Possible virulence factors of \textit{Staphylococcus aureus} in a mouse septic model

M. Tao *, H. Yamashita, K. Watanabe, T. Nagatake

Department of Internal Medicine, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto machi, Nagasaki 852, Japan

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Abstract

Twenty clinical isolates of \textit{Staphylococcus aureus} were examined to elucidate the virulence factors which are directly related to lethality in a mouse septic model. Heat or formalin treatment of the organism abolished the lethal activity of the live organism during challenge intravenously administered via the tail vein. Nevertheless, injection of ten times concentrated culture supernatant fluid (SUP) showed lethal activity in the mouse. However, there was no lethality when SUP was heated at 60°C for 15 min. To examine variations of SUP lethality among strains, we collected 20 strains of \textit{S. aureus} from four different hospitals. Then, we compared several factors for SUP lethality, which were the extracellular toxins and enzymes, such as toxic shock syndrome toxin 1, enterotoxin A, B, D, and hemolysins (α, β, γ), and also cytotoxic activity to human polymorphonuclear leukocytes and Vero cells. No difference was found among these factors except cytotoxic activity to Vero cells. Furthermore, we compared two strains in a mouse septic model according to the grade of bacteremia and lethal events. We found that mortality was higher with challenge by the strain whose SUP was lethal in comparison to the strain whose SUP was not lethal, even though the viable bacteria counts in the septic blood in both strains were not significantly different. This strongly supports the possibility that extracellular products, not the cell wall components, of \textit{S. aureus} play the key role in the lethal event in this mouse septic model. In addition, among the extracellular products, those which have cytotoxic activity to Vero cells may contribute to the lethality in sepsis caused by \textit{S. aureus} in this murine model. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: \textit{Staphylococcus aureus}; Gram-positive sepsis; Animal model

1. Introduction

\textit{Staphylococcus aureus} possesses various characteristics that are virulent to human organs [1], even though this organism is well known as a commensal bacterium in human anterior nares. Among numerous exotoxins and extracellular enzymes of \textit{S. aureus}, those such as toxic shock syndrome toxin 1 (TSST-1), enterotoxins, exfoliatin and coagulase have been characterized in relation to their invasiveness and certain specific human diseases, for example, toxic shock syndrome and staphylococcal scalded skin syndrome [1–3].

This organism remains a troublesome pathogen, especially in nosocomial infections, such as pneumo-
nia, infected pressure ulcers, intravenous catheter-associated bacteremia and sepsis, which originate from endogenously carried strains [4–8]. Moreover, spreading of methicillin-resistant *S. aureus* (MRSA) presents additional difficulties in control and management of staphylococcal disease in the hospital [9,10]. In spite of much recent research on the pathogenesis of Gram-positive sepsis in vitro and in vivo, the mortality rate from *S. aureus* has remained high [11,12].

Experimental and clinical results on Gram-positive sepsis have revealed some specific factors of *S. aureus* leading to lethal effects, such as TSST-1, K-hemolysin, exofoliatin [1], and also bacterial cell wall components [13–15]. The mechanism of staphylococcal sepsis in the clinical situation is still unclear. It appears possible that the mechanism of Gram-positive sepsis is different from that of Gram-negative sepsis, in which bacterial lipopolysaccharides play the key role. Furthermore, the pathogenesis of Gram-positive sepsis might be dependent on the characteristics of each strain, even within the same species.

In this paper, we focused on the pathogenesis of *S. aureus* in a mouse model of sepsis and evaluated possible virulence factors, such as exoenzymes, exotoxins, and cell wall components, of clinical isolates of *S. aureus* from various sources. Our purpose was to elucidate the lethal factors responsible for staphylococcal sepsis. Furthermore, candidate virulence factors of MRSA compared with methicillin-sensitive *S. aureus* (MSSA) are also discussed.

