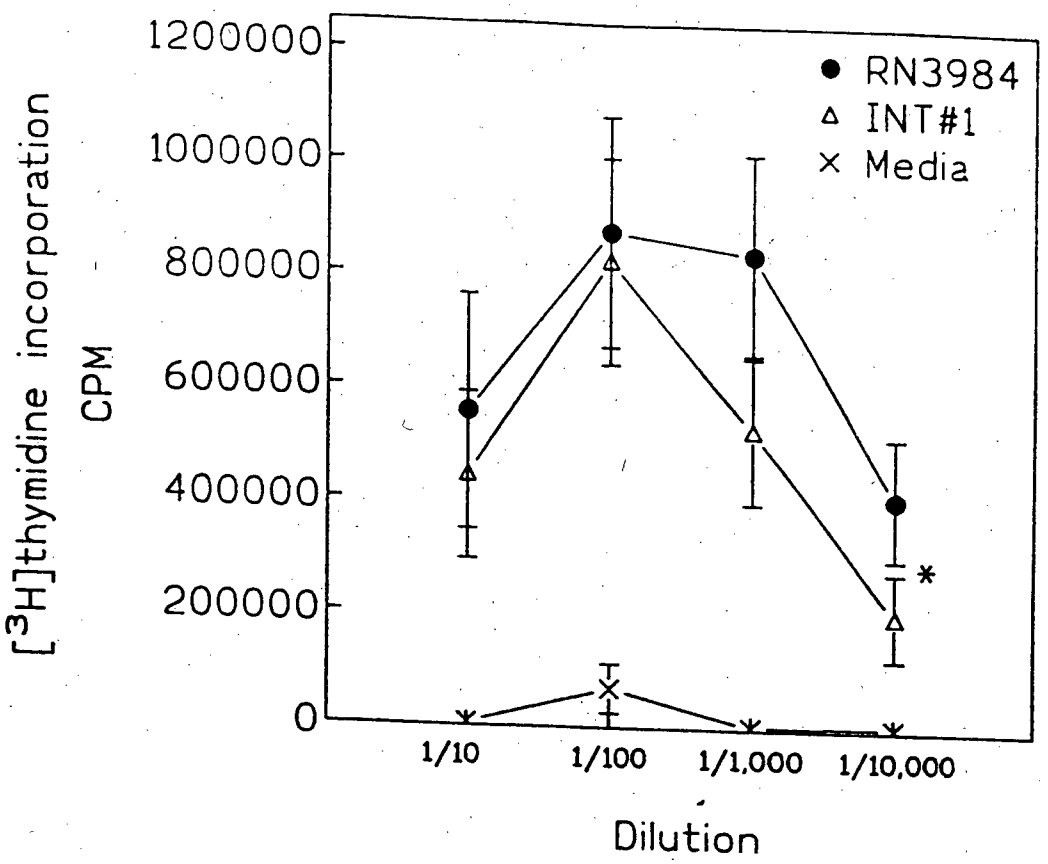


Figure 20. Mitogenic response induced by RN3984 and INT#1 culture filtrates on rabbit splenocytes. Values are expressed as the mean ± SEM measured by [^{3}H]thymidine incorporation into cellular DNA in 7 rabbit splenocyte samples each tested in a minimum of triplicate determinations. BHI served as the media control. * indicates a significant difference in proliferation compared to RN3984 (P = 0.038, paired t-test, 2-tailed)

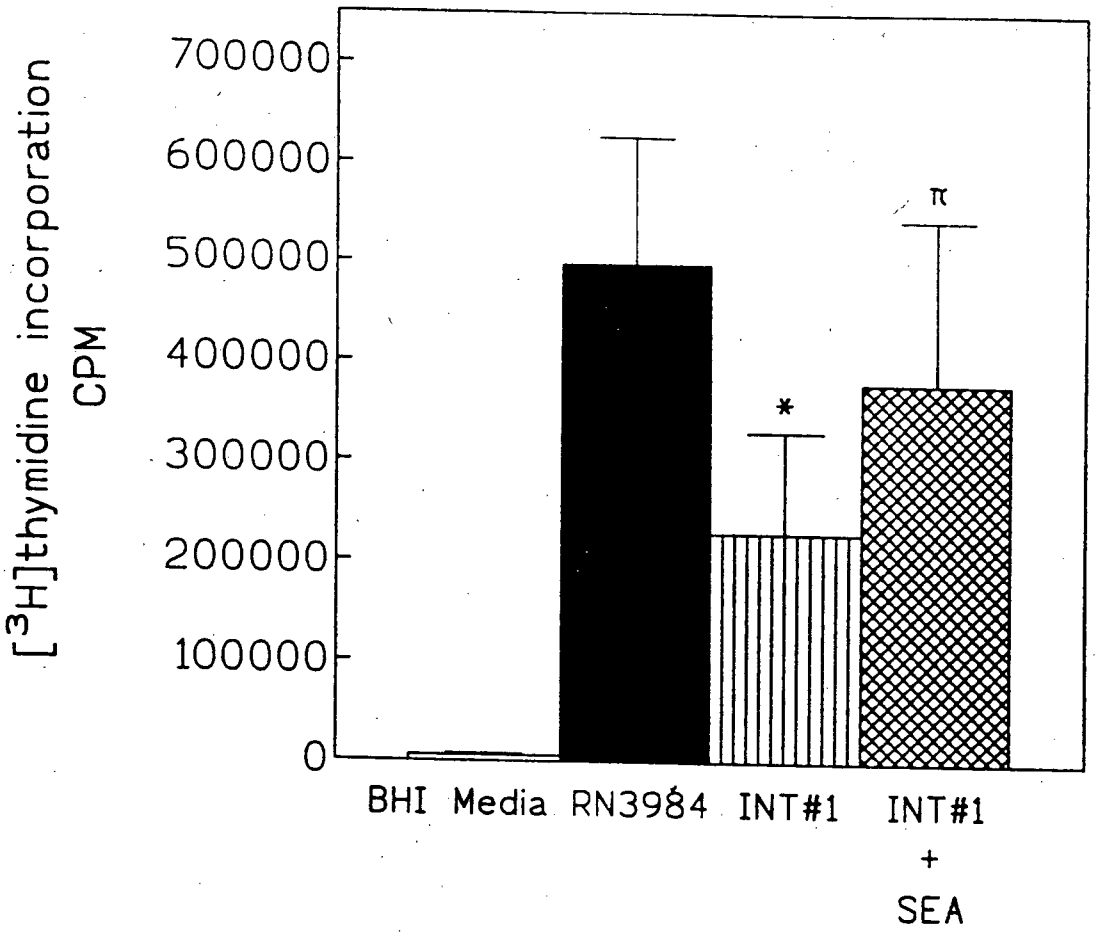


Figure 21. Increased proliferative response of rabbit splenocytes to RN3984 culture filtrate was consistent with the presence of SEA. A 1/10,000 dilution of INT#1 culture filtrate was added with purifed SEA (at a concentration that equalled the known amount found in the diluted RN3984 culture filtrate as determined by immunoassay) to rabbit splenocytes and the amount of T cell proliferation was assessed by incorporation of [^{3}H]thymidine into cellular DNA. Results are the mean ± SEM in 5 rabbit splenocyte samples each tested in a minimum of triplicate determinations. * indicates a significant difference in proliferation compared to RN3984 ($P < 0.05$, paired t-test, one-tailed); ¶ indicates no significant difference compared to RN3984 (paired t-test, one-tailed)

B. TNFα secretion

Culture filtrates of RN3984 and INT#1 stimulated rabbit splenocytes to secrete TNFα (Figure 22). No difference in the amount of TNFα secreted from rabbit splenocytes was observed upon incubation with RN3984 or INT#1 culture filtrate at any dilution examined. Two independently prepared culture filtrate samples of RN3984 and INT#1 were used for these studies.

4.2.6. Enhancement of T cell proliferation by TSST-1 and SEA in rabbit splenocytes is concentration dependent

The above observations suggest that although there was no difference between the isogenic strains in inducing mortality or morbidity *in vivo*, RN3984 induced more T cell proliferation than INT#1 *in vitro* at low concentrations (Figure 20). Since T cells are known to play a central role in mediating toxicity upon superantigen stimulation, their stimulation should have resulted in an *in vivo* effect (Marrack *et al.*, 1990; Miethke *et al.*, 1992; Miethke *et al.*, 1993). However the data also showed the ability of TSST-1 and SEA to cause an enhancement in T cell proliferation was concentration dependent since a difference in T cell proliferation observed with RN3984 culture filtrate as compared to INT#1 was found only at the highest dilution used (1/10,000). As a further test, the mitogenic response of rabbit splenocytes to TSST-1 and SEA was determined (Figure 23 and 24). Although direct comparisons between rabbit splenocyte preparations is difficult due to the variations that exist in both the amplitude and doses required to induce mitogenesis, the TSST-1 induced mitogenesis curve observed with the pooled rabbit splenoctye sample was similiar to that previously reported by Poindexter and Schlievert (1985), in that TSST-1 induced T cell

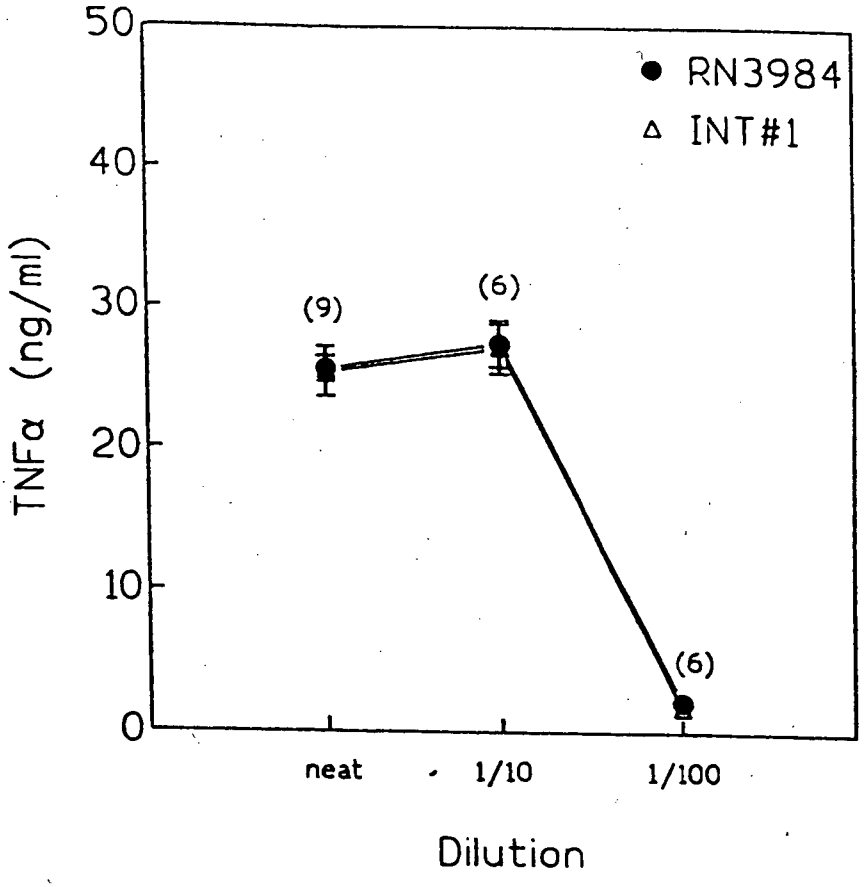


Figure 22. TNFa secretion induced from rabbit splenocytes by RN3984 and INT#1 culture filtrates. Values represent the mean ± SEM of rabbit splenocyte preparations tested in a minimum of duplicate determinations. BHI served as the media control and gave background values of <0.04 ng/ml. Numbers in brackets indicate the number of rabbits tested at each dilution.

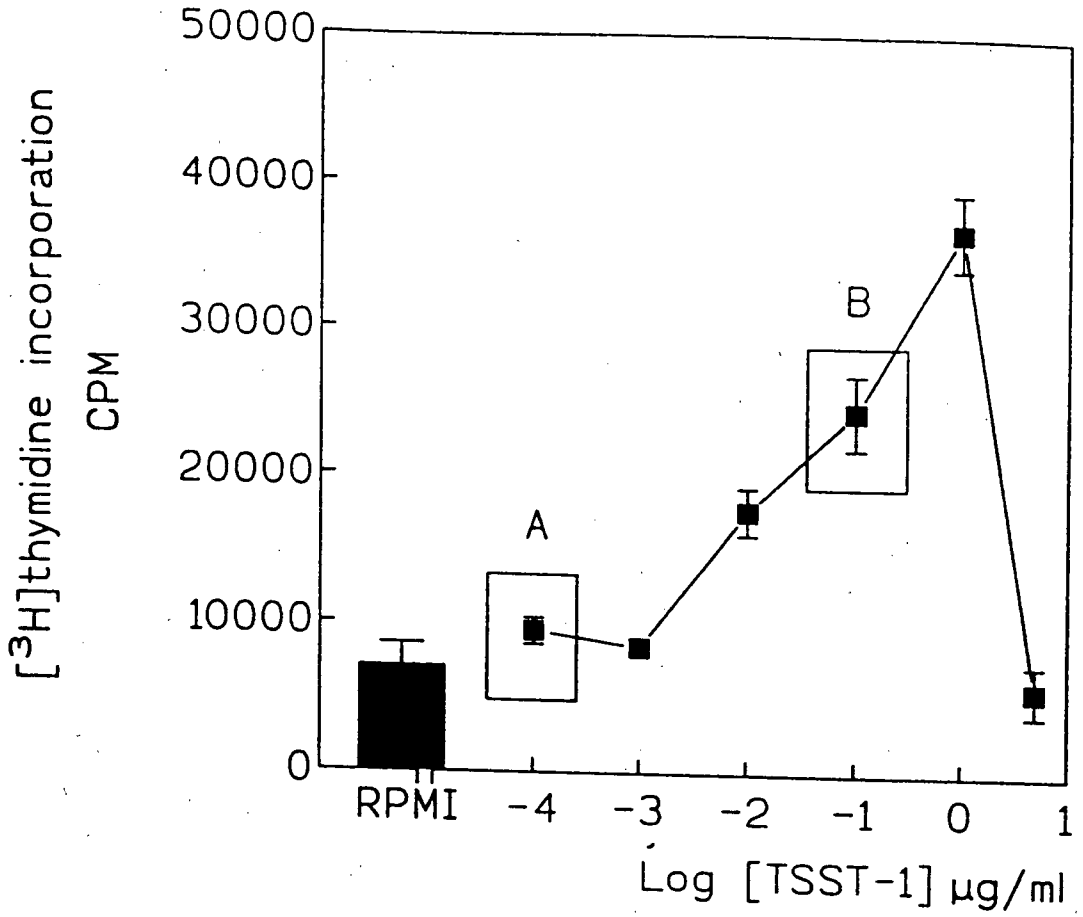


Figure 23. Mitogenic response of a pooled rabbit splenocyte preparation to purified TSST-1 as measured by [^{3}H]thymidine incorporation into cellular DNA. Each point represents the mean ± SEM from a pooled rabbit splenocyte preparation (5 rabbits) studied in a minimum of six replicate determinations. The solid bar represents the mean ± SEM observed with RPMI culture media alone. Boxes labelled 'A' and 'B' indicate the high and low concentrations used for determining whether the increase in T cell proliferation observed with RN3984 was concentration dependent.

