1. SUMMARY

Toxic shock syndrome toxin-1 (TSST-1) producing strains of *Staphylococcus aureus* isolated from 18 patients with toxic shock syndrome (TSS) and from 56 patients with other diagnoses were compared for capacity to interact with various serum and connective tissue proteins. TSS associated isolates showed significantly stronger binding of Type-I collagen (Cn-I) and Cn-II than non-TSS strains, in a particle agglutination assay (PAA) as well as in 

\[^{125}\text{I} \text{Cn uptake experiments.} \]

\[^{125}\text{I} \text{Cn-IV binding, was similar between the two groups, whereas in PAA, a stronger interaction was observed for non-TSS than TSS associated strains. The median binding of}^{125}\text{I Cn to TSS-associated strains were 52.2 (Cn-I), 30.6 (Cn-II) and 20.0 (Cn-IV) compared to 20.0 (Cn-I), 14.4 (Cn-II) and 24.4 (Cn-IV) values of non-TSS strains. A saturation with}^{125}\text{I Cn-I and Cn-II binding was established for TSS (30 min) and non-TSS (15 min) strains.}^{125}\text{I Cn-IV binding reached a saturation in 10 min and 90 min with TSS and non-TSS strains respectively. Finally, the binding profiles of TSS associated and non-TSS strains to fibronectin, fibrinogen, laminin and IgG did not differ in both PAA and radioisotope assays. In scanning electron microscopy, cells of TSS associated strains bound to the reprecipitated native Cn-I fibrils. In contrast, most cells of non-TSS strains were localized to the distal end or were trapped between the Cn fibrils. The stronger interaction with Cn-I and II in particular, shown by TSS associated strains, might enhance submucosal localization, thereby facilitating entry of toxins into the blood and establishment of TSS.

2. INTRODUCTION

Local infection or mucosal colonization by toxic shock syndrome toxin-1 (TSST-1) producing strains of *Staphylococcus aureus* has been proposed as one important factor in the pathogenesis of both menstrual and nonmenstrual toxic shock syndrome (TSS) [1–3]. Nosocomial transmission of
TSS due to staphylococcal skin and nasal carriage among hospital personnel has been demonstrated in over 200 postoperative cases [4,5]. The role of local conditions and/or duration of staphylococcal carriage has been suggested in the clinical presentation of TSS infection with TSST-1 producing S. aureus [1,6]. However, the molecular mechanisms of adhesion and colonization of TSS strains to host mucosal surface is yet unknown.

Bacterial adherence to host mucosal cell surfaces is an initial step in many infectious processes, and in carriage and transmission of certain pathogens [7,8]. Binding ability of S. aureus to various serum and connective tissue proteins has been suggested in promoting these functions [9]. Type I and II collagen isolates from patients with osteomyelitis, endocarditis, septic arthritis and bacteraemia associated with infected intravenous catheters demonstrate higher collagen binding ability than isolates from patients with uncomplicated bacteraemia or infections with coagulase negative staphylococci [11]. In an earlier study, we have shown that TSS causing staphylococci have distinct ability to bind to collagen immobilized on solid phase [12].

To elucidate this phenomenon, we have investigated the binding properties of TSST-1 producing S. aureus strains isolated from TSS and non-TSS infections, with Types I, II and IV collagen immobilized on a solid phase in a particle agglutination assay, and in a 125I-labelled protein binding assay. Furthermore the binding of laminin, a major basement protein besides Cn-IV, and other serum proteins such as fibronectin, fibrinogen and immunoglobulin G was tested. A strong interaction of TSS associated strains with Cn-I in particular, and Cn-II was found. The interaction of strains of two groups also found to differ with reprecipitated native Type-I collagen fibrils as visualized by scanning electron microscopy.

3. MATERIALS AND METHODS

3.1. Bacterial strains

A total of 74 TSST-1 positive S. aureus strains (18 isolates from TSS according to the CDC criteria [13] and 56 isolates from various non-TSS infections), previously characterized [Naidu, A.S. et al., in press], were studied. S. aureus strain Cowan 1, Newman and Wood 46. Micrococcus luteus and Micrococcus lysodeikticus laboratory reference strains for connective tissue and serum protein binding are from our collection [12].