2. Materials and methods

2.1. Bacterial strains

Twenty strains of *S. aureus* were obtained from four hospitals situated in different locations of Nagasaki prefecture, Japan, in order to avoid the possible prevalence of a particular strain in a particular hospital. Those hospitals were (1) Department of Internal Medicine, Institute of Tropical Medicine in Nagasaki University Hospital, (2) Aino Memorial Hospital, (3) Kyourin Hospital and (4) Iki General Hospital. Most strains were isolated from sputum of patients with respiratory diseases, such as chronic bronchitis and bronchiectasis. To examine the inter-strain variability, several strains were collected from subcutaneous pus, infected pressure ulcers, bacteremic blood, and also from anterior nares of healthy carriers. Bacteria were stored in 25% sterile glycerol-containing Mueller-Hinton broth (MHB, Becton Dickinson, Cockeysville, MD, USA) and kept at −80°C until use.

2.2. Detection of β-lactamase production and determinations of minimal inhibitory concentrations of antimicrobial agents

For studies of antimicrobial susceptibility, minimal inhibitory concentrations (MICs) of several antimicrobial agents were determined by the standard agar dilution method according to the Japanese Society of Chemotherapy. Briefly, Mueller-Hinton agar plates containing appropriate concentrations of antimicrobial agents were inoculated with $10^4$ colony-forming units (CFU) of the bacteria. Plates were incubated for 18–20 h at 37°C in an incubator, and MICs were determined as the lowest concentration of the antimicrobial that inhibited macroscopic growth. The strains were tested against methicillin (DMPPC), cefotiam (CTM), cefmenoxime (CMX), imipenem (IPM), arbekacin (ABK), minocycline (MINO), erythromycin (EM), and ofloxacin (OFLX). MRSA was determined when the MIC to methicillin was equal to or greater than 12.5 mg ml$^{-1}$.

β-Lactamase production was determined by a disc impregnated with nitrocefin (Cefinase, BBL Microbiology System, Cockeysville, MD) according the instructions of the manufacturer.

2.3. Preparation of culture supernatant fluid (SUP) of *S. aureus*

Each *S. aureus* strain was grown in 5 ml of MHB overnight at 37°C, then 500 μl of cultured bacteria was inoculated in 100 ml of freshly prepared MHB, and incubated at 37°C for 18 h with shaking. Culture supernatant was recovered by centrifugation at $1000\times g$ for 20 min at 4°C, and was filtered through a 0.2-μm Millipore filter (Bedford, MA, USA). If needed, this culture supernatant fluid was concentrated by an ultrafiltrated concentrator, Minicom-B.
(Amicon, Inc., Beverly, MA, USA). These filtrated culture supernatants (described as SUPs in this paper) of each staphylococcal strain were stocked at −40°C for further examination of exotoxins, exoenzymes, and animal experiments. Similarly treated MHB without bacteria was used as a control for culture supernatant fluid.

2.4. Animal model (septic model)

2.4.1. Intravenous challenge of S. aureus-associated preparations

Overnight-cultured staphylococcal strains in MHB were washed twice in physiological saline (0.9% saline) by centrifugation at 3000 rpm at 4°C. The bacteria were adjusted to 1 × 10⁸ CFU ml⁻¹ by comparison with a known value at OD₆₆₀nm. Formalin was added to the cell pellet (final 7% in 0.9% saline) and incubated for 1 h at room temperature. After washing out the formalin three times with 0.9% saline by centrifugation, bacteria were suspended in 0.9% saline and used as formalin-treated bacteria. The bacterial cell suspension was heated for 20 min at 100°C and used as heat-killed bacteria.

Six-week-old, white female Institute of Cancer Research, USA (ICR) mice weighing 23–26 g (from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan) were intravenously challenged via the caudal vein with live S. aureus strain, formalin-treated or heat-killed S. aureus, and SUP. After intravenous challenge with each preparation, the numbers of surviving animals were tabulated at regular intervals.

2.4.2. Mouse septic model

Live strain SA-k, an MRSA strain, was injected via the caudal vein of white female ICR mice. At specified intervals, mice were anesthetized with ether and blood was drawn by cardiac puncture. Blood (100 µl) was streaked onto Mueller-Hinton agar plates to determine the viable bacterial count.

2.4.3. Determination of lethal inoculum for 50% of group (LD₅₀)

The LD₅₀ was calculated employing the method of Reed and Muench [16].

