Acta Medica et Biologica
Vol. 51, No.4, 141-147, 2003

A Multiple Superantigenic Toxin Pattern of Methicillin-resistant *Staphylococcus aureus* (MRSA) as a Risk Factor in the Development of Toxic Shock Syndrome (TSS)

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Received December 26 2003; accepted January 9 2004

Summary. **Methicillin-resistant *Staphylococcus aureus* (MRSA) , an important cause of nosocomial infections, produces a number of superantigenic toxins such as toxic shock syndrome toxin (TSST)-1 and staphylococcal enterotoxin (SE) C in many cases. However, toxic shock syndrome (TSS) only rarely occurs in MRSA infections. In this study, we isolated MRSA strains from the stool and sputum of a patient with TSS and characterized the molecular nature of the TSS-associated MRSA strains. The two MRSA strains were indistinguishable from each other, as demonstrated by pulsed-field gel electrophoresis. The coagulase type was type 2. The two MRSA strains manifested a multiple superantigenic toxin (MST) pattern of TSST-1, SEA, SEC, SEG, SEH, SEI, and SET in the PCR assay, unlike the previously characterized MRSA strains that lacked (e.g.) SEA. When human peripheral blood mononuclear cells were stimulated with superantigenic toxins, a combination of TSST-1, SEA, and SEC induced much higher levels of cytokine production than did the individual toxin or combination of (e.g.) TSST-1 and SEC. The data suggest that the MST pattern of MRSA could be a risk factor in the development of TSS.**

Key words—methicillin-resistant *Staphylococcus aureus* (MRSA), multiple superantigenic toxin (MST), toxic shock syndrome (TSS), risk factor.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major agent in nosocomial infections. It not only manifests drug resistance, but also in many cases produces a number of exotoxins, such as superantigenic toxins, e.g., toxic shock syndrome toxin (TSST) -1 and staphylococcal enterotoxins (SEs). TSST-1 is the major causative toxin of toxic shock syndrome (TSS), which is a life-threatening staphylococcal infection[1], although the development of TSS is rare in hospitals. In infants, TSST-1 is frequently associated with neonatal TSS-like exanthematous disease (NTED)[2].

TSST-1 binds directly to the major histocompatibility complex (MHC) class II molecules on antigen-presenting cells and to the Vβ elements of the antigen receptor of T cells. This trimolecular interaction leads to the activation of a vast number

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Abbreviations—ET, exfoliative toxin; IL, interleukin; MRSA, methicillin-resistant *Staphylococcus aureus*; MST, multiple superantigenic toxin; NTED, neonatal toxic shock syndrome-like exanthematous disease; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; SE, staphylococcal enterotoxin; SSSS, staphylococcal scalded skin syndrome; TSS, toxic shock syndrome; TSST-1, toxic shock syndrome toxin-1.

of Vβ2$^+$ T cells to produce an excess amount of inflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-2, and interferon-γ, resulting in an abnormal reaction throughout the body[3].

In addition, some MRSA strains produce another type of exotoxin, exfoliative toxin, and cause staphylococcal scalded skin syndrome (SSSS). SSSS offen appears in infants and children[1].

We recently encountered a rare case of TSS. In this study, we investigated the MRSA strains isolated from a patient with TSS at the molecular DNA levels and examined the effect of a combination of superantigenic toxins on the cytokine production in human peripheral blood mononuclear cells (PBMCs).

METHODS

Case

A 59-year-old man with a history of hypertension and hyperuricemia admitted to our hospital for gastric cancer (Borman IV, adenocarcinoma, stage1B). He had undergone a Spleno-total gastrectomy and partial resection of colon for treatment of the cancer. On the first day after surgery, he developed a temperature of 38.0～39.0°C.

Three days after surgery, diarrhea developed. Four days after surgery, a fecal sample was obtained for culture and an MRSA (strain T1) was isolated from the sample. He exhibited origuria and dyspnea. He was placed in an ICU with mechanical ventilation support, but became hypotensive and required vasopressor support. His serum creatinine level reached 3.7 mg/dl. Polymixin B-immobilized fiber (PMX) treatment and continuous hemodiafiltration (CHDF) were performed. Thrombocytopenia was seen; his platelet count was 82000 cells/mm^3. Asparta aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (T-Bil.), and creatinine phosphokinase (CPK) respectively reached 1238 IU/l, 524 IU/l, 2.0 mg/dl, and 3227 IU/l.