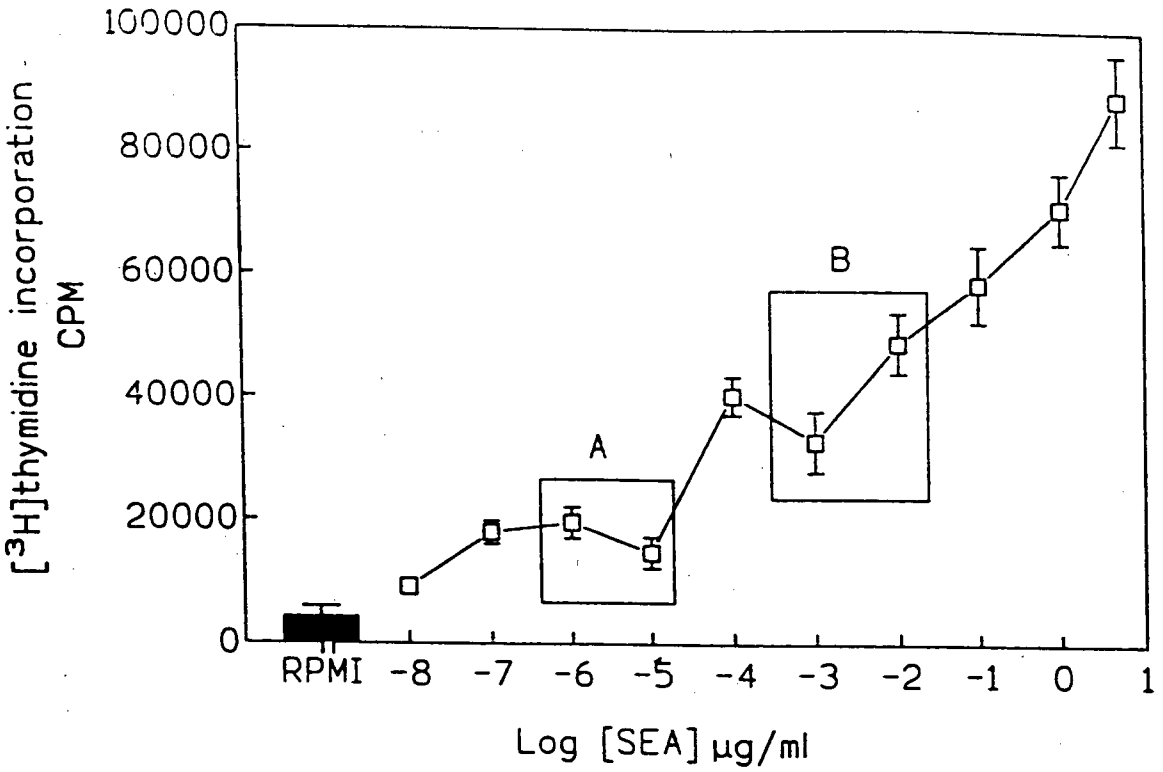


Figure 24. Mitogenic response of a pooled rabbit splenocyte preparation to SEA as measured by [^{3}H]thymidine incorporation into cellular DNA. Each point represents the mean ± SEM from a pooled rabbit splenocyte preparation (5 rabbits) studied in a minimum of six replicate determinations. The solid bar represents the mean ± SEM observed with RPMI culture media alone. Boxes labelled 'A' and 'B' indicate the high and low concentrations used for determining whether the increase in T cell proliferation observed with RN3984 was concentration dependent.

proliferation over a broad dose range. To determine if there was a concentration dependent effect of RN3984 to induce T cell proliferation, concentrations of TSST-1 and SEA that induced high and low mitogenic responses on rabbit splenocytes were chosen (Boxes 'A' and 'B' respectively, Figures 23 and 24). Minimally effective concentrations of TSST-1 (100 pg/ml) and SEA (1 or 10 pg/ml) (Box 'A') were co-incubated in 9 rabbit splenocyte preparations in 13 combinations (refer to Table 9). Next, higher concentrations of TSST-1 (100 ng/ml) and SEA (1 or 10 ng/ml) (Box 'B') were co-incubated in 5 rabbit splenocyte preparations in 9 combinations (refer to Table 10). A significant increase in proliferation was observed in 8 of 13 rabbit splenocyte preparations co-incubated with TSST-1 and SEA at the minimally effective dose (Box 'A') as compared to incubation with either toxin alone ($P < 0.05$, Mann-Whitney test, 2-tailed; Table 9). In contrast, only 1 of 9 rabbit splenocyte preparations co-incubated with higher concentrations of TSST-1 and SEA (Box 'B') exhibited a significant increase in T cell proliferation when compared to incubation with either toxin alone ($P < 0.05$, Mann-whitney test, 2-tailed; Table 10). The difference between these ratios was significant, in that low doses of TSST-1 and SEA combined enhanced T cell proliferation more often than did high doses (8/13 vs 1/9; $P < 0.05$, Fisher's test, 2-tailed) when compared to each toxin independently.

These results suggest that the ability of TSST-1 and SEA combined to cause an enhancement in T cell proliferation over either toxin alone is dependent on toxin concentration. In light of these results it may be proposed that although RN3984 culture filtrate induced greater T cell proliferation than INT#1 *in vitro* (at a 1/10,000 dilution), perhaps this effect was not observed *in vivo* because TSST-1 and SEA are co-produced at

Table 9. Effect of Low Concentrations of TSST-1 (100 pg/ml) and SEA (1 or 10 pg/ml) on the Mitogenic Response of Rabbit Splenocytes.

Rabbit #		CPM		
		SEA (0 pg/ml)	SEA (1 pg/ml)	SEA (10 pg/ml)
1	TSST-1 (0 pg/ml)		41,852 ± 12,441	135,724 ± 18,486
	TSST-1 (100 pg/ml)	120,038 ± 12,928	249,781 ± 35,877*	431,375 ± 63,815*
2	TSST-1 (0 pg/ml)		180,132 ± 31,790	534,063 ± 9,866
	TSST-1 (100 pg/ml)	514,924 ± 15,984	708,278 ± 18,006*	716,240 ± 38,641*
3	TSST-1 (0 pg/ml)		32,144 ± 8,735	105,688 ± 7,831
	TSST-1 (100 pg/ml)	67,511 ± 3,129	119,161 ± 48,364	276,069 ± 72,444*
4	TSST-1 (0 pg/ml)		61,156 ± 14,889	186,993 ± 22,945
	TSST-1 (100 pg/ml)	67,603 ± 13,682	181,146 ± 21,492*	344,527 ± 45,387*
5	TSST-1 (0 pg/ml)			353,950 ± 28,871
	TSST-1 (100 pg/ml)	509,956 ± 66,382		605,904 ± 103,545
6	TSST-1 (0 pg/ml)			387,078 ± 5,249
	TSST-1 (100 pg/ml)	502,040 ± 55,044		392,011 ± 10,380
7	TSST-1 (0 pg/ml)			519,176 ± 63,684
	TSST-1 (100 pg/ml)	177,951 ± 21,930		721,119 ± 14,559*
8	TSST-1 (0 pg/ml)			1,024,037 ± 50,859
	TSST-1 (100 pg/ml)	1,142,607 ± 71,615		974,751 ± 16,029
9	TSST-1 (0 pg/ml)			130,556 ± 17,076
	TSST-1 (100 pg/ml)	122,145 ± 20,490		80,480 ± 8,882

Values represent the mean ± SEM of a minimum of triplicate determinations for each rabbit splenocyte sample as measured by [^{3}H]thymidine incorporation into cellular DNA. Background activity has been subtracted from the reported values.
* significantly different from TSST-1 or SEA alone ($P < 0.05$, Mann-Whitney test, 2-tailed)
8 of 13 of the splenocyte preparations incubated with TSST-1 plus SEA at these low concentrations resulted in an increase in [^{3}H]thymidine incorporation compared to incubation with either TSST-1 or SEA alone.

Table 10. Effect of High Concentrations of TSST-1 (100 ng/ml) and SEA (1 or 10 ng/ml) on the Mitogenic Response of Rabbit Splenocytes.

Rabbit #		CPM SEA (0 ng/ml)	SEA (1 ng/ml)	SEA (10 ng/ml)
1	TSST-1 (0 ng/ml)		1,120,459 ± 108,681	1,228,781 ± 43,913
	TSST-1 (100 ng/ml)	1,139,926 ± 110,351	1,544,148 ± 59,919*	1,385,426 ± 39,627
2	TSST-1 (0 ng/ml)		1,430,891 ± 94,877	1,650,852 ± 209,961
	TSST-1 (100 ng/ml)	1,758,285 ± 207,126	2,113,746 ± 132,998	1,839,685 ± 103,174
3	TSST-1 (0 ng/ml)		1,616,225 ± 161,095	1,412,731 ± 177,789
	TSST-1 (100 ng/ml)	1,476,934 ± 130,258	1,713,181 ± 126,480	1,800,552 ± 93,967
4	TSST-1 (0 ng/ml)		793,592 ± 92,768	941,641 ± 130,016
	TSST-1 (100 ng/ml)	825,240 ± 49,470	918,398 ± 60,132	895,694 ± 75,390
5	TSST-1 (0 ng/ml)		790,089 ± 22,661	
	TSST-1 (100 ng/ml)	689,898 ± 26,489	762,301 ± 124,543	

Values represent the mean ± SEM in a minimum of five replicate determinations for each rabbit splenocyte sample as measured by [^{3}H]thymidine incorporation into cellular DNA. Background activity has been subtracted from the reported values.

* significantly different from TSST-1 or SEA alone ($P < 0.05$, Mann-Whitney test, 2-tailed)

1 of 9 of the rabbit splenocyte preparations incubated with TSST-1 plus SEA at these high concentrations resulted in a significant increase in [^{3}H]thymidine incorporation compared to incubation with either TSST-1 or SEA alone.

higher levels than that found in the RN3984 culture filtrate at this specific dilution. It should be noted that under the *in vitro* conditions used in this study, the undiluted culture filtrate of RN3984 contains ~100 ng/ml of TSST-1 and ~30 ng/ml of SEA. At these inputs of toxin, an additive effect in T cell proliferation in comparison to the effect observed with each toxin individually would not be predicted.

4.3. Discussion

The importance of TSST-1 in the pathogenesis of menstrual TSS was examined initially in these studies by introducing isogenic mutants in TSST-1 production into a tampon-associated vaginal infection model. The results showed that the TSST-1$^+$ strain of *S. aureus* was more lethal in the animal model than its TSST-1$^-$ isogenic derivative, suggesting that TSST-1 does play a causative role in MTSS. Confounding these results however was the decreased production of SEA by the TSST-1$^-$ mutant. If SEA plays an important role in the pathogenesis of MTSS, then the results surrounding the importance of TSST-1 in MTSS that generated from the use of these isogenic mutants *in vivo* would not be conclusive. This concern prompted studies to determine whether or not SEA plays a role in conjunction with TSST-1 in causing TSS. It was hypothesized that it might contribute to pathogenesis since TSST-1 and SEA is a clinically relevant toxin combination, and SEA is a superantigen. Co-production of two superantigens by *S. aureus* might increase the virulence of the organism through enhancing the induction of T cell mitogenesis and cytokine secretion that are believed to be involved in pathogenesis. Since binding of TSST-1 and SEA to the MHC Class II molecule is a prerequisite for T cell mitogenesis and cytokine induction, it was reasonable to assume that at minimally effective concentrations of both toxins, an enhanced

effect in these responses would be observed upon co-incubation. Consistent with this was the observation that co-incubation of TSST-1 and SEA with human PBMC significantly enhanced both T cell proliferation and TNFα secretion compared to the effect observed with either toxin alone. These results were consistent with the hypothesis that production of SEA by TSST-1$^+$ strains may increase the virulence of the organism, and further analysis of the effects of this toxin combination *in vivo* was performed.

To determine if a TSST-1$^+$/SEA$^+$ strain was more virulent than a TSST-1$^+$/SEA$^-$ strain, introduction of the strains into two animal models of lethal shock was performed. Results in both the tampon-associated vaginal infection model and the D-galactosamine sensitized mouse model indicated that the TSST-1$^+$/SEA$^+$ strain did not cause more mortality or morbidity than the TSST-1$^+$/SEA$^-$ strain. Stability of the SEA$^-$ phenotype of INT#1 after *in vivo* growth in both BALB/c mice and NZW rabbits was verified.

Although the animal models used in this study were quite different, both the mouse model and the rabbit infection model are relevant for analysing the role of staphylococcal exoproteins in TSS since the mechanisms leading to shock are similiar in the two cases. TSS is believed to be caused by the overstimulation of T cells by superantigens followed by excessive secretion of cytokines such as TNF, which can cause shock *in vivo* (Okusawa *et al.*, 1988; Dinarello *et al.*, 1989). Monoclonal antibodies against TNF are protective in a rabbit model of TSS (Parsonett and Gillis, 1988). T cells have been shown to be important in the rabbit model since TSST-1 administration to rabbits caused a significant increase in PBMC counts as compared to non-toxin treated control rabbits. In addition, the increased PBMC counts correlated with enhanced lethality in the study (Lee *et al.*, 1991). Lethal

shock in D-galactosamine sensitized mice upon superantigen stimulation also occurs via a T cell/TNF-dependent pathway since SCID mice lacking T cells were resistant to the lethal effects of either TSST-1 or SEB (Miethke *et al.*, 1992; 1993). In addition, cyclosporin A treatment (a reagent that suppresses cytokine secretion by T cells) and anti-TNF monoclonal antibodies were protective against superantigen-mediated lethality in this mouse model (Miethke *et al.*, 1992; 1993).

In support of the *in vivo* studies, incubation of rabbit splenocytes with RN3984 or INT#1 culture filtrates *in vitro* produced no difference in the amount of TNFα secreted. Although RN3984 culture filtrate induced more T cell proliferation from rabbit splenocytes *in vitro* than INT#1, this effect was concentration dependent. A concentration dependent effect in T cell proliferation would be expected with human cells since TSST-1 and SEA bind to the same receptor, the Class II antigen, on human peripheral blood mononuclear cells (Mollick *et al.*, 1989; Scholl *et al.*, 1989), and superantigen binding to Class II molecules is a prerequisite for the T cell proliferative response (Fleischer and Schlezenmeier, 1988; Fischer *et al.*, 1989; See *et al.*, 1992a). Thus although minimally effective concentrations of TSST-1 and SEA combined significantly increased T cell proliferation in human PBMC compared to incubation with either toxin alone, due to the overlapping receptors these toxins share, at higher concentrations of toxin this additive response would not be anticipated. Whether the concentration dependent effect of T cell mitogenic enhancement from rabbit splenocytes upon TSST-1 and SEA co-incubation may be mediated by the binding of a common receptor is only conjecture at this time.

As with T cell mitogenesis, TSST-1 and SEA induced cytokine release from human PBMC

is also Class II dependent (Grossman *et al.*, 1990; Matsuyama *et al.*, 1993; Akatsuka *et al.*, 1994), and as such would be expected to be concentration dependent. Therefore, although the combination of TSST-1 and SEA at the specific concentrations used in this study caused a significant increase in TNFα secretion from human PBMC compared to incubation with either toxin alone, it is predicted that this would not occur at higher concentrations of toxin. The ability to show a concentration dependent effect in TNFα secretion from rabbit splenocytes upon stimulation with the culture filtrates of the TSST-1^+/SEA^+ and TSST-1^+/SEA^- strains was restricted by the sensitivity limitations of the rabbit TNFα immunoassay. Therefore, since the amount of TSST-1 and SEA produced by *S. aureus in vivo* is not known, it can not be predicted whether or not an enhancement of T cell mitogenesis and TNF secretion occurs during human disease. The evidence presented here suggests that this effect would not be observed, since the pathogenicity of the strains was not different.

The *in vitro* studies of toxin induced cytokine secretion from human PBMC revealed variability in cytokine responses (Figures 12, 13 and 14). In contrast to TNFα induction, an increase in either IL-1ß or IL-6 levels in the culture supernatants of human PBMC stimulated with both TSST-1 and SEA as compared to incubation with either toxin alone could not be demonstrated. It is plausible that TSST-1 and SEA enhance the secretion of one cytokine but not others, since these cytokines appear to be differentially regulated (See and Chow, 1992; Trede *et al.*, 1994). Cytokine induction may occur via different signal transduction pathways (See *et al.*, 1992b). Whether the different activation pathways induced by these toxins are related to the unique epitopes which TSST-1 and SEA bind on human PBMC (Karp *et al.*, 1990; See *et al.*, 1990; Herman *et al.*, 1991; See *et al.*, 1992a) is not

known, but it does introduce another degree of complexity in superantigen induced cytokine expression.

These results suggest that when TSST-1 is produced by *S. aureus*, the concomitant production of SEA is irrelevant in terms of mediating overall enhanced lethality or morbidity. SEA may contribute to the virulence of *S. aureus* in a manner that was not monitored in these studies. For example, SEA production by TSST-1$^+$ strains may provide benefit in either colonization or survival. Furthermore, TSST-1 could interact specifically with a determinant associated with SEA in this particular clone to cause enhanced virulence. For example, SEA is carried by a triple converting phage that mediates positive conversion for staphylokinase and SEA expression, and negative conversion for ß-lysin (Coleman *et al.*, 1984). It is possible that it is staphylokinase, a plasminogen activator, and not SEA, that interacts with TSST-1 to cause enhanced virulence, or that inactivating ß-lysin gives some advantage to this clone. An association between TSST-1 and SEA production may have arisen entirely by chance. Alternatively, the phage encoding SEA may prefer some aspect in the menstrual TSST-1$^+$ host background that favours its insertion, and while no biological significance ensues, a correlation between TSST-1 and SEA production was made.

In summary, these results provide evidence to suggest that production of TSST-1 by *S. aureus* appears to be the primary toxin involved in causing menstrual toxic shock syndrome. Although the TSST-1$^-$ mutant (RN7043) was found to produce less SEA than the TSST-1$^+$ strain in Chapter 3, the results in this chapter suggest that SEA does not enhance the virulence of this particular *S. aureus* strain. The differences observed *in vivo* and *in vitro* with RN3984 and RN7043 in Chapter 3 therefore was not likely due to decreased production

of SEA by RN7043. It does remain a possibility however that production of SEA with TSST-1 among MTSS isolates aids the organism in other properties not investigated in this study. Exploring other putative benefits SEA may provide to TSST-1 producing *S. aureus* would be of clinical relevance due to the observed frequency of this toxin combination among MTSS isolates.

Chapter 5. Demonstration of whether the mutually exclusive production of TSST-1 and SEB in *S. aureus* is mediated by lack of structural genes

5.1. Introduction

Upon establishing a causal role for TSST-1 in menstrual TSS, subsequent objectives of this research was to examine the interaction of TSST-1 with other toxins, specifically SEA and SEB. TSST-1 and SEA was a toxin combination of interest due to its observed frequency among menstrual TSS isolates. Although this suggested that a *S. aureus* strain producing both TSST-1 and SEA may be more virulent than a strain producing TSST-1 alone, this did not appear to be the case when TSST-1$^+$/SEA$^+$ and TSST-1$^+$/SEA$^-$ strains were introduced into two tests of lethal shock in animals.

In contrast to the frequent co-production of TSST-1 and SEA, TSST-1 and SEB co-production appears to be mutually exclusive at the phenotypic level in *S. aureus*. At the genotypic level, results of various studies that have examined whether TSST-1 and SEB structural genes exist in the same isolate have been inconclusive. Because of this, and in light of my continued interest in the underlying mechanisms that act to favour or exclude certain toxin combinations in organisms, I explored possible mechanisms that may be mediating mutual exclusivity of TSST-1 and SEB in *S. aureus*.