2.5. Serum bactericidal assay and opsonophagocytic assay

Human serum (NHS) was collected from five healthy volunteers, pooled and stored until use at −80°C. The NHS was employed for bactericidal assay and opsonophagocytic assay as a serum source (75% final concentration) or complement source (5% final concentration). Complement activity in the serum was inactivated by heating at 56°C for 30 min, when required.

For the preparation of bacterial inocula, overnight-cultured S. aureus strain was inoculated in 100 ml of fresh MHB, then incubated at 37°C for 18 h with continuous vigorous shaking. After washing three times with 0.9% saline by centrifugation, inoculum size was adjusted by reference to a standard curve correlating CFU with optical density (OD₆₆₀nm).

Serum bactericidal assay was done using NHS at a final concentration of 75% v/v, which was taken up in sterile 12×72 mm plastic tubes (Becton Dickinson Labware). Medium 199 (Gibco BRL) was used as diluent buffer. 1-ml reaction mixtures, containing 2×10⁶ CFU of the bacteria, and NHS were then incubated at 37°C for 2 h with gentle rotation by a vertical rotor. Immediately after the incubation, all tubes were vigorously vortexed and kept in an ice bath, and viable bacterial counts were determined by quantitative culture.

For opsonophagocytic assay, human polymorphonuclear leukocytes (PMN) were prepared from 30 ml of blood drawn from a healthy donor by 6% dextran sedimentation followed by the Ficoll-Hypaque extraction method. PMNs were suspended in Medium 199. After counting the viable PMNs by staining with trypan blue (Sigma), the number of PMNs was adjusted to 5×10⁶ cells ml⁻¹. The reaction mixtures for the opsonophagocytic assay consisted of bacterial suspension, PMNs and NHS (final ratio of bacteria:PMNs was 1:10). Finally 1 ml of the reaction mixture was incubated at 37°C for 2 h with continuous rotation with a vertical rotor. After the reaction, each tube was vigorously vortexed to disrupt the PMNs. Then viable staphylococcal cell numbers were determined by quantitative culture.
With each method, samples were tested in triplicate, and the results are given as mean ± S.D.

2.6. Detection of exoenzymes and exotoxins of S. aureus

Enterotoxins A, B, C, D and TSST-1 were detected by the reversed passive latex agglutination kit (SET-RPLA, TST-RPLA, Seiken, Japan). Determination of production and typing of coagulase was done using rabbit immune serum (staphylococcal coagulase-typing kit, Seiken, Japan). The production of hemolysins was determined by the assays described by Cutle [17], and Abramson and Friedman [18] with modification. Namely, blood agar plates containing 5% rabbit, sheep, or horse red blood cells were prepared for the estimation of the hemolysis by α-, β-, δ-toxin, respectively. 50 μl of SUP of each staphylococcal strain was poured onto three different blood agar plates, and after a 12-h incubation, hemolysis was estimated as the indicator for the detection of α-, β-, δ-hemolysins, respectively.

2.7. Cytotoxicity of SUP to human PMNs and Vero cells

2.7.1. Toxicity of SUP to human PMNs

The toxicity of SUP was examined against human PMNs. Human PMNs were prepared from 30 ml of blood by the same procedure as for the opsonophagocytosis assay which is described above. 150 ml of PMN suspension (5 × 10⁶ cells/ml) with Medium 199 was kept in 96-well microplates (Nunc-immuno plates, Bedford, MA, USA). 50 ml of filtrated SUP was added to each well and incubated for 4 h at 37°C in a CO₂ incubator (Napco, Model 6300, Precision Scientific, Chicago, IL, USA). Immediately after the incubation, viable cell numbers were counted by trypan blue staining. Cytotoxicity to PMNs was determined if the viable cell number was reduced more than 90% in comparison to the control.

2.7.2. Toxicity of SUP to Vero cells

Vero cells were maintained as a monolayer in 96-well microplates with Eagle’s MEM (Gibco BRL) containing 5% fetal bovine serum (FBS) and 5% calf serum. To each well, 50 μl of SUP was added and the degenerative effects on Vero cells were estimated by microscopic examination up to 24 h.