Five days after surgery, a rash appeared on his inferior limbs (Fig. 1.). This subsequently changed to desquamation. Six days after surgery, he received intravenous teicoplanin (TEIC) for treatment of the MRSA sepsis, but results of a blood culture were negative. Supportive treatment was continued, and the patient recovered and discharged.

This case illustrates several salient features of TSS[4]: the criteria for the diagnosis of TSS for this case were fever, rash, desquamation, hypotension, and abnormalities in the gastrointestinal, renal, hepatic, and hematologic systems.

MRSA strains used as a reference

The E6 MRSA strain was isolated from an infant with NTED[5]. The H5 MRSA strain was isolated from a patient with SSSS[5]. The E6 and H5 strains were employed as a reference in pulsed-field gel electrophoresis and in the PCR assay.

Pulsed-field gel electrophoresis

Total bacterial DNA was purified and subjected to pulsed-field gel electrophoresis, as previously described[5]. Briefly, bacterial DNA was digested with *Sma*I, electrophoresed in 1.2% agarose with the molecular size standard-lambda ladder (Bio-Rad, Hercules, CA, USA), and stained with ethidium bromide.

PCR assay

The primer sets used for the PCR assay were: TST-3 and TST-6 (generating a 445-bp product) for the TSST-1 gene[6], SEA-3 and SEA-4 (generating a 127-bp product) for the SEA gene[6], SEB-1 and SEB-4 (generating a 477-bp product) for the SEB gene[6], SEC-3 and SEC-4 (generating a 271-bp product) for the SEC gene[6], SED-3 and SED-4 (generating a 319-bp product) for the SED gene[6], SEE-3 and SEE-2 (generating a 178-bp product) for the SEE gene[6], SEG-1 and SEG-2 (generating a 642-bp product) for the SEG gene[7], SEH-1 and SEH-2 (generating a 375-bp product) for the SEH gene[7], SEI-1 and SEI-2 (generating a 576-bp product) for the SEI gene[7], ZID and ZDR (generating a 1731-bp product) for the SEJ gene[8], SEK-1 and SEK-2 (generating a 1400-bp product) for the SEK gene[9], PSE-2 and PSE-4 (generating a 790-bp product) for the SEU gene[10], set1-a and set1-b (generating a 733-bp product) for the SET gene[11], ETA-3 and ETA-4 (generating a 119-bp product) for the exfoliative toxin A gene[6], and ETB-3 and ETB-4 (generating a 262-bp product) for the exfoliative toxin B gene[6]. Cycling conditions were denaturation for 30s at 95°C, annealing for 120s at 55°C, and polymerization for 120s at 72°C (30 cycles). Amplified PCR products were analyzed by gel electrophoresis with 1 or 2% agarose and stained with ethidium bromide. $\phi\times$174 RF DNA/*Hae*III fragments (Life Technologies, Gaithersburg, MD, USA) were used as molecular size standards.

Toxin production assay

Bacteria were grown for 18 h at 37°C in brain heart infusion broth (Difco Laboratories, Detroit, MI, USA), and adjusted to a bacterial concentration of

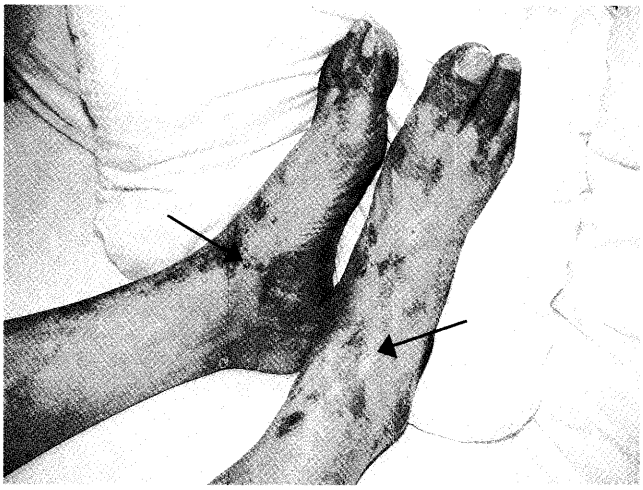

Fig. 1. Spread of a diffuse rash over the limbs and soles of the patient with TSS. Arrows point to the rash.