3.2. Chemicals

Fibronectin (Fn) purified from porcine plasma on gelatin Sepharose® was a kind gift from BioInvent International, AB, Lund, Sweden. Vitrogen-100™ a collagen (Cn) preparation containing 95% Type-I and 5% Type-III Cn, was purchased from Collagen Corp., Palo Alto, California, U.S.A. (Lot no.87H18.3). Type II Cn, purified from bovine nasal septum according to Strawich and Nimmi [14] was a kind gift from P. Speziale, Institute of Biological Chemistry, University of Sassari, Sassari, Italy. Type-IV Cn purified from basement membrane of the Engelbreth-Holm-Swarm transplantable mouse tumor was purchased from Collaborative Research Inc., Bedford, MA, USA (Lot no. 88-1403). Bacto-Gelatin (Gel) (Control-665903) was purchased from Difco Laboratories, Detroit, Michigan, U.S.A. Laminin (Lm) purified from the mouse EHS sarcoma was purchased from E.Y. laboratories, Inc., San Mateo, California, U.S.A. (Lot no.060616). Immunoglobulin G and fibrinogen (Fg) (Batch 61857) purified from human plasma were kind gifts from Kabi Vitrum, Stockholm, Sweden. Bovine serum albumin (Lot 41F-0061), was purchased from Sigma Chemicals Co., St. Louis, MO, U.S.A. Staphaurex® was purchased from Wellcome Diagnostics, Dartford, England. All chemicals used for the preparation of buffer solutions were of analytical grade.

3.3. Particle agglutination assay (PAA)

Types I, II and IV Cn-, Gel-, Fn-, Lm- and Fg-PAA standard reagents were prepared and evaluated for binding according to Naidu et al. [12]. Briefly, strains were grown on blood agar at 37°C for 18 h, harvested and washed in 0.02 M potassium phosphate (PP) buffer, pH 6.8 and re-suspended in the same buffer to a density of 10^10 cells/ml. The agglutination reaction was performed on glass slides by mixing 20 µl of
staphylococcal cell suspensions containing $5 \times 10^9$ cells with equal volumes of respective PAA reagent. Clumping was scored after two minutes as high (+++ and ++ reactions) or low (+ reaction) and negative reactions. Strains were checked for autoaggregation by mixing one drop of cell suspension with a drop of PP buffer. Ovalbumin coated latex particles were used as reagent control. *S. aureus* laboratory reference strains Cowan 1, Newman and *S. aureus* strain Wood 46, *Micrococcus luteus*, M. lysodeikticus were used as positive and negative controls respectively. All of the isolates were also tested with Staphaurex for non-immune IgG (presence of protein A) and fibrinogen binding.

### 3.4. $^{125}$I-labeled protein binding assays

Types I, II and IV Cn, Fn, Fg and IgG were labelled with $^{125}$Iodine according to the chloramine-T method [15]. Binding of $^{125}$I-labelled proteins to staphylococcal cells was quantitated according to Froman et al. [16] with minor modifications. Briefly, bacteria were grown in trypticase soy broth (TSB) at 37°C for 18 h with constant shaking. Cells were washed once in 0.15 M PBS, pH 7.2, and the density was adjusted photometrically to $10^{10}$ cells/ml. $^{125}$I proteins were diluted (to a radioactivity measurement between 15000–25000 cpm) in PBS, pH 7.2, containing 1% bovine serum albumin. Labelled protein (50 μl) was mixed with 100 μl of bacterial suspension in a polystyrene centrifuge tube and kept at room temperature for 1 hour. After adding 2 ml of ice cold PBS containing 0.05% azide and 0.1% Tween-20, tubes were centrifuged at 4500 x g for 10 minutes and the supernatants were aspirated. Radioactivity of the pellet (bacterial cells) was measured in a 1260-Multigamma (LKB-Wallac, Turku, Finland). *S. aureus* reference strains Cowan 1, Newman and *S. aureus* strain Wood 46, *Micrococcus luteus*, M. lysodeikticus were used as positive and negative controls respectively during the binding assay. The binding assays with strains were done in triplicate and repeated four times.

### 3.5. Time course experiment

*S. aureus* strain T-5993 (source TSS) and strain D-2562 (source staphylococcal septicemia), both positive for TSST-1 production, were tested for binding of radioactive Types I, II and IV Cn as described above, in time courses from 1 to 180 min.

### 3.6. Preparation of reprecipitated native-type collagen fibrils

A solution of Type-I Cn in 0.012 N hydrochloric acid was dialysed against 0.02 M sodium phosphate, pH 7.4 at 4°C for 48 hours. During dialysis buffer was changed eight times and finally a precipitate of collagen fibrils was obtained [17]. The structure of fibrils and their interaction with staphylococci was investigated in scanning electron microscopy.

### 3.7. Scanning electron microscopy (SEM)

Samples for SEM (Philips-420 T) analysis were fixed for 1 h with 4 ml of 5% glutaraldehyde in 0.0634 M sodium cacodylate buffer, pH 7.4, containing 5.5% sucrose. Samples were washed twice for 15 min in sodium cacodylate buffer without sucrose and postfixed with 1% OsO₄ in the same buffer for 1 h and washed in distilled water to remove OsO₄. Specimens were dehydrated overnight with acidified 2,2-dimethoxypropane followed by treatment with acetone for 15 min twice, and then placed and allowed to dry on grids. Finally, specimens were placed in an argon atmosphere at $10^{-6}$ to $10^{-7}$ torr in a vacuum evaporator (Polaron) and coated with approximately 20 nm of metal from gold using a sputter coater (Polaron) and viewed in SEM mode.