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**Fig. 1.** Survival curves after intravenous challenge with live S. aureus SA-k. Six mice, respectively, were challenged with live S. aureus SA-k via the caudal vein using different inoculum sizes: (●) 1.0 × 10⁷ CFU, (□) 3.3 × 10⁶ CFU, (▲) 1.1 × 10⁶ CFU, and (▲) 4.7 × 10⁵ CFU. Lethal effects of live organisms were inoculum dose-dependent. The highest concentration of live S. aureus resulted in marked lethality within 24 h. The LD₅₀ value of strain SA-k was 5.9 × 10⁶ CFU determined 24 h after intravenous challenge with live bacteria.

**Fig. 2.** Comparative mouse survival curves after intravenous challenge with S. aureus SA-k and its derivatives. There were six mice in each group. The figure shows the survival curves after injection of each derivative into the caudal vein: (●) live S. aureus SA-k; (●) formalin-killed S. aureus SA-k; (▲) heat-killed S. aureus SA-k at 100°C for 30 min; (▲) 100 μl of 10-fold concentrated SUP of S. aureus SA-k; and (▲) 100 μl of heat-treated SUP at 60°C for 15 min. Number of live or killed organisms in those derivatives was 6.0 × 10⁶ CFU. Formalin treatment and heating of live organisms completely abolished their lethal effect. However, intravenous injection of SUP of the same S. aureus SA-k strains resulted in almost the same lethality as that of live bacteria.
2.8. Statistics

Student’s $t$-test and the Fisher exact test were applied for analysis of data. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Characterization of $S. aureus$ strains of different origins

The strains of $S. aureus$ used in this experiment are summarized in Table 1. Coagulase types of 20 strains were as follows: type II (7 strains), types II, III (2 strains), type V (3 strains), type VII (3 strains) and undifferentiated type (5 strains). According to the antibiotic sensitivity to methicillin, eight of 20 strains were MRSA.

3.2. Intravenous challenge with $S. aureus$

Survival curves of mice challenged with the live $Staphylococcus$ strain are shown in Fig. 1. The highest inoculum size, $1.0 \times 10^7$ CFU, resulted in complete lethality within 24 h. There was less lethality...
with a lower inoculum size. These results indicate a dose-dependent effect of inoculum on mouse lethality. Other strains, strain SA-f (MSSA), SA-j (MRSA), and SA-l (MSSA), also showed the same dose-dependent lethality in relation to inoculum size (data not shown).

3.3. Comparative lethality of intravenous challenge with live S. aureus and its derivatives

As shown in Fig. 2, when mice were challenged with $6 \times 10^6$ CFU of live S. aureus, strain SA-k, all mice died within 72 h. However, when mice were challenged with formalin-treated or heat-killed organisms of the same bacterial concentration, lethal activity was completely abolished. Furthermore, SUP showed the same lethality as challenge with live strain. This result indicates that the lethal activity of live S. aureus upon challenge in mice by intravenous injection was not due to cell wall surface components but mainly to extracellular products or soluble factors present in the SUP. In addition, this lethality of SUP was completely inactivated by heating at 60°C for 15 min (Fig. 2).

To estimate inter-strain differences, the lethal activity of SUP of each of the 20 strains was examined by injection via the caudal vein. As shown in Table 1, lethality of SUP was clearly strain-dependent. The SUPs prepared from strains SA-k and SA-q represented high lethality which caused death of the mice within 1 h after intravenous injection of 200 μl of SUP. In contrast, no lethality was shown with SUPs of many strains.

3.4. Production of exotoxins and exoenzymes

For the examination of biologically active properties of extracellular products, exotoxins (enterotoxin A, B, C, D and TSST-1) and hemolysins were determined in SUPs of all 20 strains. The results are shown in Table 1. All strains whose SUPs possessed lethality in mice had the ability to produce either α-, β-, or δ-toxin (SA-e, f, k, l, q, r in Table 1). On the other hand, not all SUPs from strains which pro-
duced either α-, β-, or δ-toxin were lethal to mice. For example, although strains SA-c, g, j, s produced all three hemolysins (α-, β-, and δ-toxin), those SUPs were not lethal to mice by intravenous injection. In addition, TSST-1 production was not correlated with lethality.