2.0×10^9 cfu/ml. The amount of toxin in the culture supernatants was determined by passive latex agglutination using TST-RPLA, SET-RPLA, and EXT-RPLA kits (Denka Seiken Co. Ltd., Tokyo). The assay kits for SEE, SEG, SEH, SEI, SEJ, SEK, SET, and SEU were not available in this study.

Coagulase typing

The coagulase type of the MRSA strains was examined using a staphylococcal coagulase antiserum kit (Denka Seiken) in accordance with the manufacturer's instructions.

Preparation of human PBMCs

Human PBMCs were isolated as previously described[12]. Briefly, peripheral blood from healthy adults was diluted 2-fold with phosphate-buffered saline (PBS), and 20 ml was stratified in 10 ml of Ficoll-Conray solution (specific gravity: 1.077). Then the solution was centrifuged at 1500 rpm at room temperature for 30 min. Mononuclear cell fractions were collected and washed with approximately 20 ml of PBS. The solution was additionally centrifuged at 1800 rpm at 4°C for 8 min. This washing was repeated twice. The cells were suspended in 20 ml of the RPMI-1640 culture medium (Gibco-BRL, Grand Island, NY). The cell suspension was centrifuged at 1800 rpm at 4°C for 8 min, and the cells were finally suspended in the RPMI-1640 culture medium supplemented with 10% fetal bovine serum (Gibco-BRL) at a cell concentration of 1×10^6/ml.

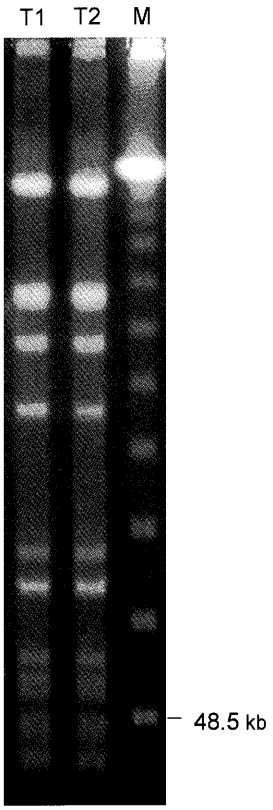

Fig. 2. Pulsed-field gel electrophoresis of the MRSA strains derived from the patient with TSS. Lanes, T1, MRSA strain T1 isolated from stool; T2, MRSA strain T2 isolated from sputum; M, molecular size standards (lambda ladder). Bacterial DNA (from MRSA strains T1 and T2) was digested with *Sma*I. The digestion patterns of E6 (isolated from a patient with NTED) and H5 (isolated from a patient with SSSS) MRSA strains were obviously distinct from those shown in Figure (data not shown).

Table 1. Coagulase and toxin types of the TSS-, NTED-, and SSSS-associated MRSA strains[a)]

Coagulase and toxin types	MRSA strains			
	TSS-associated		NTED-associated	SSSS-associated
	T1 [b)]	T2 [c)]	E6 [d)]	H5 [e)]
Coagulase	Type 2	Type 2	Type 2	Type 1
Toxin				
TSST-1	+ (256) [f)]	+ (256) [f)]	+ (64) [f)]	− (<1) [f)]
SEA	+ (2048) [g)]	+ (1024) [g)]	− (<1) [g)]	− (<1) [g)]
SEB	− (<1) [g)]	− (<1) [g)]	− (<1) [g)]	− (<1) [g)]
SEC	+ (16) [g)]	+ (8) [g)]	+ (8192) [g)]	− (<1) [g)]
SED	− (<1) [g)]	− (<1) [g)]	− (<1) [g)]	− (<1) [g)]
SEE	−	−	−	−
SEG	+	+	+	−
SEH	+	+	−	−
SEI	+	+	+	−
SEJ	−	−	−	−
SEK	−	−	−	−
SET	+	+	+	+
SEU	−	−	−	−
ETA	− (<1) [h)]	− (<1) [h)]	− (<1) [h)]	− (<1) [h)]
ETB	− (<1) [h)]	− (<1) [h)]	− (<1) [h)]	+ (4096) [h)]

[a)]The toxin genes were assayed by PCR. The toxin production levels (ng/ml) are shown in parentheses. [b)]MRSA isolated from stool in this study. [c)]MRSA isolated from sputum in this study. [d)]MRSA described previously[5]. [e)]MRSA described previously[5]. [f)]The amount of toxin in the culture supernatants (ng/ml) was determined by passive latex agglutination using TST-RPLA kits. [g)]The amount of toxin in the culture supernatants (ng/ml) was determined by passive latex agglutination using SET-RPLA kits. [h)]The amount of toxin in the culture supernatants (ng/ml) was determined by passive latex agglutination using EXT-RPLA kits.