5.2. Results

5.2.1. Detection of *tst* in TSST-1$^+$ and TSST-1$^-$ *S. aureus* isolates

To begin, Southern hybridization analysis was used to determine whether the TSST-1 and SEB toxin determinants could be found within the same *S. aureus* isolate. Six TSST-1$^+$

clinical isolates (7140, 7051, 8270, 7294, 7616, VI-5) chosen from our clinical collection based on their known status of TSST-1 and SEB production as determined by ELISA (Lee *et al.*, 1992) and two TSST-1$^+$ reference isolates [RN3984, FRI1169 (Kreiswirth *et al.*, 1989)], were confirmed to contain the TSST-1 structural gene by Southern hybridization analysis of either *Cla*I digested (Figure 26) or *Hin*dIII digested (Figure 27) genomic DNA using a 297 bp *Hin*cII-*Bam*HI *tst*-specific gene probe (Kreiswirth *et al.*, 1989) (Refer to Figure 25). One *S. epidermidis* isolate and six *S. aureus* isolates found to be TSST-1$^-$ by ELISA (RN450, 0507, 7660, 8527, 8528 and 8530) served as negative controls, and as expected, none hybridized with the *tst* probe (Figures 26 and 27). *Cla*I is a restriction enzyme that has been reported to not cut within the 4 to 7 kb TSST-1 genetic element [Kreiswirth *et al.*, 1989]. However the presence of fragments less than 4 kb (Figure 26) in 2 TSST-1$^+$ isolates (7051, 7294) could indicate the presence of a *Cla*I restriction site within the TSST-1 genetic element of these isolates, suggesting that there is a restriction fragment length polymorphism (RFLP) in this region. Alternatively, there could have been a deletion in the TSST-1 genetic element. The presence of 3 *Cla*I-*tst* hybridization patterns in the remaining 6 isolates is consistent with previous observations that *tst* is found in multiple loci in the *S. aureus* chromosome as determined by protoplast fusion and transformation mapping (Chu *et al.*, 1988).

*Hin*dIII digestion also revealed three *tst* hybridization patterns among the TSST-1$^+$ clinical isolates (Figure 27). RN7043 is an isogenic derivative of RN3984 derived by the insertion of a tetracycline resistance determinant (with an internal *Hin*dIII site) within the *tst* locus. Expectedly, hybridization with the *tst* probe revealed a smaller *Hin*dIII-*tst* fragment in

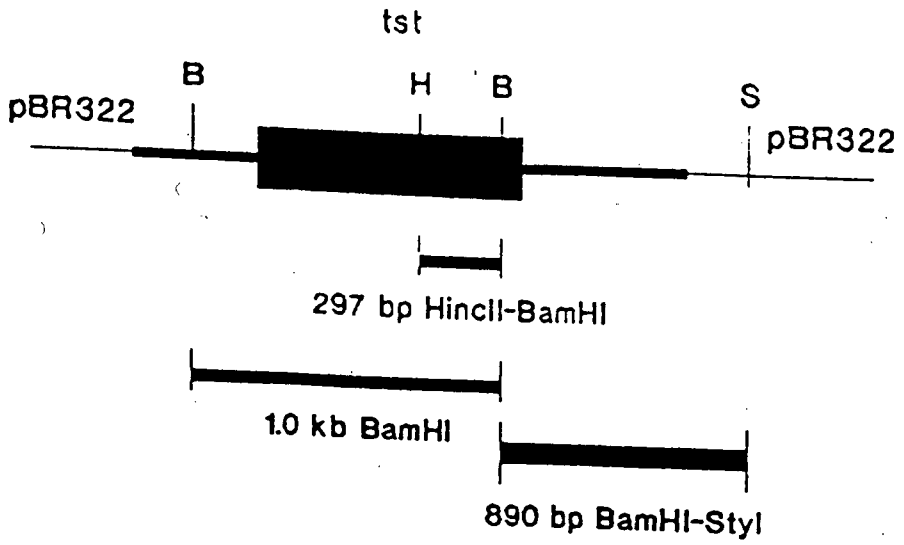


Figure 25. Restriction map of pRN6550 (pBR322 carrying *tst* and associated TSST-1 genetic element as depicted by Kreiswirth et al., 1989) and location of fragments used as probes.
B, *Bam*HI; H, *Hin*cII; S, *Sty*I

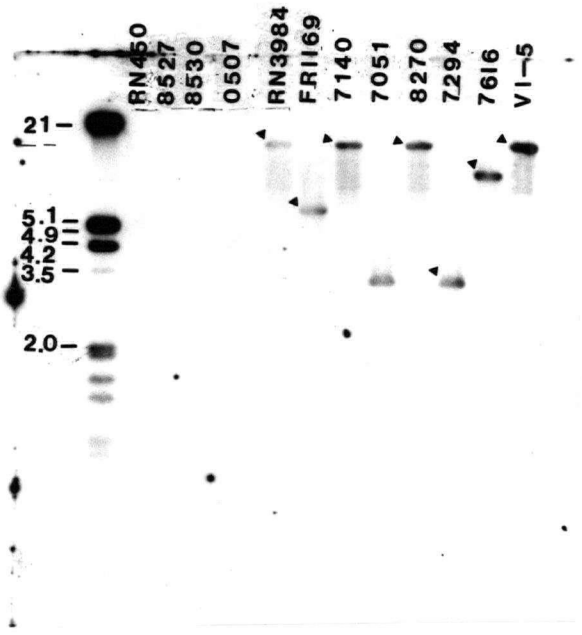


Figure 26. Southern hybridization analysis of TSST-1$^-$ (RN450, 8527, 8530, 0507) and TSST-1$^+$ (RN3984, FRI1169, 7140, 7051, 8270, 7294, 7616, VI-5) *S. aureus* isolates digested with *Cla*I and probed with a 297 bp *Hinc*II-*Bam*HI *tst* specific gene probe. Numbers at left are digoxigenin-labelled DNA molecular size standards (kb). The arrows indicate the common *Cla*I fragment that hybridized to both the *tst* probe and the 150 bp *Taq*I *seb* specific gene probe in Figure 29. Only *S. aureus* 7051 did not have a common *Cla*I fragment that hybridized to the *tst* and *seb* probes.

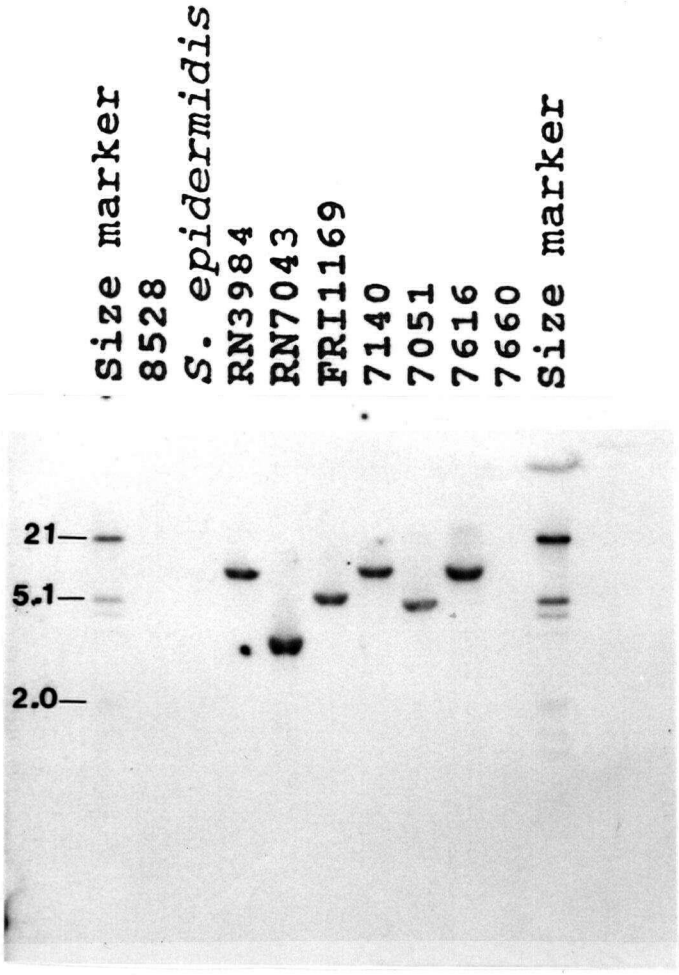


Figure 27. Southern hybridization analysis of TSST-1$^+$ isolates digested with *Hin*dIII and probed with a 297 bp *Hin*cII-*Bam*HI *tst* specific gene probe. *S. aureus* 8528 and 7660 and *S. epidermidis* served as the negative controls. Numbers at left are the positions of the digoxigenin-labelled DNA molecular size standards (kb).

RN7043 as compared to RN3984. Of special note are isolates 0507 (Figure 26) and 8528 (Figure 27), both of which are TSST-1$^-$/SEB$^+$. The *Cla*I- and *Hin*dIII-digested chromosomal DNA of these isolates did not hybridize with the *tst* probe, confirming that *tst* and *seb* do not share DNA homology.

5.2.2. Detection of *seb* among TSST-1$^+$ and TSST-1$^-$ *S. aureus* isolates

To examine if TSST-1$^+$ isolates do or do not contain the SEB structural gene, Southern hybridization analysis was performed on the 8 *Cla*I digested TSST-1$^+$ isolates using a 150 bp *Taq*I *seb*-specific gene probe (Johns and Khan, 1988; Figure 28). The *Cla*I digested chromosomal DNA of all 8 TSST-1$^+$ isolates hybridized with the *seb* gene probe (Figure 29). The two well-characterized TSST-1$^-$/SEB$^-$ reference isolates RN450, 8530 did not hybridize, while the SEB$^+$/TSST-1$^-$ reference isolate 0507 did.

Of special interest is the observation that in all isolates but one (7051), the 150 bp *Taq*I *seb*-specific gene probe hybridized to a similiar sized *Cla*I fragment that also hybridized to the 297 bp *Hin*cII-*Bam*HI tst-specific gene probe in Figure 26. This suggested that *tst* and *seb* may be located close to each other in the chromosome of these isolates. Also, although all of the TSST-1$^+$ isolates appeared to contain the *Taq*I fragment from *seb*, none produced SEB in their culture supernatants as determined by noncompetitive ELISA (Lee *et al.*, 1991). The presence of more than one *Cla*I fragment that hybridized to the *seb* gene probe in some isolates indicates that *seb* may be present in multiple copies in these isolates. Alternatively, it could also suggest the presence of frequent RFLPs in the region of *seb*, such that in some isolates there may be an additional *Cla*I site within *seb* in the region of the 150 bp *Taq*I fragment.

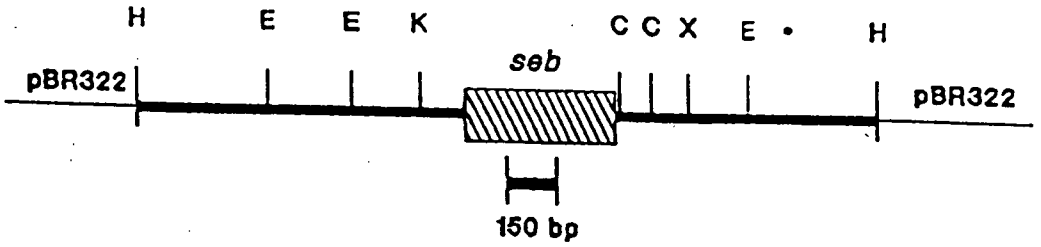


Figure 28. Restriction map of pSK155 (pBR322 carrying *seb* and SEB genetic element sequences as depicted by Johns and Khan, 1988) and the location of the 150 bp *Taq*I seb specific gene probe. H, *Hind*III; E, *Eco*RI; K, *Kpn*I; C, *Cla*I; X, *Xba*I
*junction of SEB genetic element and common chromosomal DNA as suggested by Johns and Khan (1988)

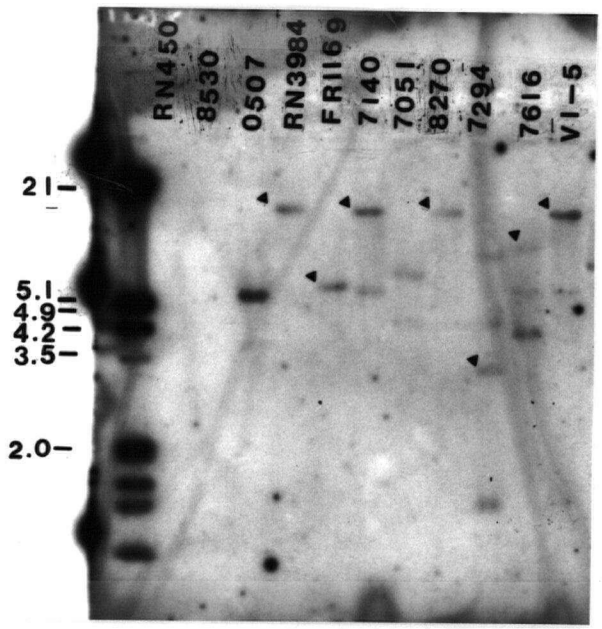


Figure 29. Southern hybridization analysis of TSST-1$^+$ (RN3984, FRI1169, 7140, 7051, 8270, 7294, 7616, VI-5) and TSST-1$^-$ (RN450, 8530, 0507) *S. aureus* isolates digested with *Cla*I and probed with a 150 bp *Taq*I *seb*-specific gene probe. 0507 is a SEB$^+$/TSST-1$^-$ strain. Numbers at left are the positions of the digoxigenin-labelled DNA molecular size standards (kb). The arrows indicate the common *Cla*I fragment that hybridized to both the 297 bp *Hinc*II-*Bam*HI *tst* probe (Figure 26) and the 150 bp *Taq*I *seb* probe in each isolate *S. aureus* 7051 did not have a common *Cla*I fragment that hybridized to both probes.

5.2.3. PCR analysis of TSST-1$^+$ isolates with oligonucleotide primers specific for *seb*

The observation that all TSST-1$^+$ isolates examined contained sequences homologous to *seb* appeared to be at variance with reports of other investigators who rarely found *seb* among TSST-1$^+$ isolates using oligonucleotide probes (Bohach *et al.*, 1989; Neill *et al.*, 1990; Johnson *et al.*, 1991). It is possible that the discrepancy in results arises from the use of probes located at different positions in *seb*. The probes used in previous studies were targeted to sequences located futher downstream in the *seb* nucleotide sequence than the 150 bp *Taq*I fragment located at nucleotide positions 40-195 of the *seb* structural gene of 798 nucleotides. To resolve this discrepancy and hopefully provide further support for the Southern hybridization results, the *seb* nucleotide primers SEB1 and SEB2, that targeted sequences downstream from the 150 bp *Taq*I fragment at positions 310-329 and 634-657 respectively of the SEB structural gene (see Figure 30) were used to perform PCR analysis on 7 of the TSST-1$^+$ isolates (as described in Materials and Methods). Two reference SEB$^+$ isolates (0507, 8528), and 3 SEB$^+$ clinical isolates (7405, 7431, 8292) served as positive controls, while 4 SEB$^-$ *S. aureus* isolates (8527, 8530, 8531, RN450) and 5 non-*S. aureus* isolates (*S. epidermidis, S. haemolyticus, S. pyogenes, B. subtilis* and *E. coli*) were used as negative controls. Even though all of the TSST-1$^+$ isolates hybridized with the 150 bp *Taq*I *seb*-specific gene probe, only 1 of these 7 isolates yielded the expected 478 bp PCR product after amplification with the SEB1 and SEB2 primers (Figure 31). Six of the seven isolates showed no product. Thus the apparent discrepancy between the present results and those of others could be explained in part by the sequence location of the *seb* oligonucleotide probes used, in conjunction with the possibility that only partial SEB structural gene sequences may

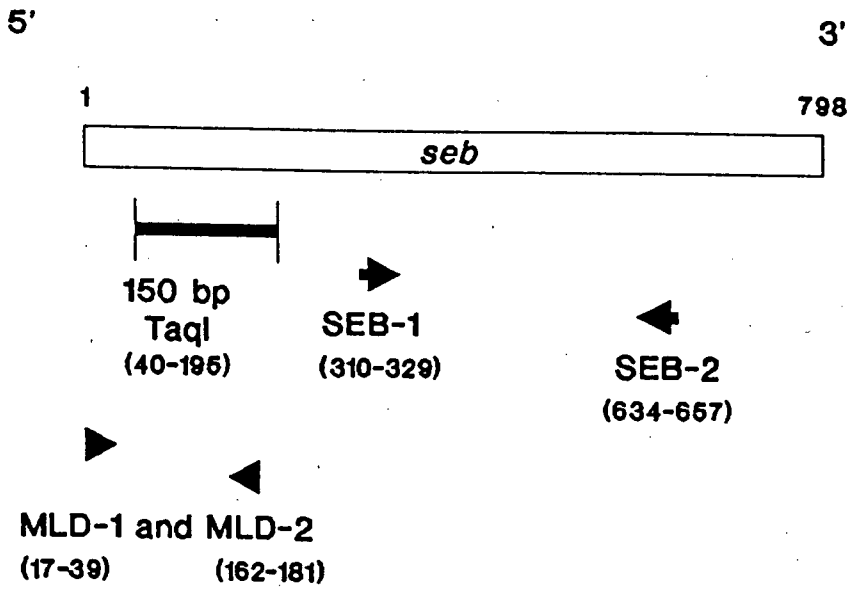


Figure 30. Location of the 150 bp *Taq*I *seb* specific gene probe used for Southern hybridization analysis and the oligonucleotide primers used for PCR analysis with respect to the nucleotide sequence of the SEB structural gene. The numbers in brackets indicate the nucleotides that comprise the probe and primers.

