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Superantigens in *Staphylococcus aureus* isolated from prosthetic joint infection

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ABSTRACT

Staphylococcus aureus is a common cause of prosthetic joint infection (PJI). The prevalence of superantigens (SAgs) among PJI-associated *S. aureus* is unknown. Eighty-four *S. aureus* isolates associated with PJI isolated between 1999 and 2006 were studied. SAg genes, *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *tst*, were assayed by PCR. Seventy-eight (92.9%) isolates carried at least 1 SAg gene studied, with 61 (72.6%) harboring more than 1. *seg* was most commonly (70.2%), and *seh* was least frequently (4.8%) detected. *tst*-positive isolates were associated with early infection and increased erythrocyte sedimentation rate at diagnosis ($P = 0.006$ and $P = 0.021$, respectively). *seg* and *sei* were associated with methicillin resistance ($P = 0.008$ and $P = 0.002$, respectively). A majority of PJI-associated isolates studied produced biologically active SAgs in both planktonic and biofilm growth modes. SAg genes are prevalent in S. aureus causing PJI.

1. Introduction

With the increasing numbers of primary arthroplasty surgeries being performed, complications associated with prosthetic joints are becoming increasingly frequent (Kim, 2008). Of the postoperative complications associated with prosthetic joints, prosthetic joint infection (PJI) is the most detrimental (Harris and Sledge, 1990). PJI is often caused by staphylococci, including coagulase-negative staphylococci and *Staphylococcus aureus* (Zimmerli et al., 2004). PJI-associated bacteria grow as biofilms on prosthetic joints (Gallo et al., 2003), and as a result, management of PJI requires a combination of antimicrobial agents and surgery.

Clinical features and outcomes of staphylococcal PJI, including associated symptomatology, chronicity, tendency to relapse, and even mortality, may be influenced by virulence factors, such as exotoxins, produced by the infecting organisms (Cunningham et al., 1996). Among the exotoxins of *S. aureus*, staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin (TSST-1) are well-known superantigens (SAgs). They cause robust activation of T cells expressing certain T-cell receptor β chain variable region families, irrespective of antigenic specificity. SAgs may also contribute to establishing *S. aureus* infection by causing immune evasion or immune subversion (Gaus et al., 1994; Kawabe and Ochi, 1990; Llewelyn and Cohen, 2002; Novick, 2003; O'Hehir and Lamb, 1990; Taylor and Llewelyn, 2010).

The prevalence of SAgs has been mostly investigated among *S. aureus* strains isolated from diseases such as septic shock (Ferry et al., 2005), endocarditis (Nienaber et al., 2011), and toxic shock syndrome (DeVries et al., 2011). To our knowledge, the prevalence of SAgs in *S. aureus* associated with PJI has not been investigated. Staphylococcal SAgs may help in establishment of PJI and contribute to its clinical features. We investigated the prevalence of SAgs in *S. aureus* associated with PJI and related the presence or absence of SAgs to clinical findings. We also examined whether *S. aureus* in in vitro biofilms produces functional SAgs and correlated the presence of SAgs with methicillin resistance.

2. Materials and methods

2.1. Collection of bacterial isolates

A collection of 84 *S. aureus* isolates from patients diagnosed with PJI at Mayo Clinic (Rochester, MN) from 1999 to 2006 were studied. *S. aureus* were isolated from periprosthetic tissues, synovial fluid, or the explanted prosthetic joints themselves (Trampuz et al., 2007). Medical records of corresponding subjects were retrospectively reviewed for demographic characteristics, clinical course, and outcome. This study was approved by the Mayo Clinic Institutional Review Board.

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2.2. Clinical definitions

PJI was defined using diagnostic criteria outlined by the Infectious Diseases Society of America (Osmon et al., 2013). Timing of infection was classified according to time since the most recent prosthesis implantation, defined as early (<3 months), delayed (3–12 months), and late (>12 months). Duration of symptoms before admission was categorized by 1-month intervals. Treatment strategies were categorized as chronic suppression, debridement and implant retention, resection and reimplantation, permanent resection, and disarticulation. Diagnosis of recurrence was confirmed by reisolation of *S. aureus* from the same joint after a treatment strategy had been applied.

2.3. Preparation of genomic DNA and PCR

S. aureus was grown on sheep blood agar, and genomic DNA extracted using the DNeasy blood & tissue kit (Qiagen, Hilden, Germany). Genes for staphylococcal enterotoxins A, B, C, D, E, G, H, and I and TSST-1 were assayed by PCR using a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA). Primers were synthesized by Integrated DNA Technologies® (Coralville, IA); primer sequences and PCR conditions are shown in Table 1 (Blaiotta et al., 2004; Johnson et al., 1991; Letertre et al., 2003; Lovseth et al., 2004).