Stimulation with superantigens

The PBMCs (5×10^5 cells) in the RPMI-1640 culture medium, prepared as above, were added to each well of 48-well plates (Becton Dickinson, Labware Oxnard, CA, USA). TSST-1 (Toxin Technology, Sarasota, Florida, USA) was then added at 10 ng/ml, as described previously[12]. SEA (Denka Seiken) and SEC (Denka Seiken) were added at 20 ng/ml and 1 ng/ml, respectively. The plates were incubated at 37°C for 12 h in the presence of 5% CO_2. The levels of immunoreactive IL-2 in the cell-free culture supernatants were measured by ELISA (Genzyme TECHNE, Minneapolis, MN, USA), following the manufacturer's instructions. The data were presented as the mean± SD of triplicate experiments.

Table 2. Summary of the TSS-associated superantigenic toxins, which were described previously

Type of TSS	Superantigenic toxins associated with TSS	Reference
Menstrual-TSS (MTSS)	TSST-1	Bergdoll MS, et al.[15,16]
Nonmenstrual-TSS (NMTSS)	TSST-1, SEB, SEC	Bohach GA, et al.[17]
Menstrual-TSS (MTSS)	TSST-1, SEA	Kain KC, et al.[18]
TSS*)	SEG, SEI	Jarraud S, et al.[7]
Nonmenstrual-TSS (NMTSS)	SEA, SEC	John S, et al.[19]

*TSS type was not reported.

Statistical analysis

The data were evaluated with the Student's t test. The level of significance was a P value of less than 0.05.

RESULTS

The two MRSA strains (T1 and T2) isolated from the stool and sputum of a patient with TSS were indistinguishable from each other when bacterial DNAs were digested with *Sma*I and analyzed by pulsed-field gel electrophoresis (Fig. 2.). The digestion pattern of the T1 and T2 strains was apparently distinct from those of the NTED-associated or SSSS-associated MRSA strains (E6 and H5), which were used as a reference strain (data not shown).

The T1 and T2 MRSA strains produced type 2 coagulase and were positive for TSST-1, SEA, SEC, SEG, SEH, SEI, and SET in the PCR assay; they were negative for SEB, SED, SEE, SEJ, SEK, SEU, and exfoliative toxin A or B (Table 1). The TSST-1 gene (705-bp) and SEA gene (774-bp) of the T1 and T2 strains were amplified, and the amplified sequences were determined. The determined sequences were in complete agreement with the previously described gene sequences for MRSA[13]. The toxin production levels were 256 ng/ml for TSST-1, 2048 ng/ml for SEA, and 16 ng/ml for SEC.

In the case of NTED-associated MRSA (e.g., strain E6), results were positive for TSST-1, SEC, SEG, SEI, and SET, but negative for SEA and SEH in the PCR assay (Table 1). Also, SSSS-associated MRSA (e.g., strain H5) was negative for all the previous superantigenic toxin genes (except for the SET gene) (Table 1).

When human peripheral blood mononuclear cells were stimulated with TSST-1, SEA, or SEC for 12 h, a marked production of IL-2 was observed (Fig. 3). The simultaneous addition of TSST-1 and SEA or TSST-1 and SEC resulted in higher levels of cytokine induction, and a combination of TSST-1, SEA, and SEC resulted in much higher levels of IL-2 induction (Fig. 3.).