### 4. RESULTS

#### 4.1. Interaction studies with proteins immobilized on solid phase

The ability of 18 TSS and 56 non-TSS isolates of *S. aureus* (all positive for TSST-1 production) to interact with Types I, II and IV Cn immobilized on solid phase was tested with PAA and compared with that of Fn-, Fg- and Lm-PAA (Table 1). Type-I Cn-PAA reactivity was higher among TSS isolates (83.3%) than non-TSS isolates (30.4%). However, 72.2% of the TSS isolates demonstrated a low binding interaction with the base-
Fig. 1. Binding of \(^{125}I\) Type I, II and IV collagens and various serum proteins to TSST-1 producing \(S. aureus\) isolated from TSS (○) and non-TSS (●) infections.

4.2. Binding studies with \(^{125}I\)-Cn and other proteins

TSS and non-TSS isolates of \(S. aureus\) were tested for binding to Type I, II and IV Cn, Fn,
Table 1

Serum and connective tissue protein interactions with TSST-1 producing *Staphylococcus aureus* isolated from TSS and non-TSS clinical sources in a particle agglutination assay

<table>
<thead>
<tr>
<th>PAA</th>
<th>TSS strains</th>
<th>Non-TSS strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Cn-I</td>
<td>15 (83.3)</td>
<td>3 (16.7)</td>
</tr>
<tr>
<td>Cn-II</td>
<td>9 (50.0)</td>
<td>8 (44.4)</td>
</tr>
<tr>
<td>Cn-IV</td>
<td>4 (22.2)</td>
<td>13 (72.2)</td>
</tr>
<tr>
<td>Gel</td>
<td>17 (94.4)</td>
<td>1 (5.6)</td>
</tr>
<tr>
<td>Fn</td>
<td>3 (16.7)</td>
<td>8 (44.4)</td>
</tr>
<tr>
<td>Lm</td>
<td>0</td>
<td>1 (5.6)</td>
</tr>
</tbody>
</table>

A total of 18 TSS and 56 non-TSS strains (all positive for TSST-1 production) were tested with standard PAA reagents. Clumping was scored as high (+++ and ++ reactions) or low (+ reactions) and negative. All strains demonstrated high reactivity to Fibrinogen-PAA and Staphaurex®. Strains were grown on blood agar at 37°C for 18 h, harvested and washed once in 0.02 M potassium phosphate buffer, pH 6.8 and resuspended to a density of 10^10 cells/ml and tested for serum and connective tissue protein interaction according to a standard particle agglutination assay [12].

Fibrinogen and IgG (Fig. 1). The median ^125^I-Cn binding values for TSS strains were 52.2 and 30.6 and to non-TSS strains were 20.0 and 14.4 for Type-I and Type-II respectively. Thus TSS associated strains demonstrated significantly higher binding to Type-I and Type-II collagens compared to non-TSS strains as measured by uptake of ^125^I-protein. In contrast, the binding to Type-IV Cn was 24.4 and 20.0, respectively and did not differ between the two groups of strains. Thus, the difference between TSS and non-TSS associated strains observed in Type-II and IV Cn PAA was not revealed in the corresponding radio-binding tests. No significant differences were found in

---

![Graph](image-url)

**Fig. 2.** Time course of [125] collagen binding to TSST-1 positive *S. aureus* strains T-5993 (TSS) and D-2562 (septicemia).
Fig. 3. Interaction of reprecipitated native Type-I collagen fibrils with cells of TSS and non-TSS strains. Reprecipitated native Type-I Cn fibrils of ~ 500 nm thickness (Plate A, ×27000); Cn bridging of TSS strain T-5993 during the early fibril formation (Plate B, ×31000); Adhesion of cells of strain T-5993 to the surface of aggregated Cn fibrils (Plate C, ×18000 and Plate D, ×9000); Binding of cells of non-TSS strain D-2562 to the distal end of fibrils (Plate E, ×18000); Trapping of cells of strain B-3413 (TSST-1 positive isolate from an eye infection) between Cn fibrils (Plate F, ×15000).
binding of \(^{125}\text{I-Fn}, \ ^{125}\text{I-Fg} \) and \(^{125}\text{I-IgG} \) binding to TSS and non-TSS strains, which was in agreement with the PAA.

