Coagulase-negative staphylococci isolated from two cases of toxic shock syndrome lack superantigenic activity, but induce cytokine production

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Abstract

Two strains of Staphylococcus epidermidis isolated from patients with toxic shock symptoms have been reported to carry genes related to S. aureus enterotoxins B and C by dot-blot hybridisation, although the corresponding superantigenic toxins were not detected immunologically. We here show that these strains produce no superantigens capable of stimulating proliferation of human mononuclear leukocytes or rabbit splenocytes, and that no DNA homologous to the seb or sec genes can be detected by PCR. However, stimulation of human monocytes by whole killed bacteria induced dose-dependent production of the cytokines TNFα, IL-1β and IL-6, which may be responsible for the clinical symptoms in these patients.

Keywords: Tumor necrosis factor-α; Interleukin-1β; Interleukin-6; Staphylococcus epidermidis

1. Introduction

Coagulase-negative staphylococci are increasingly being recognised as important pathogens, especially for patients in surgical, haematological or intensive care units [1]. Staphylococcus epidermidis is frequently responsible for nosocomial infections and can cause shock symptoms which are clinically se-

vere [2] in up to 30% of patients with bacteraemia; half of these patients die [3]. Shock may be induced by several distinct mechanisms. Cases associated with Gram-negative bacteria result from the overstimulation of macrophages by lipopolysaccharide (LPS) present in the outer membrane. The activated macrophages produce excessive amounts of cytokines such as tumor necrosis factor alpha (TNFα) and interleukins 1β and 6 (IL-1β; IL-6) [4] which contribute to the shock symptoms. Certain Gram-positive bacteria can induce shock through a similar mechanism where peptidoglycan and teichoic acid cell wall components stimulate cytokine production by monocytes. The shock thus
produced by *S. aureus* and *S. epidermidis* in experimental animals closely resembles that induced by *Escherichia coli* [5,6]. Other Gram-positive bacteria induce shock through the production of extracellular toxins with superantigenic properties. These induce polyclonal MHC-dependent T cell proliferation with, again, release of TNFα, IL-1β, IL-6 and γ-interferon, generally accounting for the observed symptoms of toxic shock syndrome (TSS) [7,8].

Two strains of *S. epidermidis* isolated from patients whose symptoms fitted the CDC definition for toxic shock syndrome (TSS) induced by superantigen [9] gave a positive signal on dot-blot hybridisation with oligoprobes recognising *S. aureus* genes coding for enterotoxins -B and -C (SEB and SEC), but antisera to SEB or SEC failed to recognise components in the culture supernatants [10]. An antigenically novel, but genetically related toxin was envisaged. In this paper we re-examine the evidence for toxin production by these two *S. epidermidis* strains, and test the alternative hypothesis of cytokine induction in macrophages through cell wall components.

### 2. Materials and methods

#### 2.1. Bacterial strains

*S. epidermidis* strains N860094 and N900057 derive from blood cultures of two patients with TSS symptoms (fever, hypotension, rash and multiple organ failure leading to death in one case); strain 354 was used as an unrelated control. Defined strains of *S. aureus* were used to evaluate enterotoxin production: RN450 (sea-, seb-, sec-); FRIS6 (sea-, seb+, sec+); FRI137 (sea-, seb-, sec+). Strains were stored at -80°C until used.

#### 2.2. PCR amplification of seb and sec

Genomic DNA was extracted from staphylococcal cultures [11] and used as template for amplification using primers previously shown to be specific for seb and sec [12,13]. Seb1 (5'-GAGAGTCACAACGATCTAAACCAG-3'), seb2 (5'-ATACCAAAAGCTATTCTCATTTTCT-3'), sec1 (5'-GACATAAAAGCTAGGAATTT-3') and sec2 (5'-AAATCGGATTAACATTATCC-3') were synthesised on a Applied Biosystems 391 DNA synthesiser (Perkin-Elmer, Montagny-le-Bretonneux, France). After amplification for 30 cycles (1 min denaturation at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C) the products were analysed by electrophoresis through 1% agarose gels (Sigma, Saint Quentin Fallavier, France).

### 2.3. Mitogenic activity of staphylococcal supernatants

Exoproteins were prepared from overnight cultures of staphylococci in brain-heart infusion by precipitation with 5% vols. of 98% ethanol for 2 days at 4°C. After drying at 20°C, the precipitate was resuspended in 1/50 vol. pyrogen-free distilled water, filtered through Ultrafree UFCCTHK (100 kDa cutoff) cartridges and concentrated on MC UFC3-LGC (10 kDa cutoff) filters (both from Millipore, Molsiteln, France).

Human peripheral blood mononuclear cells were prepared from blood of healthy donors (Centre de Transfusion, Lyon, France) on ficoll Hypaque (Lymphoprep, Nycone, Norway) gradients [14] and resuspended in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated foetal calf serum at 10⁵ cells ml⁻¹. Individual wells of 96-well microtitre plates were seeded with 100 μl of cell suspension and 100 μl of staphylococcal supernatant to give final concentrations of 0, 1/100, 1/10 and 1/1 relative to the initial staphylococcal culture. After incubation for 48 h at 37°C in humidified 5% CO₂ in air, 1 μCi of [³H]thymidine (1 Ci mM⁻¹; CEA, Saclay, France) in 10 μl was added to each well and the culture was continued for 24 h. Cells were harvested on glass-fibre filters and their radioactivity was evaluated in a Packard scintillation counter. Results are expressed as cpmp ± standard error of the mean in triplicate cultures.