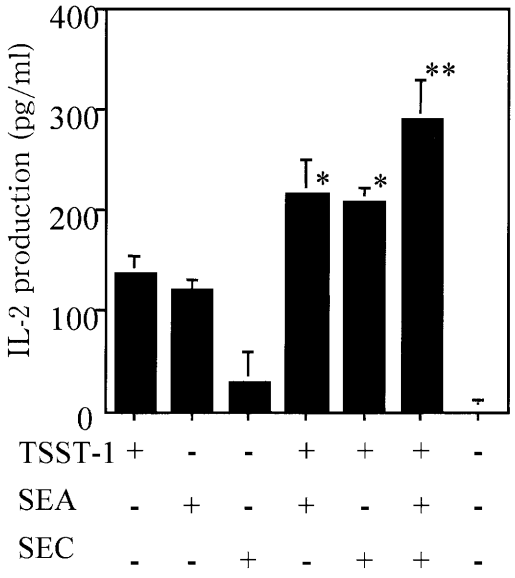

Fig. 3. Effects of a combination of superantigenic toxins, TSST-1, SEA, and SEC, on the IL-2 production in human peripheral blood mononuclear cells (PBMCs). PBMCs were stimulated with TSST-1 (10 ng/ml) , SEA (20 ng/ml), or SEC (1 ng/ml) for 12 h. The levels of IL-2 released from the cells were determined by ELISA. The results are presented as mean±SD of triplicate experiments. *$P<0.05$ versus TSST-1 only. **$P<0.05$ versus TSST-1 plus SEC (or TSST-1 plus SEA).

DISCUSSION

The initial report of TSS was made by Todd et al.[14], and supertoxigenic antigens have come to be associated with TSS. It has been reported that there are two types of TSS development: menstrual TSS (MTSS) associated with menstruation, and non-menstrual TSS (NMTSS) not associated with menstruation.

Regarding the associations of supertoxigenic antigens with TSS (Table 2), Bergdoll et al. reported that TSST-1 was associated with MTSS[15,16], and noted in 1990 that a combination of TSST-1, SEB, and SEC was associated with NMTSS[17]. In 1993, Kain et al. reported that a combination of TSST-1 and SEA was associated with MTSS[18]. In 1999, the development of TSS by a combination of SEG and SEI[7] and a combination of SEA and SEC[19] was proposed.

It has been reported that about 50% of MRSA isolates in hospitals in Japan possess TSST-1[20]. MRSA recently isolated at Niigata University Hospital was investigated, and almost all isolates had TSST-1 (unpublished data), while SEC was frequently linked with TSST-1. Although many hospital isolates of MRSA possess TSST-1, the development of TSS is very rare, and the reason for this is unknown. However, when TSS develops, it is very severe, and may lead to death[4].

In this study, the supertoxigenic antigen pattern was investigated in MRSA isolated from a TSS case, and the data were compared with those of previously described MRSA strains associated with NTED or SSSS.

TSS-associated MRSA isolates possessed seven types of superantigen genes: TSST-1, SEA, SEC, SEG, SEH, SEI, and SET. In addition, the expression of the TSST-1, SEA, and SEC genes were experimentally confirmed. These TSS-associated MRSA isolates possessed most of the supertoxigenic antigen genes reported to be associated with the development of TSS (Table 2).

In the case of NTED-associated MRSA strains, a lesser number of superantigenic toxins were found; e. g., strain E6 lacked SEA and SEH. Also, the SSSS-associated MRSA strain (H5) did not possess any important superantigenic toxins (except for SET). The role of SET as a virulence factor remains uncertain because all *S. aureus* isolates so far examined had the SET gene, irrespective of their isolation. For instance, *S. aureus* isolates from food poisoning and from examination of nasal mucosal swabs in routine practice to monitor *S. aureus* infection for medical staff and for medical students had the SET gene (unpublished data). Therefore, the SET gene seems to be a kind of "housekeeping gene" or "silent gene" of *S. aureus*, and may not be associated with the development of a well-defined clinical syndrome such as TSS.

Superantigenic toxins, such as TSST-1 and SEs, bind directly to the major histocompatibility complex (MHC) class II molecules on antigen-presenting cells and to the Vβ elements of antigen receptor of T cells (TCR). This trimolecular interaction leads to the activation of a vast number of V$\beta 2^+$ T cells to produce an excess amount of proinflammatory cytokines such as IL-2[3].

The IL-2 production in PBMCs was much higher when the cells were stimulated with a combination of TSST-1, SEA, and SEC simultaneously than when stimulated with the superantigenic toxins individually and with a combination of TSST-1 and SEC (or TSST-1 and SEA). The effect of the combination was at least additive. Although the details are being analyzed, it was clarified that the addition of SEA to a combination of TSST-1 and SEC further increased the supertoxigenic antigen activity of MRSA. SEA is considered to be related to food poisoning in Japan, and is less frequently detected in hospital MRSA isolates.

The results of this study suggest that the MST pattern consisting of TSST-1, SEA, SEC, SEG, SEH, and SEI are a risk factor in the development of TSS. Preparation of mutants of (e.g.) the SEA gene and analysis by animal experiments using the mutant strains will be necessary.

Acknowledgments. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, and a grant from Kampou Science Foundation, Japan.

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