4.3. Time course binding of Type-I, II and IV Cn to bacterial cells

\textit{S. aureus} strain T-5993 isolated from TSS and D-2562, a non-TSS strain isolated from septicemia were tested for the uptake of \(^{125}\text{I}-\text{labelled Type I, II and IV collagens} \) over a time course of 5 to 180 min (Fig. 2). Binding equilibrium for the TSS strain was established after 30 min contact with \(^{125}\text{I}-\text{labelled Type I and Type-II Cn} \), compared to 15 min for that of the non-TSS strain. During the kinetics of \(^{125}\text{I}-\text{labelled Type-IV Cn} \) binding the TSS strain reached a saturation within 10 min of contact time course while the non-TSS strain reached saturation at 90 min.

4.4. SEM studies on bacterial interaction with reprecipitated native Type-I collagen fibrils

Reprecipitated native Type-I Cn fibrils of ~500 nm thickness were spontaneously formed after 48 h (Fig. 3, Plate A). These fibrils showed Cn-Cn interactions and aggregated. When TSS strain T-5993 was incubated with Type-I Cn preparation before the fibril formation, (during early 30–60 min), Cn interacted between cells and bridged staphylococci into a fine network (Plate B). Three different patterns of reprecipitated native Type-I Cn interactions with staphylococci were observed. Most of the T-5993 cells adhered to the surface of the fibrils throughout the length (Pattern 1, Plates C and D), while a few cells attached to the distal end or tip of the fibril. On the contrary, most of the cells of non-TSS strain D-2562 bound to the distal end (Pattern 2, Plate E), while a very few cells on the surface length of the fibril. Non-TSS strains binding less than 15\% to \(^{125}\text{I} \) Type-I Cn demonstrated a trapping between fibrils (Pattern 3, Plate F) and did not or scarcely adhered on to the surface. Pattern 1 was more prominent among other TSS strains although very few cells of these strains also demonstrated patterns 2 and 3.

5. DISCUSSION

TSST-1 producing strains of \textit{S. aureus} occur at a high frequency among various clinical infections, however, such manifestations seldom lead to the onset of TSS, probably due to the prevalence of anti-TSST-1 antibodies [18,19]. Factors such as focal conditions and/or duration of staphylococcal carriage were suggested in the clinical presentation of infection with TSST-1 producing strains. Our data suggest that \textit{S. aureus} strains isolated from patients with TSS are distinct in their binding to Type-I and Type-II Cn. Certain strains of staphylococci also bind to fibronectin and laminin present in the basement membrane [20]. In our study, TSS strains did not demonstrate such ability to bind basement membrane proteins, Type-IV Cn and laminin or to plasma proteins, fibronectin and fibrinogen in PAA.

Quantitative studies with \(^{125}\text{I}-\text{labelled proteins} \) showed that TSS strains bound Type-I and II Cn higher than non-TSS strains. Moreover, a longer time to reach a binding equilibrium with \(^{125}\text{I} \) Type-I and II Cn suggests more Cn interacting sites on the cell surface of TSS than on non-TSS strains. Specific binding of different types of Cn to \textit{S. aureus} and coagulase negative staphylococci has been described [21–25]. These observations are based on binding of soluble Cn to staphylococci. In vivo Cn exists as a polymer in fibrillar forms [10]. To establish the physiological relevance of Cn-bacterial cell interaction, we have also examined adhesion of TSS strains to reprecipitated native Type-I Cn fibrils. During SEM studies on the Cn fibril interaction with cells of three non-TSS strains, binding was generally at the distal end while one non-TSS strain with high cell surface hydrophobicity was found trapped within the fibrils. Thus the results from PAA, rather than radioisotope assay, hypothetically reflect physiological conditions. Capsular polysaccharides of \textit{S. aureus} are shown to interfere with IgG binding to protein A and binding of fibronectin [26,27]. Our recent finding that TSS strains show low cell surface hydrophobicity (Naidu et al., in press) suggests that Cn interaction may not be of nonspecific hydrophobic nature and also the presence of capsule seems less likely.

Therefore, the enhanced ability of TSS associated strains to bind connective tissue proteins may provide a mechanism for submucosal establish-
ment. In particular, vaginal mucosal ulcerations commonly occurring among tampon users [28], may expose connective tissues, providing Cn for interaction with TSS strains. It is of interest that bacterial pathogens such as Vibrio cholerae and toxigenic Escherichia coli seem to require colonization factors (adhesins) as well as enterotoxins in the development of enteric infections [29,30]. In analogy, our present finding might suggest a synergism between Type-I Cn binding and TSST-1 production in the development of TSS.

ACKNOWLEDGEMENTS

Authors wish to thank Dr. P. Speziale for providing Type-II collagen. This work was supported by grants from the Swedish Medical Research Council (B88-16X-08294-01, 16X-04723-12C) and the National Swedish Board for Technical Development (DNR 86-4332).

REFERENCES