2.4. Preparation of culture supernatants and quantitation of planktonic and biofilm cultures

For planktonic cultures, *S. aureus* was grown to 10^8 CFU/mL in trypticase soy broth (TSB) for 24 hours. After centrifugation at 4000 rpm for 5 min, supernatants were collected, filtered through a 0.22 μm syringe filter (MILLEX®GP; Millipore, Billerica, MA) and stored at −80 °C until further analysis. Biofilms were grown on Teflon® discs in 2 stages. During the first stage, the discs were placed in 24-well flat bottom plates with 2 mL of TSB containing a 1×10^6 CFU/mL inoculum. After 24 hours of incubation, each disc was removed, rinsed with sterile saline to remove planktonic cells, and transferred to new 24-well flat bottom plates, with each well containing 2 mL of TSB containing 4 μg/mL of vancomycin (to inhibit the planktonic growth). After incubation for an additional 24 hours, culture media was collected, filtered through 0.22-μm syringe filters, and frozen at −80 °C until further testing was performed. Quantitative cultures of biofilms on the discs were performed in duplicate after the initial incubation stage without vancomycin and following incubation with vancomycin. Prior to culture, biofilms were dislodged and disaggregated using vortexing and sonication, as previously described (del Pozo et al., 2009). Quantitative culture results were expressed as the average biofilm density ($\log_{10}$CFU/cm^2) or, for planktonic cells, $\log_{10}$CFU/mL for comparison of the biologic activity of supernatants from biofilm and planktonic cultures, respectively. To specifically investigate the correlation between methicillin susceptibility and biofilm growth in the presence of vancomycin, a convenience sample of 8 methicillin-susceptible *S. aureus* (MSSA) and 7 methicillin-resistant *S. aureus* (MRSA) isolates was first grown on Teflon discs as described above. Then, during the second stage, the discs were cultured in TSB with and without vancomycin. After 24 hours of reincubation, the colony counts were determined.

2.5. T-cell proliferation assay with HLA-DR3 transgenic mouse splenocytes

To measure the biological function of SAgs produced by *S. aureus* grown in planktonic as well as biofilm states, a splenocyte proliferation assay was performed using HLA-DR3 transgenic mice. HLA-DR3 mice expressing functional HLA-DRA1*0101 and HLA-DRB1*0301 transgenes on an MHC class II–deficient background (AE°) have been previously described (Rajagopalan et al., 2003). Splenocytes harvested from naive HLA-DR3 mice were cultured with 100 μL of serial 2-fold dilutions of the culture supernatant (from 1:2 to 1:256) prepared as above. Supernatants from isogenic *S. aureus* strains, RN6734 containing pRN5543::seb (pRN7114) and RN6734 containing pRN5543::seb(b.2) (pRN7116), which produce SEB only or no SAgs (generous gifts from Richard Novick, New York University Medical Center, New York, NY, USA), were used as positive and negative controls, respectively (Vojtov et al., 2002). Splenocytes were cultured at 10^5 cells per well in 100 μL of HEPES-buffered RPMI 1640 containing 5% fetal calf serum, serum supplement, and streptomycin and penicillin. After 48 hours of incubation, 1 μg of tritiated [^{3}H] thymidine was added to the splenocytes and incubated for an additional 17 hours. The extent of proliferation was determined by measuring incorporated radioactivity using standard procedures. The dilution that induced maximal proliferation was identified and used to compare mitogenic activity. Mean radioactivity counts per minute (CPM) with SD was recorded in triplicate wells. Supernatants from planktonic and biofilm cultures of a convenience sample of 38 isolates were tested by this method. Among those 38 isolates, 15 isolates were

Table 1
Nucleotide sequences of primers and references.

Gene	Primer name	Oligonucleotide sequence (5′-3′)	Amplicon size (base pair)	Ta(°C)[a]	Control strain	Reference
sea	SEA-1	GCA GGG AAC AGC TTT AGG C	521	55	IDRL-7971[b]	(Lovseth et al., 2004)
	SEA-2	GTT CTG TAG AAG TAT GAA ACA CG				
seb	SEB-1	TCG CAT CAA ACT GAC AAA CG	478	55	RN6734, pRN7114[c]	(Johnson et al., 1991)
	SEB-2	GCA GGT ACT CTA TAA GTG CC				
sec	SA-U	TGT ATG TAT GGA GGT GTA AC	102	55	ATCC 19095[d]	(Letertre et al., 2003)
	SEC-2	AAT TGT GTT TCT TTT ATT TTC ATA A				
sed	SED-1	CTA GTT TGG TAA TAT CTC CT	317	45	ATCC 23235	(Johnson et al., 1991)
	SED-2	TAA TGC TAT ATC TTA TAG GG				
see	SA-U	TGT ATG TAT GGA GGT GTA AC	213	50	ATCC 27664	(Letertre et al., 2003)
	SEE-2	GCC AAA GCT GTC TGA G				
seg	SEG-1	TGC TAT CGA CAC ACT ACA ACC	704	53	ATCC 19095[d]	(Blaiotta et al., 2004)
	SEG-2	CCA GAT TCA AAT GCA GAA CC				
seh	SA-U	TGT ATG TAT GGA GGT GTA AC	245	53	ATCC 51811	(Letertre et al., 2003)
	SEH-2	TCT CTA GGA GTT TTC ATA TC				
sei	SEI-1	GAC AAC AAA ACT GTC GAA ACT G	630	52	ATCC 19095[d]	(Blaiotta et al., 2004)
	SEI-2	CCA TAT TCT TTG CCT TTA CCA G				
tst	TSST-1	GCT TGC GAC AAC TGC TAC AG	559	55	ATCC 51651	(Lovseth et al., 2004)
	TSST-2	TGG ATC CGT CAT TCA TTG TTA T				

[a] PCR was performed using a total volume of 20 μL and 35–40 cycles: denaturation for 2 min at 94 °C, annealing for 2 min, and primer extension for 1 min at 72 °C. Platinum® PCR SuperMix (Invitrogen™, NY, USA) was used. Ta represents annealing temperature.
[b] *S. aureus* IDRL-7971, isolated from human nares, was confirmed by enzyme-linked immunosorbent assay to produce staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin B (SEB).
[c] RN6734, pRN7114 is a generous gift from Richard Novick (New York Medical Center, NY) and is known to produce only SEB.
[d] *S. aureus* ATCC 19095 is known to have both *sec* and the enterotoxin gene cluster (*egc*).