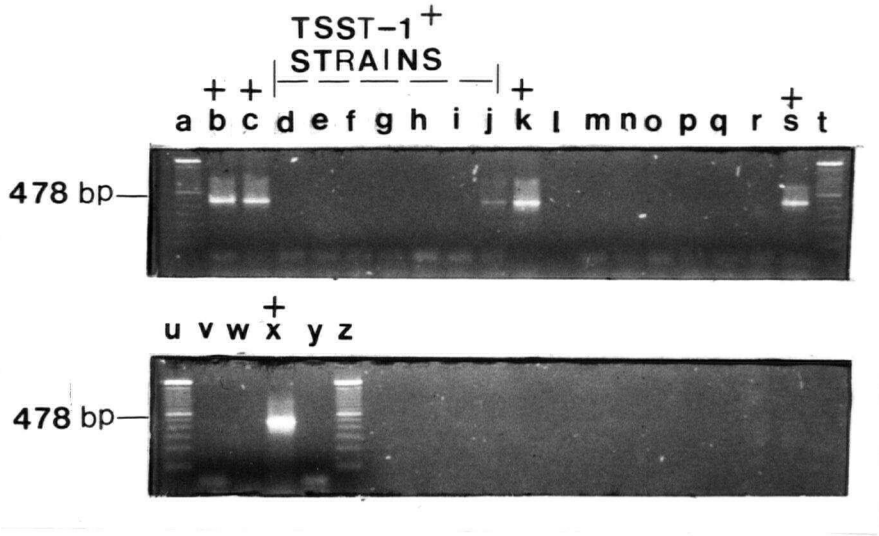


Figure 31. Agarose gel electrophoresis of PCR products after amplification using oligonucleotide primers (SEB1 and SEB2) specific for *seb* located at positions 310-329 and 634-657 respectively in the SEB nucleotide sequence (Jones and Khan, 1986).

Lanes:

a,t,u,z: 100 bp ladder

SEB$^+$ isolates
b,c,k,s,x: 0507, 8292, 7405, 8528, 7431

TSST-1$^+$ test isolates
d,e,f,g,h,i,j: RN3984, FRI1169, 7140, 7051, 8270, 7294, 7616

SEB$^-$ isolates
l,m,n,o,p,q,r,v,w: 8527, 8530, 8531, RN450, *S. pyogenes, S. epidermidis, S. haemolyticus, B. subtilis, E. coli*

y: Reagent blank

be present in the isolates studied.

To confirm the results observed with Southern hybridization analysis, primers were designed that targeted sequences in the 150 bp *TaqI* region of *seb*. Two primers designated MLD-1 and MLD-2, located at nucleotide positions 17-39 and 162-181 of the SEB structural gene (Figure 30) were used to perform PCR analysis on 7 of the 8 TSST-1$^+$ test isolates that hybridized to the 150 bp *TaqI* probe in Figure 29. Two reference SEB$^+$ isolates (0507 and 8528) and 1 SEB$^+$ clinical isolate (8292) served as positive controls, while 5 SEB$^-$ isolates (8527, 8530, 8531, 8587, 7690) served as negative controls. One non-*S. aureus* isolate (*S. pyogenes*) also served as a negative control. Even though all TSST-1$^+$ isolates hybridized to the 150 bp *TaqI* probe, none gave the expected 165 bp PCR product after amplification with the MLD-1 and MLD-2 primers (Figure 32). Although this experiment did not help to corroborate the Southern hybridization results, it indicated that the sequences targeted by either the MLD-1 or MLD-2 primer, or both, were not present in the TSST-1$^+$ isolates and suggested that sequences surrounding the 150 bp *TaqI* region in these isolates are not completely homologous to *seb*.

5.2.4. Immunoblot analysis of putative SEB$^+$/TSST-1$^+$ *S. aureus* isolates

Our laboratory had previously reported the existence of 5 putative SEB$^+$/TSST-1$^+$ isolates among 344 clinical *S. aureus* isolates studied phenotypically by ELISA (Lee *et al.*, 1992). In light of the present findings, it was of interest to reanalyse these putative SEB$^+$/TSST-1$^+$ isolates to determine if they did in fact coexpress both toxins. Immunoblot analysis of concentrated culture supernatants of the isolates revealed that in fact none of them produced both toxins; 3 produced TSST-1 only, 1 produced SEB only, and 1 produced neither TSST-1

Figure 32. Agarose gel electrophoresis of PCR products after amplification using oligonucleotide primers (MLD-1 and MLD-2) specific for *seb* located at positions 17-39 and 162-181 respectively in the SEB nucleotide sequence (Jones and Khan, 1986).
Lanes:

a,s: 100 bp ladder

SEB$^+$ isolates
b,c,q: 0507, 8528, 8292

TSST-1$^+$ test isolates
d,e,f,g,h,i,j,k: RN3984, FRI1169, 7140, 7051, 8270, 7294, 7616, VI-5

SEB$^-$ isolates
L,m,n,o,p,t: 8527, 8530, 8531, *S. pyogenes*, 8587, 7690

r: Reagent blank

nor SEB. The SEB ELISA (as described in Materials and Methods) was then repeated for these 5 isolates, and the results were consistent with the observations made upon immunoblot analysis.

5.2.5. Examination of SEB$^+$ isolates with probes derived from sequences flanking *tst*

Since all TSST-1$^+$ *S. aureus* isolates analysed were SEB$^-$, even though all hybridized with the 150 bp *Taq*I *seb* probe, it was of interest to determine whether *S. aureus* isolates that did produce SEB also contained the TSST-1 genetic element. Nine *S. aureus* isolates were chosen that were found by hybridization analysis to be *seb*$^+$, and by ELISA to be SEB$^+$ (0507, 7371, 7372, 7407, 7429, 7513, 7694, 7836, 8385). When these isolates were hybridized with the 297 Hinc*II*-Bam*HI tst* probe, no homology was evident (not shown). In addition, when these isolates were probed with a 1.0 kb *Bam*HI fragment consisting of sequences upstream of *tst* and a 890 bp *Bam*HI-*Sty*I fragment consisting of sequences downstream of *tst* (refer to Figure 25), no homology was observed (Figure 33 and 34 respectively). These two probes consist of DNA sequences within the region that has been reported to be unique to TSST-1-producing isolates (Kreiswirth *et al.*, 1989).

5.3. Discussion

The results presented here suggest that TSST-1$^+$ isolates do contain sequences that are homologous to the SEB structural gene, but none of the isolates produce SEB. In contrast, *S. aureus* isolates that do produce SEB do not contain *tst*. These observations are intriguing because it is known that little amino acid sequence homology exists between TSST-1 and SEB (Blomster-Hautamaa *et al.*, 1986; Marrack and Kappler, 1990). Furthermore, there was no cross-hybridization between the 297 bp *Hinc*II-*Bam*HI *tst*-specific probe and the

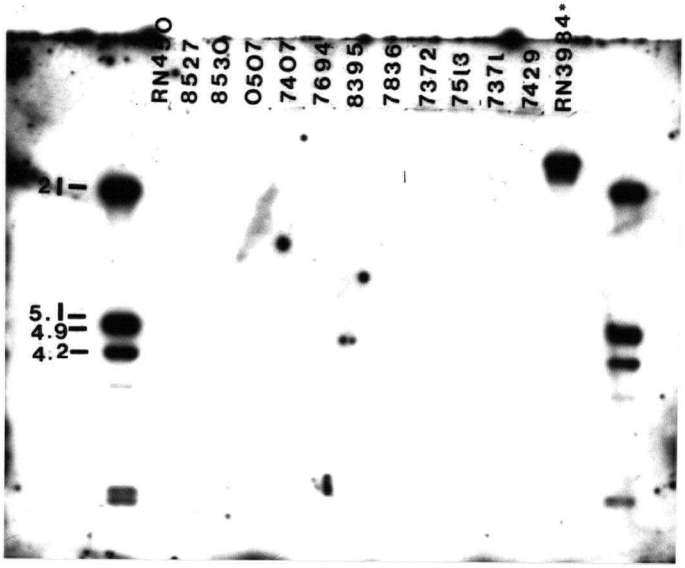


Figure 33. Southern hybridization analysis of *Cla*I digested SEB$^+$ genomic DNA probed with a 1.0 kb *Bam*HI fragment derived from pRN6550 (see Figure 25). RN450, 8527 and 8530 served as the TSST-1$^-$/SEB$^-$ control isolates. RN3984 was the TSST-1$^+$ control isolate.

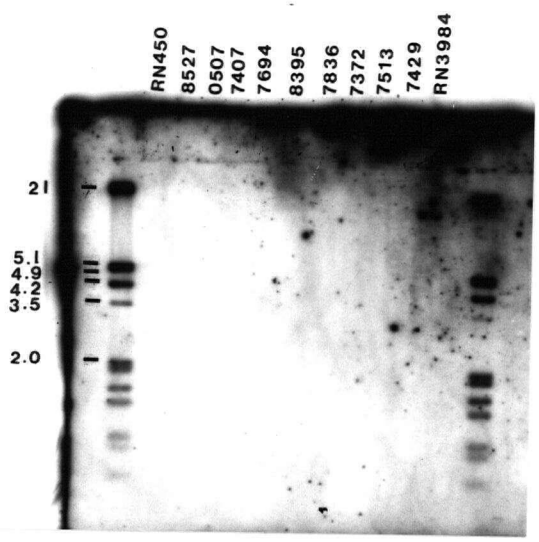


Figure 34. Southern hybridization analysis of *Cla*I digested SEB$^+$ genomic DNA probed with a 890 bp *Bam*HI-*Sty*I fragment derived from pRN6550 (see Figure 25). RN450 and 8527 served as the TSST-1$^-$/SEB$^-$ control isolates. RN3984 was the TSST-1$^+$ control isolate.

chromosomal DNA of the SEB$^+$ reference isolates (0507, 8528, Figure 26 and 27).

With relative confidence, it can be stated that these results do not appear to be due to nonspecificity of the *seb* probe for the following reasons. First, hybridization reactions were performed under conditions of high stringency so the possibility of false-positive hybridization signals was unlikely. Second, none of the control isolates that did not contain *seb* hybridized to the 150 *Taq*I probe (RN450, 8530, Figure 29). Third, if the *seb* probe hybridized nonspecifically to *tst* or the TSST-1 genetic element, it would be expected that the *tst* probe or TSST-1 genetic element probes would also show hybridization signals with the SEB$^+$ isolates. This was not observed in any of the 9 SEB$^+$ isolates examined. The possibility that the 150 bp *Taq*I *seb* probe hybridized with coexisting SEC genetic determinants (known to share amino acid homology with SEB) was unlikely because the *seb* probe did not hybridize the SEC$^+$ reference isolate 8530 (Figure 29).

The observation that 8 of 8 *Cla*I digested TSST-1$^+$ *S. aureus* isolates examined also contained SEB structural gene sequences appears at first to be at variance with results reported by other investigators. Using PCR analysis with primers located at positions 310-329 and 634-657 in the SEB structural gene, Johnson *et al.* (1991) found that 3 (11%) of 28 TSST-1$^+$ isolates contained both TSST-1 and SEB genes. Neill *et al.* (1990) did not find any evidence of *seb* in TSST-1$^+$ isolates with an oligonucleotide probe located at positions 312-329 of the *seb* structural gene sequence. Bohach *et al.* (1989), using an SEC1 probe derived from sequences internal to the gene and having 68% nucleotide sequence homology with the analogous region of *seb*, also concluded that TSST-1$^+$ isolates did not contain *seb*. It is possible that in our study various deletions within the SEB structural gene have occurred. In

an attempt to resolve this discrepancy, PCR analysis was done on the TSST-1$^+$ isolates with the same primers as those used by Johnson *et al.* (1991), located to the right of the 150 bp *Taq*I *seb* probe in Figure 30. As expected, most (6 of the 7) isolates did not show the 478 bp PCR amplification product (Figure 31). These results help to explain the incongruencies in various observations between different investigators and suggest that nucleotide sequences distal to the 150 bp *Taq*I sequence are absent in the TSST-1$^+$ isolates. The presence of a 478 bp PCR product in 1 (11%) of 7 TSST-1$^+$ isolates in this study correlated with the observed frequency (11%) of TSST-1$^+$ isolates positive for *seb* reported by Johnson *et al.* (1991) using the same olignucleotide probes and PCR technique.

Although TSST-1$^+$ isolates hybridized to the 150 bp *Taq*I *seb* probe, this region did not appear to be completely homologous with the corresponding region of *seb* since PCR primers that targeted this region failed to amplify the expected PCR product. Deletions extending to this portion of *seb* sequences in these isolates is possible. Hybridization may have occurred in these isolates since a probe of 150 bp has a better chance of hybridizing to partial sequences than the shorter PCR primers. Alternatively, these TSST-1$^+$ isolates may contain a *seb*-like gene.

The only known report that made use of a DNA probe overlapping the 150 bp *Taq*I probe used in this study stated that 0 of 37 TSST-1$^+$ isolates hybridized in colony blots with the probe (Notermans *et al.*, 1988). The reason for the discordant results between that study and this one is not immediately clear, except that only 41 bp of the 150 bp *Taq*I sequence was present in the *seb* probe used by these investigators.

Even though all TSST-1$^+$ isolates tested contained sequences homologous to *seb*, in not

one instance did these isolates produce SEB in their culture supernatants. Many investigators have reported the observation that TSST-1 and SEB are not co-produced in *S. aureus* (Crass and Bergdoll, 1986a; Crass and Bergdoll, 1986b; Garbe *et al.*, 1985; Bohach *et al.*, 1990). However, others have provided evidence that some *S. aureus* strains do produce both SEB and TSST-1 (Ewan *et al.*, 1989; Johnson *et al.*, 1991; Lee *et al.*, 1992). In fact, one of the latter reported examples was a study performed in our laboratory, in which a total of 344 *S. aureus* isolates were analysed for SEB and TSST-1 production by noncompetitive ELISA (Lee *et al.*, 1992). It was reported that expression of SEB and TSST-1 was usually mutually exclusive, in that only 5 of 344 (1.5%) isolates were found to co-produce both toxins. Upon investigation by immunoblot however, none of these 5 putative $SEB^+/TSST\text{-}1^+$ isolates contained both toxins in their culture supernatants. The fact that determination of toxin profiles via immunological analysis can lead to false positive results is well recognized (Ewald *et al.*, 1990; Johnson *et al.*, 1991).

The observations reported here overwhelmingly support the notion that expression of SEB and TSST-1 are mutually exclusive since in the study of Lee *et al.* (1992), it can now be stated that 0 of 344 *S. aureus* isolates (119 of which were $TSST\text{-}1^+$) co-produce SEB and TSST-1 (see Table 11). Due to the important role SEB and TSST-1 play in the pathogenesis of NMTSS and MTSS respectively, further studies that address the mechanisms that underlie the acquisition and expression of these toxin determinants in *S. aureus* are justified.

Table 11. TSST-1 and SEB Production among *S. aureus* Isolates.

Patient Group (no. of isolates)	TSST-1$^+$ (%)	SEB$^+$ (%)	TSST-1$^+$/ SEB$^+$ (%)
MTSS (22)	82	5	0
NMTSS (41)	68	20	0
Non-TSS (137)	22	11	0
Carrier (144)	26	10	0

MTSS, menstrual toxic shock syndrome; NMTSS, nonmenstrual toxic shock syndrome; Non-TSS, non-toxic shock syndrome-associated *S. aureus* infection; Carrier, no infection

Data compiled from Chang *et al.* (1991), Lee *et al.* (1992) and updated with the results from this study.

Chapter 6. Possible Molecular Mechanisms for Mutually Exclusive Toxin Co-Production of TSST-1 and SEB in *S. aureus*

6.1. Introduction

In the previous chapter the observed mutual exclusivity of TSST-1 and SEB co-production in *S. aureus* was verified. Up until now, the molecular basis for this exclusivity in *S. aureus* has not been investigated. Hybridization analysis presented in Chaper 5 showed that 8 of 8 TSST-1^+/SEB$^-$ isolates contained sequences homologous to the SEB structural gene. The hybridization results also revealed that *tst* and *seb* were localized to a restriction fragment of the same size in the majority of the isolates, suggesting a close proximity in the chromosome. However PCR analysis in Chapter 5 revealed that the sequences homologous to *seb* in TSST-1^+ isolates were not consistent with the presence of an entire SEB structural gene. As a result of these studies, it appeared that perhaps a novel regulatory system was acting to inhibit the expression of SEB in these TSST-1^+ isolates. In this chapter, exploration of several possibile causes for the observed mutual exclusivity was carried out. Based on the collective data, a molecular mechanism mediating the lack of SEB and TSST-1 co-production in *S. aureus* is proposed.

6.2. Results

6.2.1. Creation of a SEB$^+$/TSST-1^+ strain of *S. aureus*

Since SEB is not produced even though both *tst* and *seb* homologous sequences are found within the same isolate, it is possible that these genes may be competing for the same transcriptional activator. As one test of this hypothesis, an attempt was made to construct a

SEB$^+$/TSST-1$^+$ strain of *S. aureus*. If TSST-1 and SEB compete for a positive regulator, then TSST-1 and SEB co-expression would not be anticipated to occur in the constructed strain. pMLD6876-1 (a plasmid that contained *seb*) and pJW1 (a recombinant *B. subtilis* plasmid which contained *tst*) were introduced into the restriction-deficient, nonenterotoxigenic strain of *S. aureus* RN4220 as described in Materials and Methods. This resulted in the production of both TSST-1 and SEB in 5 of 5 RN4220 transformants tested by immunoblot as described in Materials and Methods (see Figure 35). The location of the band for SEB in Figure 35 at approximately 14 kD as opposed to its molecular mass of 28 kD is presumably due to the presence of "nicked" protein in the commercial source used, as has been previously described (Grossman *et. al.*, 1990). These results suggest that lack of TSST-1 and SEB co-production in *S. aureus* is not mediated by competition for a common transcriptional activator.