Alternatively, splenocytes were prepared by gently teasing apart spleens aseptically removed from New Zealand rabbits (ESD, Châtillon sur Chalaronne, France). After removal of aggregates by passage through nylon wool, the suspensions were adjusted to 10⁶ cells ml⁻¹ and incubated with staphylococcal supernatant as described above.
2.4. Cytokine production after staphylococcal stimulation of human monocytes

*S. epidermidis* colonies were freshly inoculated into Muller-Hinton broth and incubated at 37°C for 18 h. Harvested cells were washed ×3 in phosphate-buffered saline, pH 7.4, counted spectrophotometrically and inactivated by heating at 60°C for 30 min. Suspensions for stimulation of human monocytes were adjusted to the equivalent of $5 \times 10^{10}$ CFU ml$^{-1}$. Incubation on blood agar plates confirmed the absence of living bacteria in the heated suspensions.

Human peripheral blood monocytes were prepared and incubated with the killed bacteria as described in [6]. Briefly, 100 µl of bacterial suspension at the indicated concentrations was added to $10^6$ adherent monocytes in 96-well flat-bottomed culture plates. After 18 h incubation, the supernatants were removed and centrifuged, then cytokine concentrations were evaluated by enzyme immunoassay as described in [14]. Measurements are expressed as ng ml$^{-1}$ of cytokine.

3. Results

PCR amplification from bacterial DNA of the two *S. epidermidis* strains associated with TSS using primers specific for the *seb* and *sec* genes from *S. aureus* produced no detectable signal, whereas the control *S. aureus* strains gave a clearly positive result (Fig. 1). This is in contradiction with observations that DNA from the *S. epidermidis* strains can hybridise with oligonucleotide probes for these genes [10]. We attempted amplifications where one of the PCR primers for each gene was replaced by an oligonucleotide previously used as probe, and amplifications using lower annealing temperatures but no positive signals were obtained (data not shown). Mutations in the gene regions corresponding to the primers could nevertheless have prevented amplification, so we tested the biological activity of culture supernatants from the two strains of *S. epidermidis.*

![Fig. 1. PCR amplification of staphylococcal toxin genes seb and sec from *S. epidermidis* isolated from TSS patients and *S. aureus* reference strains. Lanes A. *S. aureus* RN450 (seb $-$; sec $-$); Lanes B. *S. aureus* FRIS6 (seb $+$; sec $-$); Lanes C. *S. aureus* FR1137 (seb $-$; sec $+$); Lanes D. *S. epidermidis* N900057; Lanes E. *S. epidermidis* N870094. seb: amplification using primers seb 1 and seb 2; sec: amplification using primers sec 1 and sec 2.](https://academic.oup.com/femspd/article-abstract/13/1/81/540604)

<table>
<thead>
<tr>
<th>Stimulant added</th>
<th>Rabbit splenocytes (cpm ± SEM)</th>
<th>PBMC (cpm ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain heart solution</td>
<td>667 ± 109</td>
<td>181 ± 21</td>
</tr>
<tr>
<td><em>S. epidermidis</em> N900057</td>
<td>889 ± 358</td>
<td>168 ± 28</td>
</tr>
<tr>
<td>1/100</td>
<td>750 ± 362</td>
<td>208 ± 8</td>
</tr>
<tr>
<td>1/10</td>
<td>724 ± 92</td>
<td>224 ± 6</td>
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<tr>
<td><em>S. epidermidis</em> N870094</td>
<td>903 ± 62</td>
<td>191 ± 16</td>
</tr>
<tr>
<td>1/100</td>
<td>837 ± 48</td>
<td>119 ± 109</td>
</tr>
<tr>
<td>1/10</td>
<td>775 ± 94</td>
<td>225 ± 17</td>
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<tr>
<td><em>S. aureus</em> FRIS6</td>
<td>5241 ± 418</td>
<td>2267 ± 187</td>
</tr>
<tr>
<td>1/100</td>
<td>16217 ± 520</td>
<td>8570 ± 596</td>
</tr>
<tr>
<td>1/10</td>
<td>24915 ± 396</td>
<td>15395 ± 1842</td>
</tr>
<tr>
<td><em>S. aureus</em> RN420</td>
<td>967 ± 102</td>
<td>182 ± 16</td>
</tr>
<tr>
<td>1/100</td>
<td>622 ± 50</td>
<td>158 ± 11</td>
</tr>
<tr>
<td>1/10</td>
<td>334 ± 6</td>
<td>214 ± 16</td>
</tr>
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</table>

Measures are the mean of three determinations. cpm, counts per minute; SEM, standard error of the mean. Strains: as in Fig. 1.
Table 2