3.5. Cytotoxicity of SUPs to human PMNs and Vero cells

The cytotoxicity of SUPs to human PMNs and cultured Vero cells was examined. No correlation was observed between SUP lethality and cytotoxicity to human PMNs (Table 1). In contrast, lethality and cytotoxicity to cultured Vero cells were strongly correlated among seven strains whose SUPs were shown to be lethal to mice, except for strain SA-1.

3.6. Antibiograms of 20 strains of S. aureus

Table 2 summarizes the MICs of 20 strains of S. aureus used in this experiment. There was no relationship between the virulence of SUP and particular antimicrobial resistance to the tested antibiotics, which were DMPPC, CTM, CMX, IPM, MINO, HBK, OFLX, and VCM.

3.7. Serum bactericidal assay and opsonophagocytic assay

3.7.1. Serum bactericidal assay

The sensitivity of S. aureus strains SA-1 (MSSA) and SA-j (MRSA) to NHS was examined. Fig. 3 shows the viable bacterial number after incubation of S. aureus with heat-inactivated NHS at 56°C for 30 min (b in Fig. 3) and fresh NHS (e in Fig. 3). Both strains are equally resistant to 75% NHS. Furthermore, SA-j (MRSA) grew more rapidly than SA-1 (MSSA), and NHS with or without heat inactivation accelerated the growth of both S. aureus strains in comparison to Medium 199 (a in Fig. 3).

3.7.2. Opsonophagocytic assay

Fig. 4 shows the opsonophagocytosis of SA-1 and SA-j strains by normal PMNs. In both strains, 5% of NHS (b in Fig. 4) had no bactericidal effect, but PMNs plus 5% NHS showed the greatest killing effect among other preparations.
3.8. Murine septic model

Mice were rendered septic by intravenous injection of live S. aureus via the caudal vein. In this experiment, two strains, SA-r and SA-t, were chosen to compare the relationship among viable bacteria counts in the blood, lethality and in vitro microbiological characteristics. Strain SA-r was lethal and strain SA-t was not lethal to mice in SUPs experiment (Table 1).

Fig. 5A shows a time course of the survival rate of 40 mice in each group. Inocula of SA-r and SA-t strains were 6.8 x 10^7 CFU and 5.0 x 10^6 CFU, respectively. At about 30 h after the initiation of infection, survival rate gradually decreased in the mice challenged with SA-r. In contrast to SA-r, a delayed and lesser degree of lethality was observed in SA-t-challenged mice. At 72 h, survival rate was 66.7% in SA-t-challenged mice and 40% in SA-r-challenged mice (P = 0.0017). Fig. 5B shows the time course of viable bacteria counts in the blood. In both groups, viable bacteria number in the blood decreased immediately after the injection. But after 30–42 h, bacteria increased up to 5 x 10^5 CFU ml^-1 and the same degree of bacteremia continued until 72 h. In contrast to survival rate (Fig. 5A), there was no significant difference between two groups as to viable bacteria counts in the blood at any time point. In these experiments two facts were observed. First, the beginning of a lethal event in the mouse is correlated with the time point at which the viable bacteria counts in the blood increase (from 30 h to 42 h). And, second, even though bacterial counts in the blood were not statistically different between the two groups, the survival rate in the SA-r-challenged mice was significantly lower than that in SA-t-challenged mice.

4. Discussion

S. aureus causes a variety of serious infections, such as pneumonia, endocarditis, abscesses, and sepsis, as well as superficial infections of the skin and mucous membrane [1,19].

Because of the commensality of this organism, both in the environment and in human beings, staphylococcal diseases are among the more troublesome nosocomial infections [7–9,11]. In addition, the recent acquisition of antimicrobial resistance as multi-resistant MRSA has raised difficulties in the management and treatment of nosocomial infections [9–12,20,21]. Furthermore, epidemiologic studies revealed that Gram-positive bacteria has markedly increased among nosocomial infections, especially caused by S. aureus, coagulase-negative S. aureus (CNS), and Enterococcus [22,23]. Thus, S. aureus should be considered a possible life-threatening organism which can lead to uncontrolled lethal bacteremia, especially in immunocompromised patients, even if it initially occurs as a superficial skin infection.