6.2.2. Analysis of TSST-1$^+$ isolates with probes derived from sequences flanking the SEB structural gene and located within the SEB genetic element

Previously, TSST-1$^+$ isolates studied were found to contain *seb* homologous sequences by Southern hybridization analysis with a 150 bp *Taq*I *seb*-specific gene probe (Figure 29). In addition, the *tst* and *seb*-specific gene probes hybridized to the same sized *Cla*I restriction fragment in these isolates, which indicated that the *tst* and *seb* genes were located near one another in the chromosome. To seek further evidence for the presence of SEB genetic element DNA in TSST-1$^+$ *S. aureus* isolates, Southern hybridization analysis using two additional probes derived from sequences upstream of *seb* was performed on the same 8 ClaI digested TSST-1$^+$ genomic DNA examined previously. All SEB$^+$ isolates are highly

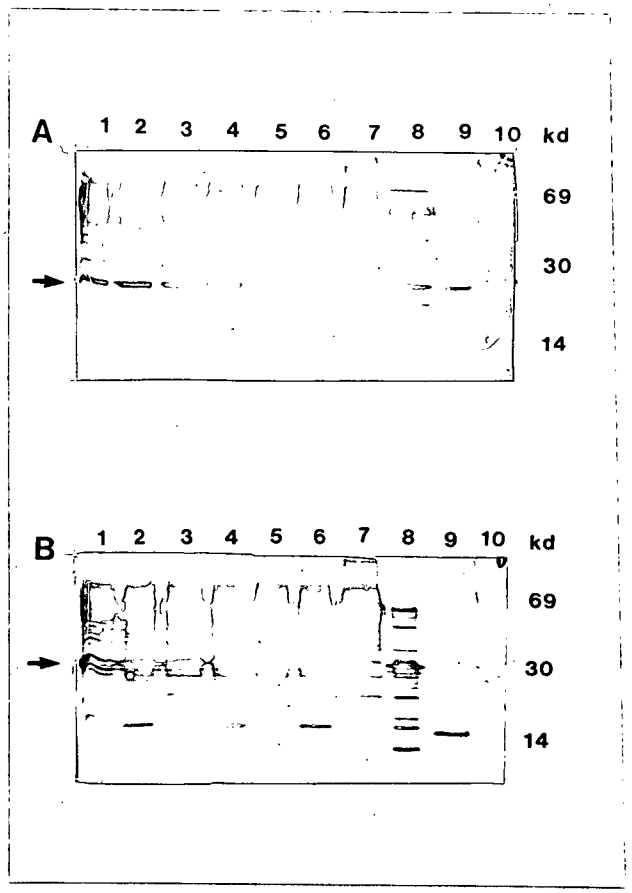


Figure 35. Immunoblot analysis of culture filtrates of Cm^r/Em^r transformants of RN4220 run on SDS-PAGE and blotted with a polyclonal anti-TSST-1 antibody (A) or a polyclonal anti-SEB antibody (B). Arrows indicate the postions of TSST-1 and SEB.

Lane(s) #

1-5: Cm^r/Em^r transformants of RN4220
6: RN4220 (pJW1) and RN4220 (pMLD6876-1) in A and in B respectively
7: RN4220
8: FRI1169 ($TSST\text{-}1^+$ control) and 0507 (SEB^+ control) in A and in B respectively
9: TSST-1 (in A) and SEB (in B) standards (Toxin Technology)

The numbers on the right indicate the positions of the molecular mass standards (kd) as determined by separating the Bio-Rad Kaleidoscope Pre-stained molecular size standards in Lane 10.

homologous in a 26.8 kb region around *seb* (termed the SEB genetic element; Johns and Khan, 1988), and the upstream probes were located in this region. The membrane that was used for the experiment described in Figure 26 was washed to remove the probe and then reprobed with the 700 bp *Eco*RI fragment of pSK155 (Figure 36) that consisted of sequences located approximately 1.1 kb upstream of *seb*. In a separate experiment, *Cla*I digested TSST-1$^+$ genomic DNA was probed with a 1.1 kb *Hin*dIII-*Eco*RI fragment located further upstream of the 700 bp *Eco*RI probe but still within the SEB genetic element (Figure 36).

The specificity of these probes was first evaluated. The 700 bp *Eco*RI SEB genetic element probe hybridized with a strong signal to *Cla*I digested RN450, a TSST-1$^-$/SEB$^-$ reference isolate cured of all known prophages (Murphy *et al.*, 1981). Faint signals were also observed with two other SEB$^-$ reference isolates (8527, 8530, Figure 37). Similiarly, the 1.1 kb *Hin*dIII-*Eco*RI probe hybridized (albeit faintly) with RN450 and 8527 (Figure 38). Hybridization of a SEB genetic element probe to DNA from SEB$^-$ cells was not totally unexpected since Johns and Khan (1988) had previously reported that some SEB$^-$ isolates may contain an incomplete or related SEB genetic element.

The most significant findings were that both flanking SEB genetic element probes hybridized to *Cla*I digested TSST-1$^+$ chromosomal DNA, indicating that TSST-1$^+$ isolates also contain sequences that are homologous to the SEB genetic element. More importantly, in 6 of the 8 TSST-1$^+$ isolates, the 700 bp *Eco*RI fragment (Figure 37) and the 1.1 kb *Hin*dIII-*Eco*RI fragment (Figure 38) hybridized to a *Cla*I fragment that was similiar in size to the fragment that hybridized to the 297 bp *Hin*cII-*Bam*HI *tst* probe shown in Figure 26. Only in isolates 7294 and 7616 was the *Cla*I fragment that hybridized to the SEB genetic

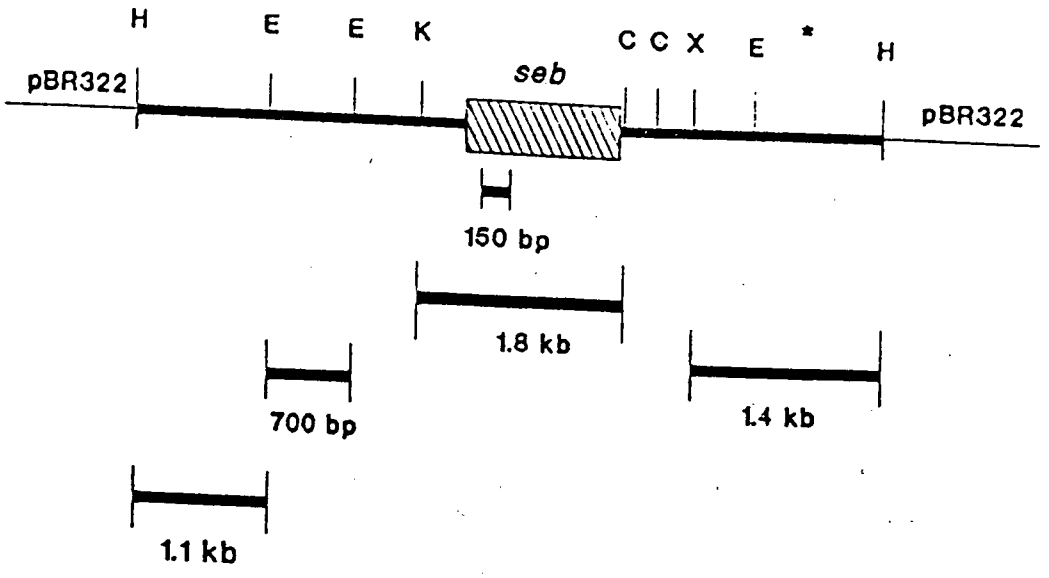


Figure 36. Restriction map of pSK155 [as depicted by Johns and Khan (1988)] and location of fragments used as probes.
H, *Hin*dIII; E, *Eco*RI; K, *Kpn*I; C, *Cla*I; X, *Xba*I
*, junction of SEB genetic element and common chromosomal DNA as suggested by Johns and Khan (1988)

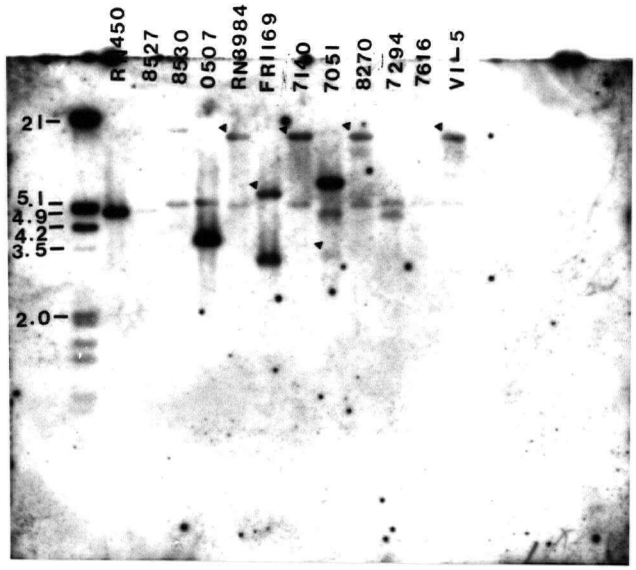


Figure 37. Southern hybridization analysis of TSST-1$^+$ *S. aureus* isolates with a 700 bp *Eco*RI SEB genetic element probe. Genomic DNA was digested with *Cla*I and hybridized with a 700 bp *Eco*RI fragment of pSK155 (see Figure 36). RN450, 8527 and 8530 are TSST-1$^-$/SEB$^-$ control isolates. Numbers at left indicate the positions of the digoxigenin-labelled DNA molecular size standards (kb). The arrows indicate the common sized *Cla*I fragment that hybridized to the 297 bp *Hinc*II-*Bam*HI *tst* probe in Figure 26. Isolates 7294 and 7616 did not have a common *Cla*I fragment that hybridized to both the tst and the 700 bp EcoRI probe.

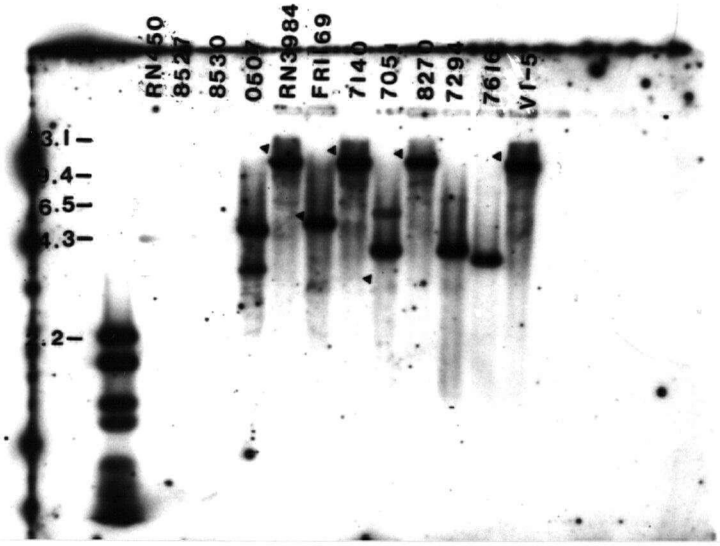


Figure 38. Southern hybridization analysis of TSST-1$^+$ *S. aureus* isolates with a 1 1. kb *Hin*dIII-*Eco*RI SEB genetic element probe. Genomic DNA was digested with *Cla*I and hybridized with a 1.1. kb *Hin*dIII-*Eco*RI fragment of pSK155 (see Figure 36). RN450, 8527 and 8530 are TSST-1$^-$/SEB$^-$ control isolates. Numbers at left indicate the positions of the digoxigenin-labelled DNA molecular size standards (kb). The arrows indicate the common sized *Cla*I fragment that hybridized to the 297 bp *Hin*cII-*Bam*HI *tst* probe in Figure 26 and the 700 bp *Eco*RI probe in Figure 37. Isolates 7294 and 7616 did not have a common *Cla*I fragment that hybridized to these probes.

element probes different in size from the fragment that hybridized with the *tst* probe (Figures 26, 37, and 38), perhaps due to RFLPs in the region upstream of *seb* in these two isolates.

To further examine the observation that *tst* and the SEB genetic element sequences are located close to each other, *Hin*dIII digested genomic DNA of the TSST-1$^+$ isolates were hybridized with the 297 bp *Hin*II-*Bam*HI *tst*-specific probe (Figure 27) and the 1.1 kb *Hin*dIII-*Eco*RI SEB genetic element probe (Figure 39). The specificities of the SEB genetic element probes were further assessed by including three SEB$^-$ isolates (7660, 8531, 8297) and three non-*S. aureus* isolates (*S. epidermidis* ATCC 14990, *S. haemolyticus* ATCC 29970, *B. subtilis* ATCC 6633) as negative controls. There was no homology between the 1.1 kb *Hin*dIII-*Eco*RI SEB genetic element probe and the three non-*S. aureus* isolates. As seen in earlier results in which *Cla*I digested SEB$^-$ isolates had weak hybridization signals with the 1.1 kb *Hin*dIII-*Eco*RI probe (RN450, 8527 and 8530 in Figure 38), the three SEB$^-$ isolates 7660, 8531 and 8297 also had a faint hybridization signal to the probe (Figure 39), again perhaps due to the presence of an incomplete or related SEB genetic element. More importantly, as can be observed in Figures 27 and 40, similiar sized *Hin*dIII restriction fragments of RN7043, FRI1169, and 7140 hybridized to both the *tst* and the 1.1 kb *Hin*dIII-*Eco*RI SEB genetic element probes. Isolates RN3984, 7051, and 7616 did not show this result likely because of incomplete digestion of chromosomal DNA, since a subsequent experiment did confirm that the *tst* and the 1.1 kb *Hin*dIII-*Eco*RI probes hybridized to a *Hin*dIII fragment of a similiar size in isolates RN3984 and 7051 (not shown). The fact that hybridization data with *Cla*I and *Hin*dIII localized *tst* and SEB genetic element DNA to similiar-sized restriction fragments provides further evidence that the TSST-1 gene is located

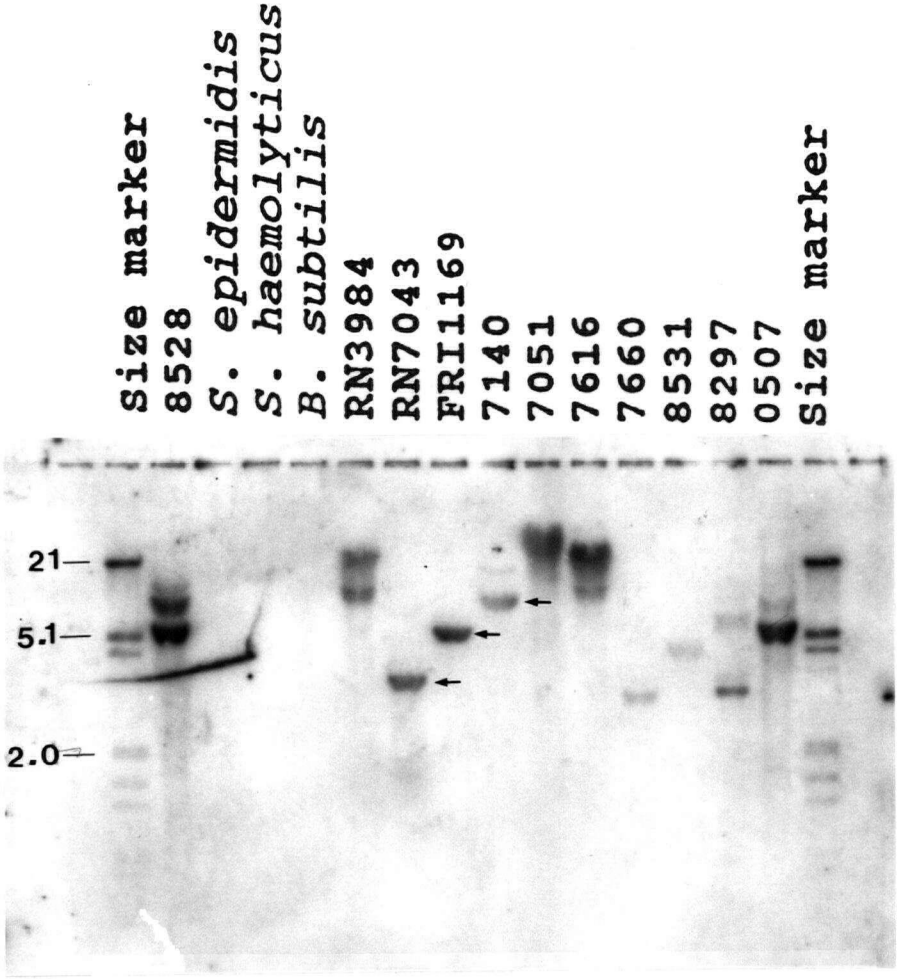


Figure 39. Southern hybridization analysis of TSST-1$^+$ isolates digested with *Hin*dIII and hybridized with a 1.1 kb *Hin*dIII-*Eco*RI fragment of pSK155. *S. aureus* 8528 and 0507 served as the SEB-producing positive control isolates. *S. aureus* 7660, 8531, 8297 and *S. epidermidis*, *S. haemolyticus*, and *B. subtilis* served as the non-SEB-producing negative control isolates. Arrows indicate the position of the common *Hin*dIII fragment that also hybridized to the 297 bp *Hin*cII-*Bam*HI *tst* probe in Figure 26. The higher migrating fragments observed in isolates RN3984 and 7616 are the result of incomplete digestion of chromosomal DNA. The DNA of 7051 was degraded. Numbers at left are the positions of the digoxigenin-labelled DNA molecular size standards (kb).

near SEB genetic element sequences in these isolates.