<table>
<thead>
<tr>
<th>Induction of TNFα, IL-1β and IL-6 expression by PBMC on incubation with killed S. epidermidis</th>
<th>TNF</th>
<th>IL-1</th>
<th>IL-6</th>
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<tr>
<td>Medium 199</td>
<td>–</td>
<td>&lt; 0.2</td>
<td>0.3</td>
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<td></td>
<td>$1 \times 10^7$</td>
<td>15.6</td>
<td>36.7</td>
</tr>
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<td>2.0</td>
<td>19.8</td>
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<td>15.0</td>
<td>36.2</td>
</tr>
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</table>

Data are expressed in ng ml⁻¹ as determined by enzyme immunoassay. Strains as in Fig. 1; S. epidermidis 354 is a reference strain.

Superantigen expression leads to proliferation of human peripheral blood monocytes or of rabbit splenocytes. When tested in these systems the S. epidermidis supernatants, at any concentration, caused no mitogenic response in the cells over controls. S. aureus FRIS6, as expected, produced a vigorous proliferative response, while S. aureus RN450 which does not produce superantigen, was inactive (Table 1). We conclude that strains N900057 and N860094 of S. epidermidis do not produce superantigens or mitogenic exotoxins.

Possible induction of cytokine expression in monocytes by components of the bacterial cell wall was therefore investigated by incubating human monocytes with suspensions of killed washed S. epidermidis. Table 2 shows that both TSS associated strains and a control strain (354) of S. epidermidis induce a dose-dependent release of TNFα, IL-1β and IL-6 into the supernatant by human monocytes. Very similar levels of release of each cytokine was observed on stimulation by the same concentration of all three strains tested.

4. Discussion

The mechanisms by which S. epidermidis causes shock are at present unclear. It has been suggested that it might produce a superantigenic toxin, and indeed it does secrete exoproteins and toxins such as δ-haemolysin, cytotoxins and Dnase which resemble their counterparts from S. aureus [15,16]. Enterotoxin-producing isolates of S. epidermidis have been reported from animal samples [17], and an initial study of 7 human cases of CNS-related TSS suggested that only TSST-1-producing strains were involved [18]. Later studies have, however, shown that these strains neither produce TSST-1 toxin nor possess the homologous genes [19,20].

The S. epidermidis strains N900057 and N870094 were initially thought to have caused TSS in the patients from which they were isolated through an exoprotein because their DNAs appeared to hybridise with oligoprobes specific for the S. aureus seh and sec genes. However, immunological assays detected no SEB or SEC in the culture supernatants, suggesting antigenic variation [10]. We have re-examined the possible presence of seh or sec DNA in these strains by PCR, and find no signal even under relaxed conditions. We therefore conclude that the previous results obtained using oligoprobes were false positives due to inadequate specificity of the method, although dot-blot hybridisation has been widely used for the detection of bacterial genes, including those for TSST-1 and enterotoxins [10,11,21].

It remained possible that our S. epidermidis isolates produced an unrelated superantigenic exotoxin, so we tested supernatants of mature cultures for mitogenic activity on susceptible human and rabbit cells. No mitogenic activity was observed at any concentration, providing strong evidence that these strains do not secrete superantigenic toxins, or indeed any other mitogenic substance.

An alternative mechanism for induction of TSS involves interaction between bacterial cell wall components and monocytes, leading to the release of cytokine mediators. Gram-negative bacteria such as E. coli induce shock through LPS in their outer membrane, and S. epidermidis can cause apparently identical experimental shock in rabbits [5]. In both cases the percentage of animals suffering shock was similar, and symptoms were accompanied by TNFα and IL-1β release. Recently peptidoglycans, teichoic acid and lipoteichoic acid from S. epidermidis cell walls have been shown to induce cytokine production in human monocytes [6,14,22]. We show here...
that whole killed S. epidermidis is a powerful inducer of TNFα, IL-1β and IL-6 expression in human adherent monocytes.

It might be questioned whether cytokine release in the absence of superantigen stimulation of T cells can provide a sufficient stimulus for TSS. It has been shown that immunisation against TNFα protects animals from SEB-induced TSS, and that TNFα and IL-1β are major mediators of shock [7,8,23]. In addition, superantigen-induced TSS requires the presence of T cells, since nude mice are not susceptible, but T cell proliferation is not required, since cyclosporin treatment does not protect [24,25]. Release of cytokines, especially TNFα and IL-1β, either through cell wall interactions with monocytes or through superantigen stimulation of T cells, thus appears to be a crucial element in the induction of TSS.

It may be noted that S. epidermidis strain 354 which was derived from a catheter-related bacteremia without known TSS, was as efficient an inducer of cytokine release as the two TSS-related strains. The incidence of TSS in human patients and in experimental animals is generally less than half of the potential cases. Although the capacity to induce excessive cytokine release appears necessary for the induction of TSS by a bacterium, other factors must intervene in the progression to clinical shock. S. epidermidis cell wall components appear to be a sufficiently powerful stimulus to cytokine release from monocytes to explain clinical TSS associated with this bacterium.

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References

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