In contrast to our understanding of the signifi-
cance of *S. aureus* in the clinical field, the mechanism of the lethality of staphylococcal sepsis is still controversial.

In the present paper we focused on the possible lethal properties of *S. aureus* in the septic state and examined the multiple pathogenic factors that apply in a murine septic model.

When live *S. aureus* was intravenously challenged in the mouse, lethality was inoculum dose-dependent (Fig. 1). In this septic model, the virulent properties seemed to be attributed not to cell wall components but to the SUP which contained heat-labile extracellular products of *S. aureus* (Fig. 2). In this model, when a larger inoculum size (more than $5 \times 10^8$ CFU ml$^{-1}$) was used, the mouse died within 5 min (data not shown). This lethality seems not to be related to the toxic effects of enzymes and cell wall components, but rather caused by other mechanisms, such as cerebral emboli. Death may have occurred too early to be caused by direct lethality or cytokine-mediated lethality.

Recent studies demonstrated that cell wall components of Gram-positive bacteria have a central role in lethality in sepsis [13–15]. Lee et al. [15] demonstrated that highly encapsulated *S. aureus* was more virulent in mice compared to unencapsulated and microencapsulated mutants. Freudenberg et al. [13] demonstrated that killed Gram-positive bacteria produced lethality in *D*-galactosamine-treated mice that were intravenously challenged. They suggested the possible role of tumor necrosis factor $\alpha$ (TNF-$\alpha$) as an endogenous mediator of lethality. Kimpe et al. [14] also reported that peptidoglycan and lipoteichoic acid synergistically initiate shock and multiple organ failure in the rat, in which the release of TNF-$\alpha$ and inducible nitric oxide synthase was critical for the lethal activity. These studies actually demonstrate that cell wall components, such as capsule, lipoteichoic acid, and peptidoglycan, may be one of the virulent factors of *S. aureus*, where sepsis is followed by the release of TNF-$\alpha$ as a mediator [24–25]. In our experiment, $6 \times 10^6$ CFU of dead *S. aureus* did not cause death in mice, whereas 10 times concentrated SUP was lethal (Fig. 2). This lack of lethality of dead *S. aureus* might be due to the relatively small amount of cell wall components. However, it seems doubtful that such a septic model in an animal which is produced by intravenous injection of a large inoculum is suitable for gaining a better understanding of sepsis in clinical situations.

Among the extracellular products of *S. aureus*, it has been proved that TSST-1, enterotoxins, and pyrogenic exotoxin A can elicit shock [26–28]. Furthermore, it is suggested that exotoxins may accelerate the ability of cell wall components to cause staphylococcal septic shock [19,29].

From those observations, including our results, it can be suggested that septic lethality in humans caused by *S. aureus* may not be simply attributed to a specific property of the organism, but is a more complicated procedure, which is caused by the cell wall components, exotoxins, and enzymes which will be affected by, for example, the severity of focal infection, the grade of bacteremia, and also the immune condition of the host. From the clinical assessment of septic shock and multiple organ failure, Ruokonen et al. [30] concluded that refractory hypotension was the main cause of early death and that subsequent multiple organ failure was a later cause of death. This result indicates that virulent factors of septic shock may differ according to the time phase of sepsis.

In recent studies, Peacock et al. [20] examined the virulence factors of an epidemic strain of MRSA. They concluded that the epidemic strain, MRSA-Va, appeared to be more virulent in comparison to the two control MSSA strains. But they could not clearly demonstrate a particular property directly associated with the death of the mouse after intraperitoneal (i.p.) challenge of the culture supernatant or after i.p. and i.v. challenge of the live organisms. In our experiments, the fact that lethal activity of SUP was completely abolished by heating at 60°C for 15 min (Fig. 2) suggested that the virulence properties in SUP seem not to be cell wall components which were released during the bacterial growth, but some other heat-labile factors.

It has been proposed by many that the specific properties of extracellular products of *S. aureus* are related to particular pathogenic states. For example, TSST-1 and staphylococcal enterotoxin B (SET-B) are associated with TSS [26,28], and $\alpha$-toxin [31], coagulase [32], and leukocidin are associated with keratitis, endocarditis and dermonecrosis, respectively [33].