6.2.3. Transduction of RN7043 [*tst::Tc*^{r}] into *S. aureus* 7690

One possible mechanism by which TSST-1 and SEB co-production could be mutually excluded in *S. aureus* is if the mobile TSST-1 genetic element inserts into the SEB genetic element to cause deletion or interruption of SEB structural gene sequences. This would explain the partial *seb* sequences and the close proximity of the *tst* and SEB genetic element sequences. In an attempt to determine if this could occur, the *tst::*Tc^r cassette of RN7043 (described in Materials and Methods) was transduced into strain 7690 as described in Materials and Methods. 7690 was a putative SEB$^+$/TSST-1$^-$ clinical isolate of *S. aureus* previously characterized by immunoassay by Lee et al. (1992). Selection of transductants for resistance to tetracycline assured that the TSST-1 genetic element of RN7043 had been successfully introduced into 7690. During the subsequent screening of Tc^r transductants of 7690 for loss of SEB production by immunoassay, it was found that the wild-type 7690 did not produce SEB, despite its original designation as a SEB-producer. Lack of SEB production in 7690 was subsequently verified by immunoblotting (as described in Materials and Methods), and absence of *seb* was verified by performing PCR analysis with the SEB1/SEB2 and the MLD-1/MLD-2 primers (see Figure 32) as described in Materials and Methods. In light of these results, it was no longer possible to examine whether the TSST-1 genetic element, when introduced into a SEB-producing strain, could mediate deletion of *seb* coding sequences to result in loss of SEB production. This experiment did provide both corroborating and enlightening results however. Successful transduction of the TSST-1 genetic element into 7690 was verified by hybridization of *Cla*I digested genomic DNA of

five Tc^r transductants with the 297 bp *Hin*cII-*Bam*HI *tst* probe (Figure 40; Lanes 1-5). Two well characterized TSST-1$^-$ reference isolates (RN450, 8528) served as negative controls, and as expected, did not hybridize with the *tst* probe, whereas the TSST-1$^+$ control isolate FRI1169 did. In addition, lack of *tst* in 7690 was verified.

Hybridization of the Tc^r isolates with the 700 bp *Eco*RI probe derived from SEB genetic element sequences (see Figure 41) corroborated previous observations that *tst* was located near SEB genetic element sequences in TSST-1$^+$ isolates, since transduction of the tst::Tc^r cassette of RN7043 into 7690 resulted in the transfer of 700 bp *Eco*RI SEB genetic element sequences on a fragment of the same size as the *Cla*I fragment that hybridized to the *tst* probe (indicated by the arrow in Figure 41; compare with Figure 40). Interestingly, the genomic DNA of all strains, including wild-type 7690, appeared to contain 700 bp *Eco*RI-related sequences, despite the fact that this isolate is SEB$^-$. Pre-existing 700 bp *EcoRI* related sequences were also observed in the nonenterotoxigenic strain RN450, and is consistent with a previous result in this study (Figure 37). Other SEB$^-$ isolates that hybridized to the 700 bp *Eco*RI probe included 8527 and 8530 (Figure 37). As mentioned previously, it is believed that the 700 bp *Eco*RI sequences in these SEB$^-$ isolates may represent an incomplete or related genetic element (Johns and Khan, 1988).

Finally, hybridization of the tst::Tc^r transductants of 7690 with the 1.1 kb *Hin*dIII-*Eco*RI SEB genetic element probe provided yet further evidence that SEB genetic element sequences are located close to *tst* since these sequences were also transduced along with the tst::Tc^r cassette into 7690. The 1.1 kb *Hin*dIII-*Eco*RI fragment hybridized to a fragment of the same size as the *Cla*I fragment that hybridized to *tst* and 700 bp *Eco*RI sequences in the Tc^r

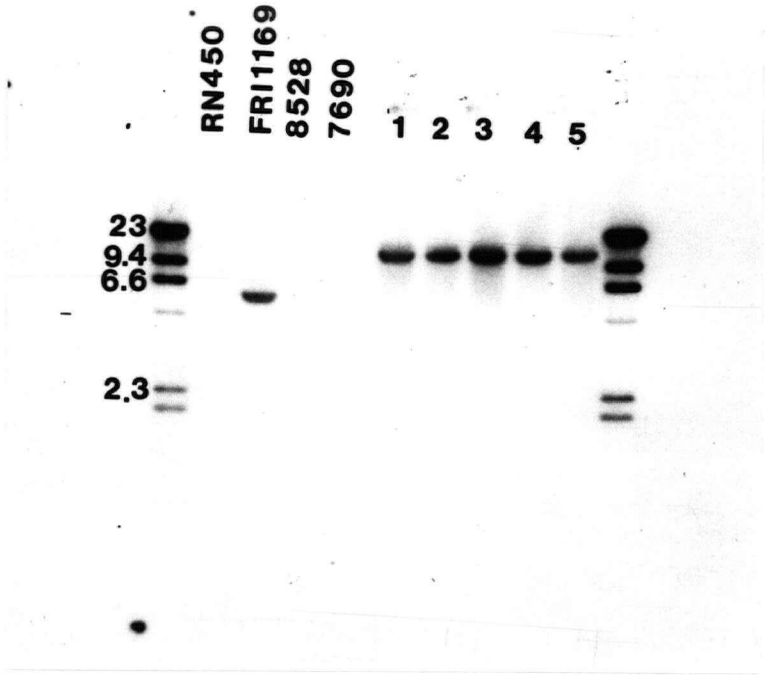


Figure 40. Southern hybridization analysis of Tc^r transductants of *S. aureus* 7690 with a 297 bp *Hinc*II-*Bam*HI *tst*-specific gene. The tst::Tc^r cassette of RN7043 was transduced into 7690 as described in Materials and Methods. Five Tc^r transductants were digested with *Cla*I and hybridized with the *tst* gene probe (Lane 1-5). FRI1169 served as the TSST-1^+ control isolate. RN450, 8528 and 7690 were the TSST-1^- control isolates. Numbers at left indicate the postions of the digoxigenin-labelled DNA molecular size markers (kb).

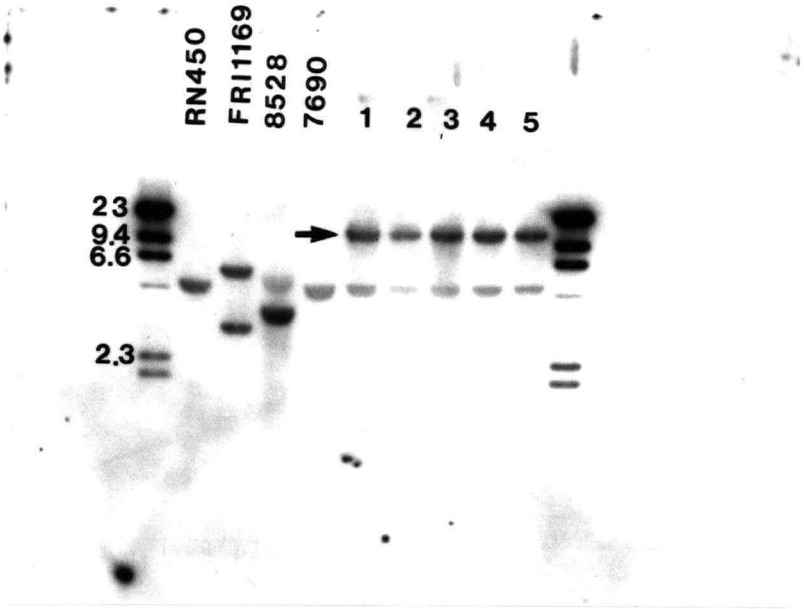


Figure 41. Southern hybridization analysis of Tc^r transductants of *S. aureus* 7690 with a 700 bp *Eco*RI fragment of pSK155 (see Figure 36). Five Tc^r transductants were digested with *Cla*I and hybridized with the 700 bp *Eco*RI probe. The arrow indicates the common-sized *Cla*I fragment that also hybridized with the 297 bp *Hinc*II-*Bam*HI *tst* gene probe in Figure 40. Numbers at left indicate the positions of the digoxigenin-labelled DNA molecular size standards (kb).

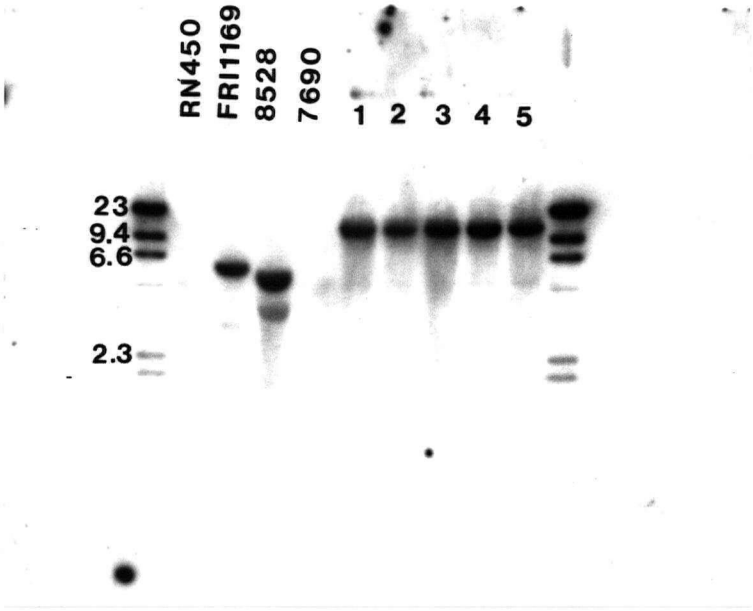


Figure 42. Southern hybridization analysis of Tc^r transductants of *S. aureus* 7690 with a 1.1 kb *Hin*dIII-*Eco*RI fragment of pSK155 (see Figure 36). Five Tc^r transductants were digested with *Cla*I and hybridized with the 1.1 kb *Hin*dIII-*Eco*RI probe. The *Cla*I fragment that hybridized to this probe appeared to be the same fragment that also hybridized with the 297 bp *Hinc*II-*Bam*HI *tst* probe (Figure 40) and the 700 bp *Eco*RI probe (Figure 41). Numbers at left indicate the positions of the digoxigenin-labelled DNA molecular size standards (kb).

transductants (Figure 42).

Although wild-type 7690, RN450, 8527 and 8530 contained sequences that were homologous to the 700 bp *Eco*RI SEB genetic element probe (Figures 37 and 41), these isolates do not contain the 150 *Taq*I fragment of *seb*. These SEB genetic element-related sequences in these isolates could represent the presence of a related or incomplete SEB genetic element. The fact that extremely weak hybridization signals were evident upon analysis of these strains with the 1.1 kb *Hin*dIII-*Eco*RI probe (Figures 38 and 42) provides further support for the supposition that these SEB genetic element-related sequences do not represent a complete copy of the SEB genetic element.

6.2.4. Analysis of SEB$^+$ isolates with the 700 bp EcoRI fragment derived from sequences located within the SEB genetic element

Two *Cla*I fragments that hybridized to the 700 bp *Eco*RI SEB genetic element probe have been observed in 7 of the 8 TSST-1$^+$ isolates (Figure 37) and the SEB$^+$ reference isolates 0507 (Figure 37) and 8528 (Figure 41). Although homologous sequences to the SEB genetic element have been observed in TSST-1$^-$/SEB$^-$ isolates such as RN450, 8527, 8530 and 7690 (Figures 37, 38, 41 and 42), the duplication was not. Since only two SEB$^+$ isolates were examined with the 700 bp *Eco*RI probe, Southern hybridization analysis was performed on several additional SEB$^+$ isolates with the 700 bp *Eco*RI probe to determine if multiple *Cla*I fragments that hybridized to this probe are as common in SEB$^+$ isolates as in TSST-1$^+$ isolates. As observed in Figure 43, 8 of 8 SEB$^+$ isolates in addition to the reference isolate 0507 revealed two *Cla*I fragments having homology to the probe. Duplication of SEB genetic element sequences among SEB$^+$ *S. aureus* isolates has been observed previously

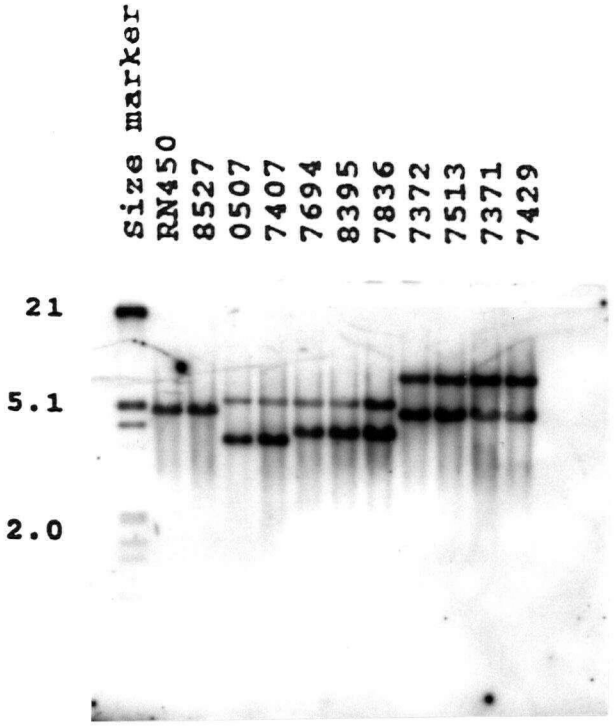


Figure 43. Southern hybridization analysis of SEB$^+$ isolates with a 700 bp *Eco*RI SEB genetic element probe. Genomic DNA was digested with *Cla*I and hybridized with the 700 bp *Eco*RI fragment of pSK155. RN450 and 8527 are SEB$^-$ control isolates. Numbers at left indicate the postions of the digoxigenin-labelled DNA molecular size standards (kb).

(Johns and Khan, 1988). Consistent with previous results (Figures 37 and 41), the TSST-1$^-$ /SEB$^-$ isolate RN450 and 8527 revealed only one *Cla*I fragment with homology to the 700 bp *Eco*RI probe.

6.2.5. Analysis of SEB$^+$ isolates with *seb*-specific probes

To identify which *Cla*I fragment in the SEB$^+$ isolates that hybridized to the 700 bp *Eco*RI probe in Figure 43 also contained *seb*, the *Cla*I digested genomic DNA of the SEB$^+$ isolates were hybridized with the 150 bp *Taq*I *seb* probe. As observed in Figure 44, 4 of the 9 SEB$^+$ isolates had *seb* located on the *Cla*I fragment of ~8.5 kb in size (7372, 7513, 7371, 7429) whereas 5 of the 9 SEB$^+$ isolates examined had both *Cla*I fragments exhibiting homology to the 150 bp *Taq*I probe (0507,7407,7694,8395,7836). This indicated that two copies of *seb* may be present in these isolates. The observation that the smaller *Cla*I fragment (~3.7 or 4.1 kb) in these isolates had a fainter hybridization signal to the 150 bp *Taq*I probe than the larger *Cla*I fragment (~6.1 kb) may indicate that these sequences contain only a part of the 150 bp *Taq*I fragment.

Based on the results in Figures 43 and 44, a depiction of the region surrounding *seb*, in addition to the molecular sizes of the *Cla*I fragments that hybridized to the probes can be proposed as shown in Figure 45. The location of the second *Cla*I fragment in the chromosomes of these isolates with respect to the SEB genetic element is not known. It could be possible that the second *Cla*I fragment is located within the 26.8 kb region associated with *seb*. In addition, it should be noted that two 150 bp *Taq*I homologous sequences could be present in the SEB$^+$ isolates 7372, 7513, 7371 and 7429 (Figure 44 and Figure 45), but the sequences may be located on the same *Cla*I fragment that contains the

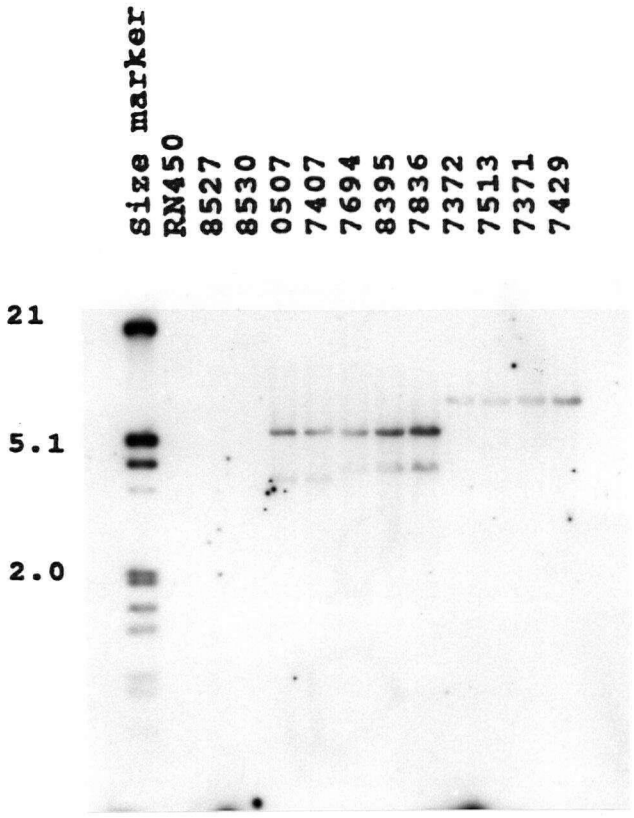
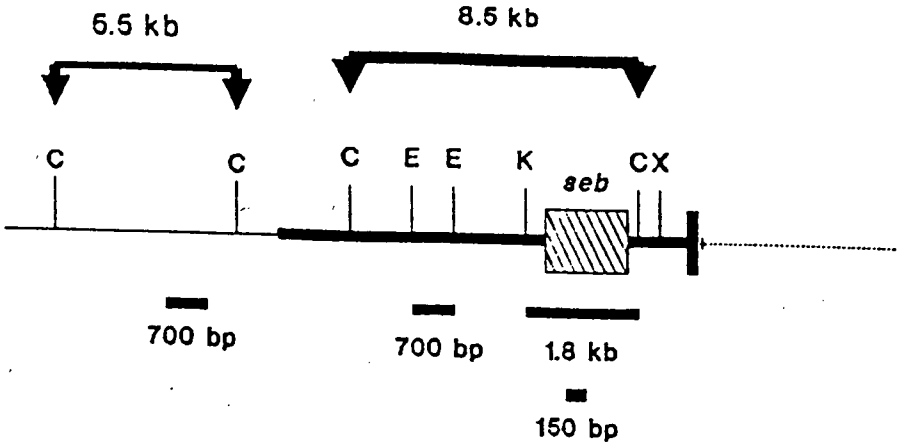


Figure 44. Southern hybridization analysis of SEB$^+$ isolates with a 150 bp *Taq*I *seb* specific gene probe. Genomic DNA was digested with *Cla*I and hybridized with the 150 bp *Taq*I fragment of pSK155. RN450, 8527 and 8530 are SEB$^-$ control isolates. Numbers at left indicate the positions of the digoxigenin-labelled DNA molecular size standards (kb).

A



AND

B

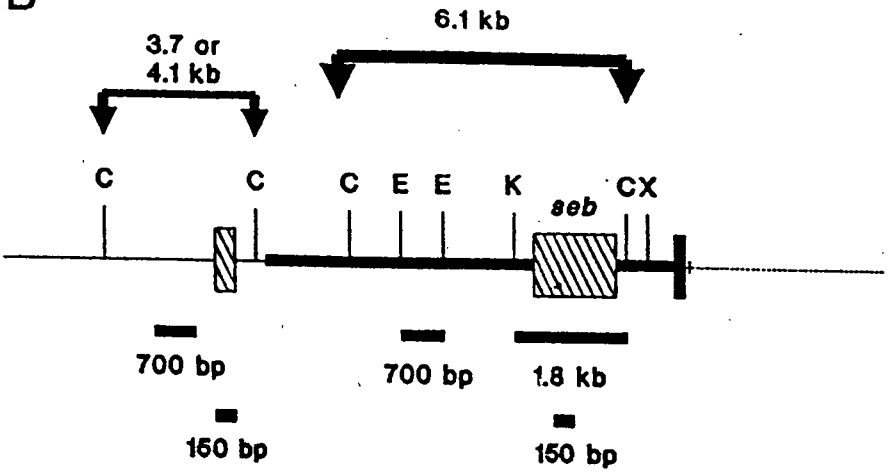


Figure 45. Illustration of the chromosomal region surrounding *seb* in *S. aureus* isolates. 'A' depicts the region surrounding *seb* in isolates 7372, 7513, 7371 and 7429 (see Figures 43 and 44). 'B' depicts the region surrounding *seb* in isolates 0507, 7407, 7694, 8395 and 7836 (see Figures 43 and 44). The SEB genetic element (thick line) with the restriction sites are shown as described by Johns and Khan (1988). The probes that were found to hybridize to each restriction fragment are shown below the SEB genetic element, and the sizes of the corresponding *Cla*I fragments that hybridized to these probes are indicated above the SEB genetic element. The hatched box represents *seb* sequences. C, *Cla*I; E, *Eco*RI, K, *Kpn*I, X, *Xba*I

complete structural gene and thus only one fragment hybridizing to the 150 bp *Taq*I probe would be observed.

Analysis of 32 SEB$^+$ isolates with a 1.8 kb *Kpn*I-*Cla*I probe (see Figure 36) that contained SEB structural gene sequences in addition to flanking sequences gave complete concordance between the size of the *Cla*I fragment that hybridized to the 1.8 kb *Kpn*I-*Cla*I probe and the 150 bp *Taq*I probe in each of the 8 SEB$^+$ isolates in Figure 44 as would be expected. However, in contrast to the results observed with the 150 bp *Taq*I probe (Figure 44), multiple *Cla*I fragments hybridizing to the 1.8 kb KpnI-ClaI probe were not observed in isolates 0507, 7407, 7694, 8395 and 7836. Results for 0507, as shown in Figure 46, are representative for these isolates. This observation supports the previous contention that the smaller ClaI fragment (3.7 or 4.1 kb) in these isolates may contain only partial *seb* sequences, since if two complete SEB coding sequences were present, the 1.8 kb *Kpn*I-*Cla*I probe should hybridize to both, yielding two *Cla*I fragments upon Southern blotting. The observation that the 150 bp *Taq*I probe hybridized to two *Cla*I fragments indicates that the smaller *Cla*I fragment in these isolates contained only partial SEB coding sequences. As was the case with TSST-1$^+$ isolates, these observations suggest that SEB$^+$ isolates appear to contain partial SEB coding sequences.

6.2.6. Analysis of SEB$^+$ isolates with a probe derived from sequences that extend past the junction of the SEB genetic element downstream of seb

The results in section 6.2.5 indicate that TSST-1$^+$ and SEB$^+$ strains have a number of DNA similiarities based upon Southern hybridization patterns with SEB genetic element probes. First, in both types of strains, *tst*, *seb* and SEB genetic element sequences appear to

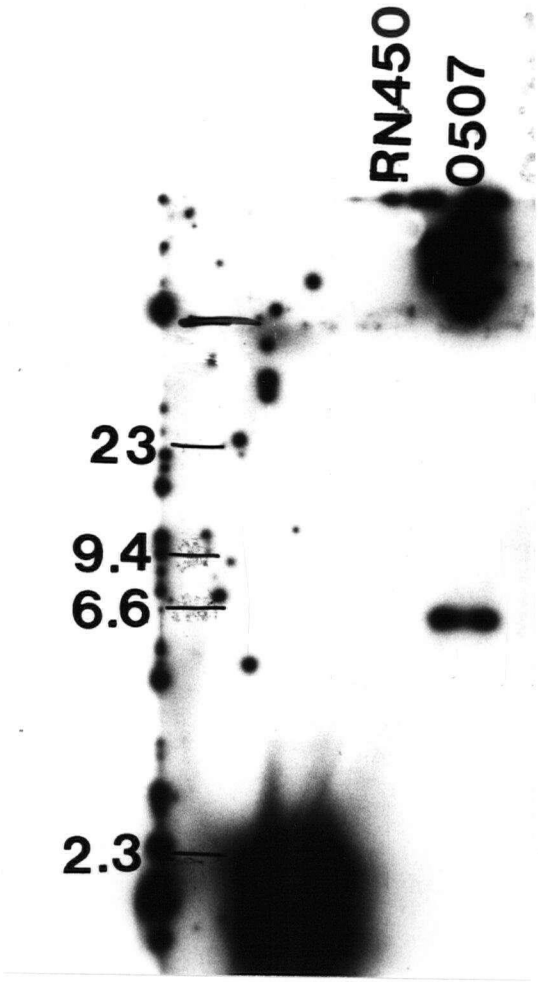


Figure 46. Southern hybridization analysis of the SEB$^+$ isolate 0507 with a 1.8 kb *Kpn*I-*Cla*I *seb* gene probe (see Figure 36). 0507 was digested with *Cla*I and hybridized with a 1.8 kb *Kpn*I-*Cla*I fragment derived from pSK155. RN450 is the SEB$^-$ control isolate. Numbers at left indicate the postions of the digoxigenin-labelled DNA molecular size standards (kb).

be located close to one another in the chromosome. Second, TSST-1$^+$ and SEB$^+$ isolates have multiple *Cla*I fragments that hybridize to the 700 bp *Eco*RI SEB genetic element probe, and both appear to have partial SEB coding sequences. TSST-1$^-$/SEB$^-$ isolates do not contain multiple *Cla*I fragments with homology to the 700 bp *Eco*RI probe, or 150 bp *Taq*I sequences. One possibility to explain these observations is that the TSST-1 and SEB structural genes are on similiar or closely related genetic elements. Alternatively, the TSST-1 genetic element may have a preferred site of insertion in the SEB genetic element. If this were true, DNA similiarities upon hybridization with SEB genetic probes would be expected. One important difference in the characteristics of genetic elements carrying TSST-1 and SEB is their reported mobility. The TSST-1 genetic element is mobile (Chu *et al.*, 1988) whereas the SEB genetic element has been found in only one chromosomal location (Johns and Khan, 1988). To further support either of the two hypotheses, I searched evidence to suggest that the SEB genetic element is mobile.

The DNA of 9 SEB$^+$ isolates analysed in Figure 44 were digested with *Kpn*I and hybridized with the 1.4 kb *Xba*I-*Hin*dIII SEB genetic element junction probe (see Figure 36). *Kpn*I cuts within the SEB genetic element and to the right past the end of the suggested junction of the element (see Figure 36; Johns and Khan, 1988). If the SEB genetic element is located in more than one chromosomal location, *Kpn*I fragments of different sizes would be expected to hybridize to the 1.4 kb *Xba*I-*Hin*dIII probe. As observed in Figure 47, three *Kpn*I fragments of different sizes were found to hybridize to the probe in the 9 SEB$^+$ isolates. The observed heterogeneity in *Kpn*I fragments indicate that the SEB genetic element is present either in a region of frequent RFLPs, or in different chromosomal

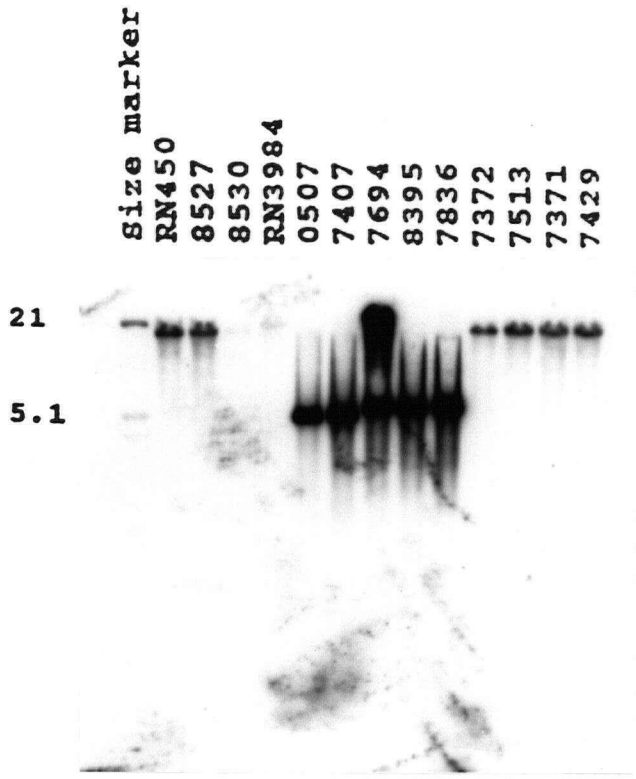


Figure 47. Southern hybridization analysis of SEB$^+$ *S. aureus* isolates with a 1.4 kb *Xba*I-*Hin*dIII SEB junction element probe (see Figure 36). Genomic DNA was digested with *Kpn*I and hybridized with a 1.4 kb *Xba*I-*Hin*dIII fragment derived from pSK155. RN450, 8527, 8530 and RN3984 are SEB$^-$ control isolates. The high molecular weight fragment that hybridized with the probe in isolate 7694 is presumed to be due to incomplete digestion of DNA. Numbers at left indicate postions of digoxigenin-labelled DNA molecular size standards (kb).

locations.

6.3. Discussion

Lack of TSST-1 and SEB co-production in *S. aureus* is an interesting phenomenon. Other infrequently observed phenotypic combinations that have been identified in *S. aureus* include hemolysin and TSST-1 production (Chow *et al.*, 1983) and staphylokinase and ß-lysin production (Winkler *et al.*, 1965). The molecular mechanisms mediating these have been fully characterized (Coleman *et al.*, 1986; O'Reilly *et al.*, 1990). Studies addressing the mechanisms that underly the mutually exclusive production of TSST-1 and SEB however have been limited due to uncertainties surrounding the nature of the genetic elements that carry both toxin determinants. In spite of these uncertainties, a number of observations have been made in this study that allow one to both rule out and extend certain possibilties. First, one putative explanation for the lack of TSST-1 and SEB co-production in *S. aureus* is that they may be competing for the same transcriptional activator. This is reasonable based on the fact that both TSST-1 and SEB are under control of the *agr*. The *agr* is a locus that is known to control the expression of a number of exoprotein genes in *S. aureus* (Kornblum *et al.*, 1990). However construction of a strain producing both TSST-1 and SEB argues against this possibility.

It has been noted that TSS-associated strains of *S. aureus* are phenotypically distinct from other *S. aureus* strains. For example, TSS-associated strains of *S. aureus* are more proteolytic than non-TSS-associated *S. aureus* strains (Barbour, 1981; Todd *et al.*, 1984). This observation has been used to suggest that the TSST-1 genetic element could encode a protease that regulates the SEB product. The ability to create a TSST-1$^+$/SEB$^+$ strain does

not specifically rule out this hypothesis since the *tst*-containing plasmid pJW1 used in the construct did not contain the entire TSST-1 genetic element. pJW1 contained only approximately 1.6 kb of the ~4-7 kb sequences associated with the TSST-1 genetic element. To determine if protease regulation is involved, a plasmid containing the entire TSST-1 genetic element could be introduced into RN4220 containing SEB-producing pMLD6876-1, and screened for TSST-1 and SEB co-production. The plasmid pRN6100, which contains the original cloned TSST-1 gene on a 10.6 kb chromosomal fragment (Kreiswirth *et al.*, 1983) would be ideal for this purpose. The ability to create a TSST-1$^+$/SEB$^+$ *S. aureus* strain using a plasmid containing the entire TSST-1 genetic element would help to determine if posttranslational modification of SEB can occur through the expression of a protease.

Southern hybridization analysis of TSST-1$^+$ isolates revealed that *tst*, *seb*, and SEB genetic element probes hybridized to the same size *Cla*I digested DNA fragments in the majority of isolates. These observations indicate that both TSST-1 and SEB-related genetic element sequences may be located close to one another in the chromosome. In addition, a second restriction enzyme, *Hin*dIII, localized *tst* and the 1.1 kb *Hin*dIII-*Eco*RI fragment of the SEB genetic element to a common-sized fragment. Moreover, transduction of the TSST-1 genetic element from the TSST-1$^+$ strain RN7043 into a TSST-1$^-$/SEB$^-$ strain of *S. aureus* (7690) resulted in the transfer of SEB genetic element sequences on the same *Cla*I fragment carrying the TSST-1 gene, as evident by a common fragment hybridizing to *tst*, the 700 bp *Eco*RI and 1.1 kb *Hin*dIII-*Eco*RI probes in all transductants. Collectively, these observations strongly support the contention that the TSST-1 structural gene is located in close proximity to SEB genetic element in these isolates. On the other hand, SEB-producing *S. aureus*

isolates did not hybridize to the *tst*-probe, or to two probes derived from upstream and downstream sequences that flanked the TSST-1 gene. This observation in conjunction with the fact that the TSST-1 genetic element is mobile, could suggest that the TSST-1 genetic element may be interfering with the expression of SEB through insertion into the SEB genetic element.

There are several possibilities to explain how insertion of the TSST-1 genetic element could interfere with SEB expression. The TSST-1 genetic element could insert into the promoter or structural region of SEB, thereby disrupting transcription. Alternatively, once inserted into the SEB genetic element, the TSST-1 genetic determinant could possibly disrupt gene function due to exertion of strong polar effects on SEB. Another possibility is that the TSST-1 determinant could carry a transcriptional terminator that does not allow the transcription of SEB. Although all of these mechanisms are possible and none can be discounted, it may also be possible that the TSST-1 genetic element could cause deletion of SEB coding sequences upon insertion. This possibility appears to be more likely than the previously described mechanisms since PCR results did not show the expected SEB amplified products when primers that targeted various regions of *seb* were used as probes in the TSST-1^+ isolates, indicating that the entire SEB coding sequence is not present. To determine if this mechanism mediates the lack of TSST-1 and SEB co-production in *S. aureus*, introduction of the TSST-1 genetic element into a SEB^+ clinical strain, perhaps via transduction, followed by the observed loss of SEB production and verification of deletion of SEB coding sequences would need to be demonstrated. Although this appears to be a straight-forward approach, especially with the availability of the strain RN7043 (*tst*::Tc^r),

two limitations of this approach are noteworthy. First is the observed difficulty of introducing foreign DNA into a clinical *S. aureus* isolate. The efficiences of this are extremely low, and thereby explains why most genetic manipulations of *S. aureus* are done in the restriction-deficient mutant RN4220. Second, to perform this experiment, one would ideally require a *rec*A SEB$^+$ mutant of *S. aureus* in order to minimize the incorporation of the TSST-1 genetic element into the host chromosome by homologous recombination. This would undoubtably enhance the probability of identifying desired transductants that have arisen via independent movement of the TSST-1 genetic element, but unfortunately this type of strain is not currently available.