From this point of view, we examined the possible
virulence factors in SUPs with 20 strains of S. aureus, such as coagulase, enterotoxins A, B, C, D (SET-A, B, C, D), TSST-1, hemolysin, and cytotoxin to human PMNs and Vero cells (Table 1). Ten-fold concentrated SUP from 20 strains of S. aureus showed different grades of lethality to mice when injected intravenously. Nevertheless, no correlation of virulent factors with SUP lethality was identified, except cytotoxic activity of SUP to Vero cells. Namely, SET-A–D and TSST-1 were not necessary to cause lethality, while most strains (strains SA-e, f, k, l, q, r, in Table 1) which had lethal SUPs produced all three hemolysins: K-toxin, L-toxin, and N-toxin. In contrast, SUPs from the strains which could produce those three hemolysins (strain SA-c, s in Table 1) did not always exhibit lethality in the mouse. Furthermore, our results showed that most lethal SUPs possessed cytotoxicity not only to human PMNs but also to Vero cells, suggesting that toxin-like leukocidin may not be necessary to elicit lethality in mice in this model. In addition, lethal SUPs were not related to antibiotic susceptibilities of original strains (Table 2), such as whether strains were MRSA or MSSA.

From our observations and those of others, two explanations are possible for the lethality of SUPs. First, extracellular products of S. aureus, including toxins and enzymes, may contribute to lethality in the mouse in some synergistic manner. The function of staphylococcal exotoxins has been well characterized. K-toxin exhibits cytolysis in many mammalian cells [1, 34–36] and causes dermonecrosis [1], keratitis [31], and mastitis [37]. L-toxin [1, 37] and N-toxin, which have a broad range of target cells [38, 39], have cytolytic activity. Despite all those known in vitro and in vivo characteristics of staphylococcal toxins, the actual virulent properties of S. aureus in sepsis remain unclear. Because we did not measure the actual concentration of α-toxin in SUP, our result that α-toxin-positive SUP was not necessary to cause death of mice might not adequately explain septic shock, in which the amount of toxin seemed to be critical.

Second, our finding that in vivo lethality is strongly correlated with the cytotoxicity to Vero cells of SUP (Table 1) suggests the possibility that some other undetermined virulent properties in SUPs may exist. Interestingly, Noda et al. [40] found a novel cytotoxin from a pathogenic strain of S. aureus which was isolated from patients suffering from serious cholera-like diarrhea. They succeeded in purification and crystallization of that toxin and revealed that this toxin was fully lethal, inducing myocardial dysfunction.

In order to confirm the virulence of SUP, we examined the grade of bacteremia and the lethal events that followed intravenous injection of live S. aureus using the murine sepsis model. When two strains, SA-r and SA-t, which possess different characteristics regarding lethality of SUP (Table 1) were compared in the in vivo septic model, the overall mortality of mice challenged with SA-r was much greater than that of mice challenged with SA-t even though the viable bacteria numbers in the blood of both strains were not statistically different (Fig. 5). This higher mortality in SA-r-challenged mice indicates that the mechanism leading to death at a relatively late phase (30 h after the intravenous challenge with live S. aureus) cannot be attributed to cell wall components but to extracellular products. On the other hand, we found that the number of surviving mice challenged with strain SA-t also gradually decreased. This indicates that the cell wall components may also, at least partially, play a role in lethality to mice in a septic state.

Recent advances in research on the actual mediators of septic shock, such as interleukins, TNF, and nitric oxides, [13, 29, 30, 41], have revealed the complexity of the mechanism of the mortality in both Gram-positive and Gram-negative septic shock. Results from studies using our septic mouse model indicate the importance of extracellular properties such as cytotoxin. Nevertheless, numerous studies searching for the virulent properties in septic mortality suggest many possible factors, including cell wall components, according to the experimental model used. With this in mind, it can be proposed that detailed studies utilizing both clinical and bacteriological analysis in staphylococcal sepsis should be done in septic patients. In addition, establishment of a suitable animal model representing the human septic state will be required.

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