Another possibilty to explain the presence of SEB genetic element sequences in TSST-1$^+$ isolates is that perhaps the TSST-1 and SEB structural genes are carried by similiar genetic elements. If the two toxin genes were carried by similiar genetic elements, it is likely that these elements would share a preferred site of insertion. Potentially, the insertion of a genetic element carrying either the TSST-1 or SEB structural gene would likely exclude the insertion of the other. Indirect support for a common genetic element is supplied by the observation that TSST-1$^+$ and SEB$^+$ isolates share several DNA similiarities in relation to hybridization results with SEB genetic element probes. It was found that in contrast to TSST-1$^-$/SEB$^-$ isolates, both TSST-1$^+$ and SEB$^+$ isolates had multiple *Cla*I fragments that hybridized to the 700 bp *Eco*RI SEB genetic element probe. In addition, both TSST-1$^+$ and SEB$^+$ isolates appeared to contain partial *seb* coding sequences. Partial *seb* coding sequences were not found in any of the TSST-1$^-$/SEB$^-$ isolates examined. If the theory that both genes are carried by similiar genetic elements were true however, one would have expected to

observe SEB-producing isolates to hybridize to probes derived from the TSST-1 genetic element. This was not observed in these studies (Figures 33 and 34). In addition the results suggest that the SEB genetic element is mobile like the TSST-1 genetic element. Although a preferred location in the chromosome may be possible if both toxins genes are carried by similiar genetic elements, if the elements are mobile, alternative sites of insertion would be expected. Thus theoretically, TSST-1$^+$/SEB$^+$ isolates should be observed. The observation that SEB$^+$ isolates do not hybridize to TSST-1 genetic element probes, in addition to the observation that the SEB and TSST-1 genetic elements are mobile, argue against the hypothesis that mutual exclusivity of TSST-1 and SEB production in *S. aureus* is mediated by the carriage of both toxin genes on similiar genetic elements that each have the same preferred site of insertion in the chromosome.

A duplication of 700 bp *Eco*RI sequences was also found with a SEC$^+$/SEB$^-$/TSST-1$^-$ isolate 8530 (ATCC 19095)(see Figure 37). As with co-production of TSST-1 and SEB, co-production of SEC1 with SEB has also been found to be rare, although co-production of TSST-1 and SEC1 is relatively frequent (TSST-1 appears to influence the site of insertion of the SEC1 gene) (Bohach *et al.*, 1989). Although the nature of the SEC determinant still remains unknown, it is possible that lack of SEB and SEC co-production among *S. aureus* isolates is mediated by a mechanism similiar to the one that mediates the mutual exclusivity of TSST-1 and SEB co-production.

In summary, a number of putative mechanisms that may underly the mutual exclusivity of TSST-1 and SEB co-production in *S. aureus* have been explored. Although none of these possibilities can be definitively ruled out, some appear to be more likely than others based on

the collective preliminary observations made by genetic analyses. The pattern observed upon Southern hybridization analysis of TSST-1$^+$ and SEB$^+$ strains with the probes used in this study lead to at least two possible mechanisms that may explain the mutual exclusivity of TSST-1 and SEB co-production in *S. aureus* as illustrated in Figure 48. The TSST-1 genetic element could have a preferred site of insertion in the SEB genetic element. Upon insertion, the TSST-1 genetic element may interfere with the expression of SEB in as yet some unindentified manner (Figure 48A). Alternatively, the TSST-1 and SEB genetic elements may each have the same preferred site of insertion in the *S. aureus* chromosome. Insertion of one genetic element carrying one of the toxin genes would inhibit the insertion of the other genetic element carrying the alternate toxin gene. It may be further speculated that the shared point of insertion may be linked to a partial *seb* coding sequence associated with a DNA sequence that shares homology with the SEB genetic element (Figure 48B). Whether the partial *seb* coding sequence that has homology to the 150 bp *Taq*I probe represents a *seb*-like gene, a remnant of a *seb* sequence, or an ancestral sequence that was involved in creating the *seb* gene found today, is only speculative at present. Future studies in this area are clearly needed in order to determine which, if any, of these possible mechanisms may be mediating the mutual exclusivity of TSST-1 and SEB co-production in *S. aureus*.

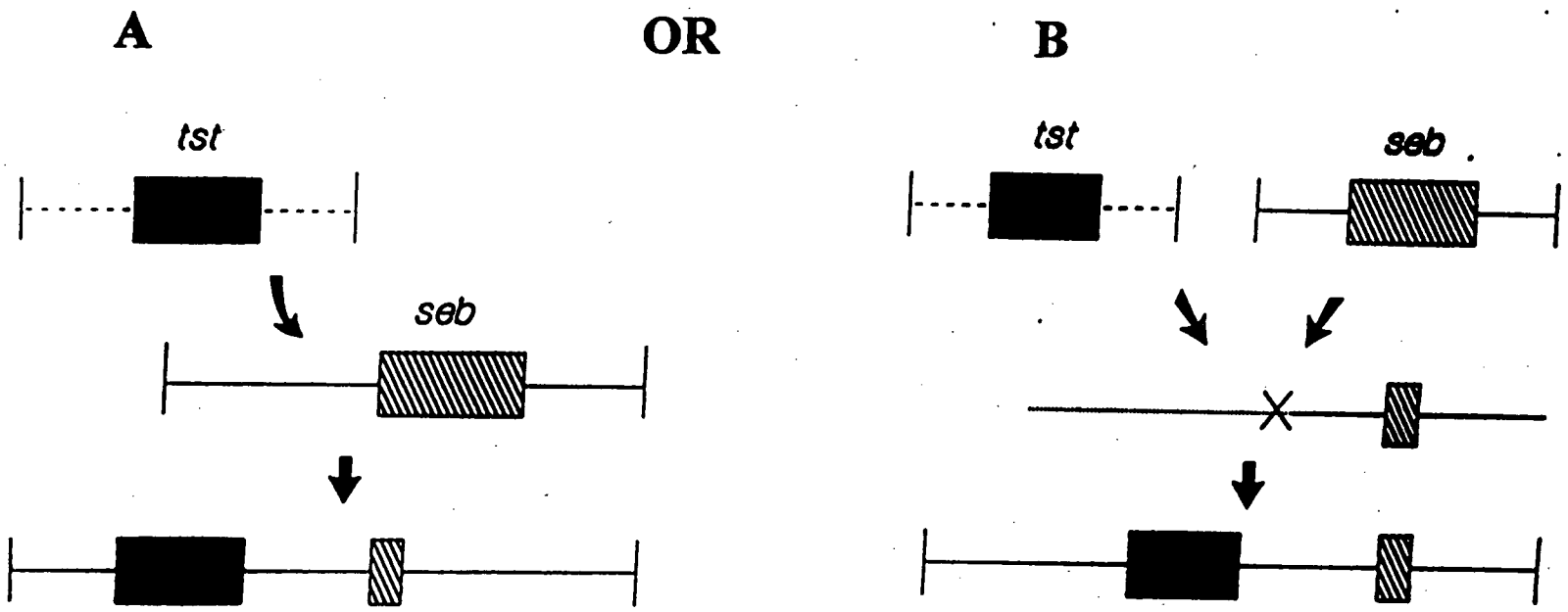


Figure 48. Mechanisms that may mediate the mutual exclusivity of TSST-1 and SEB co-production in *S. aureus*. Illustrated are the possible mechanisms (A or B) that may mediate the observed lack of TSST-1 and SEB co-production in *S. aureus* isolates as described in the text.

Chapter 7. Summary of Scientific Contributions and Future Avenues of Research

7.1. Summary of Results and Scientific Contributions

The main objective of this research was to study the interaction of the staphylococcal superantigen TSST-1 with SEA, and SEB. In addition to belonging to the superantigen family of proteins, these three toxins are carried by genetic elements, of which only the element carrying *sea* has been fully characterized. Specific interactions may be occurring with regards to the expression of these toxins in *S. aureus* since certain of these toxin combinations are more prevalent than others. TSST-1 and SEA is a toxin combination frequently found co-produced among menstrual TSS isolates, whereas in contrast, TSST-1 and SEB are rarely (if ever) found co-produced. These two observations provided the framework for the research thesis in that questions pertaining to the functional and molecular aspects of these toxin combinations were formulated and examined.

SUMMARY

1. Demonstration that TSST-1 plays an important role in the pathogenesis of menstrual TSS

Although TSST-1 has been implicated in the pathogenesis of TSS, a causal role for this toxin in MTSS had not yet been verified. A causative role for TSST-1 in MTSS was

definitively established in this study by introducing a pair of isogenic mutants in TSST-1 production into a tampon-associated vaginal infection model of TSS in the NZW rabbit. The TSST-1$^+$ strain induced more lethality and fever than the TSST-1$^-$ isogenic derivative. The *in vivo* studies were corroborated by the *in vitro* data which showed that the culture filtrate from the TSST-1$^+$ strain induced more T cell proliferation and TNFα secretion from rabbit splenocytes than the culture filtrate from the TSST-1$^-$ strain. These effects are believed to play an important role in the pathogenesis of superantigen-mediated shock. Although the amount of SEA produced by the TSST-1 isogenic mutants differed, SEA was subsequently shown not to enhance the virulence of the TSST-1$^+$ strain. This strengthens the premise that TSST-1 plays an important role in the pathogenesis of MTSS and suggests that the observed difference in virulence between RN3984 and RN7043 was not a result of decreased production of SEA by RN7043.

2. Effect of TSST-1 and SEA Superantigen Co-Production in the Pathogenesis of Menstrual TSS

In light of the frequent co-expression of TSST-1 and SEA among MTSS isolates, it was hypothesized that co-production of two superantigens by a *S. aureus* strain may increase various superantigen-associated activities which may lead to more virulent strain. Consistent with this hypothesis was the observation that the combination of TSST-1 and SEA significantly enhanced T cell proliferation and TNFα secretion from human PBMC as compared to TSST-1 or SEA alone. To test if this toxin combination may have an effect *in vivo*, TSST-1$^+$/SEA$^+$ and TSST-1$^+$/SEA$^-$ strains of *S. aureus* were introduced into two animal models of lethal shock. The TSST-1$^+$/SEA$^+$ strain did not cause more lethality or

morbidity than the TSST-1$^+$/SEA$^-$ strain. This suggested that co-production of SEA with TSST-1 did not increase the virulence of this strain. This was consistent with the observation that the TSST-1$^+$/SEA$^+$ culture filtrate did not induce a higher amount of TNFα secretion from rabbit splenocytes as compared to the TSST-1$^+$/SEA$^-$ culture filtrate. Although the culture filtrate of the TSST-1$^+$/SEA$^+$ strain induced more T cell proliferation than the culture filtrate of the TSST-1$^+$/SEA$^-$ strain, the effect was concentration dependent. Since the pathogenicity of the strains was not different in two test models, these results suggest that an enhancement in T cell proliferation does not occur *in vivo*, perhaps since the amount of TSST-1 produced by the strain was already at a high level.

This was the first time to my knowledge that a toxin gene of *S. aureus* has been inactivated by the method of plasmid integration. The concern that this type of construct may be unstable *in vivo* was alleviated since the stability of the SEA$^-$ phenotype of INT#1 after growth in BALB/c mice and NZW rabbits was verified. This was also the first reported study that used isogenic mutants in animal studies to determine the effect various toxin combinations produced by *S. aureus* may play in pathogenesis.

3. Genetic Analyses Focused on the Mutual Exclusivity of TSST-1 and SEB Co-Production in *S. aureus*

i) Since TSST-1 and SEB are not co-produced, it was of interest to determine if the TSST-1 and SEB structural genes could be found within the same isolate. Upon Southern hybridization with an intragenic *seb* probe, 8 of 8 TSST-1$^+$ isolates hybridized to the probe, even though SEB was not produced. This indicated that TSST-1$^+$ isolates do contain *seb*-homologous sequences.

ii) Although TSST-1$^+$ isolates hybridized to a 150 bp *Taq*I *seb* probe, a complete copy of *seb* was not present in these isolates since PCR analysis using primers that targeted various regions of the SEB nucleotide sequence failed to amplify the expected PCR products.

iii) The TSST-1 structural gene, *seb* homologous sequences, and SEB genetic element sequences were all localized to the same sized DNA restriction fragment in the majority of TSST-1$^+$ isolates analysed. In addition, transduction of *tst* from a TSST-1$^+$ strain of *S. aureus* into a TSST-1$^-$ strain resulted in the co-transfer of SEB genetic element sequences. This suggested that *tst* was located close to SEB genetic element sequences in these isolates.

iv) SEB producing isolates of *S. aureus* did not contain the TSST-1 structural gene, or sequences that immediately flanked *tst* within the TSST-1 genetic element.

v) TSST-1$^+$ and SEB$^+$ isolates were found to contain multiple *Cla*I fragments that hybridized to the 700 bp *Eco*RI SEB genetic element probe. TSST-1$^+$ and SEB$^+$ isolates also contained partial *seb* coding sequences. This is in contrast to TSST-1$^-$ /SEB$^-$ isolates which only had one *Cla*I fragment that hybridized to the 700 bp *Eco*RI probe, and no *seb*-homologous sequences.

vi) Results of Southern hybridization analysis suggested that the SEB genetic element may be found in different chromosomal locations.

vii) In light of the initial observation that although TSST-1$^+$ isolates appeared to contain *seb*-homologous sequences, SEB is not co-produced, it was proposed that expression of both toxins may be regulated by the competition for a common transcriptional

activator. Demonstration that a TSST-1$^+$/SEB$^+$ strain of *S. aureus* could be genetically constructed argues against this possibility.

Based on the collective observations made above, possible mechanisms mediating the lack of TSST-1 and SEB co-production in *S. aureus* have been proposed. First is the possibility that the TSST-1 genetic element has a preferred site of insertion in the SEB genetic element. Upon insertion, the TSST-1 genetic element may interfere with the expression of SEB in as yet some unidentified manner. Alternatively, the genetic elements carrying either *tst* or *seb* may have a common point of insertion in the *S. aureus* chromosome that may be linked to a partial *seb* coding sequence. Insertion of one genetic element carrying one of the toxin genes may inhibit the insertion of the other genetic element carrying the alternate toxin gene.

This was the first study to address the mechanisms that may mediate the mutual exclusivity of TSST-1 and SEB co-production in *S. aureus*. As a result of these studies, this research has enabled further characterization of the region surrounding *seb* in *S. aureus* (see Figure 45).

7.2. Future Avenues of Research

The observed frequent production of SEA in conjunction with TSST-1 among MTSS isolates remains enigmatic. Although the results presented in this study appeared to suggest that this toxin combination does not enhance the virulence of this organism, it can still not be ruled out. I believe that it is realistic to assume that the presence of SEA, or as yet some unidentified protein, is responsible for causing this particular clone to be dominant among menstrual-associated TSS infections. Since it has been found that co-production of SEA with TSST-1 did not cause more lethality or morbidity *in vivo*, the constructed isogenic mutants

can next be used to assess whether TSST-1 and SEA co-production has an effect on other parameters important in establishing virulence, such as colonization or growth. Alternatively, the question of whether the expression of staphylokinase or the repression of ß-lysin may play a role in increasing the pathogenic potential of this specific clone should be examined.

A concentration dependent effect of TSST-1 and SEA-induced T cell proliferation on rabbit splenocytes was demonstrated. One follow-up to this study could be to identify the TSST-1 and SEA binding sites on rabbit splenocytes. If they bind to overlapping epitopes on the same receptor as they do on human PBMC, the usefulness of the rabbit model for dissecting out the role of staphylococcal superantigens in the pathogenesis of TSS would be supported. This would also support the prediction that a concentration effect in T cell proliferation exists upon co-incubation of TSST-1 and SEA with human PBMC.

If the concentration of TSST-1 and SEA produced *in vivo* is a critical factor that may dictate whether or not an additive response in various effects will occur, it would follow that the variability in the concentrations of toxin produced by various TSST-1^+/SEA^+ strains may be an important issue. The production of SEA by a strain of *S. aureus* that produces a minimally effective concentration of TSST-1 (for example 1 ng/ml), would be more of a benefit to the organism than production of SEA in a strain that produced a higher amount of TSST-1 (for example 100 ng/ml). RN3984 appears to produce a relatively higher amount of TSST-1 in comparison to other TSST-1^+ strains (Rosten *et al.*, 1987). It would be interesting to determine if the same results in the animal studies would occur if a TSST-1^+/SEA^- mutant was constructed from a TSST-1^+/SEA^+ strain of *S. aureus* that produced a

lower amount of TSST-1.

Despite many attempts by various investigators, the nature of the genetic elements that carry *tst* and *seb* still defy full characterization. Sequence analysis of the genetic elements that carry both *tst* and *seb* would help to determine the nature of these elements, and would be an important direction for future research.

Although studies that address the role of superantigens in disease are important, it is my hope that the underlying mechanisms that favour the acquisition and expression of certain superantigen combinations are not overlooked. The aspects governing how and why toxins associated with mobile genetic elements are introduced, maintained and excluded in a particular *S. aureus* strain in my opinion, parallel the importance of studies that address the functional consequences of these toxins. Through these studies I believe I have begun to address some of these important issues, and hopefully enough interest will be generated that such studies will continue in the future.

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Appendix 1. Ph.D. Publications To Date

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