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Staphylococcus aureus Resists Human Defensins by Production of Staphylokinase, a Novel Bacterial Evasion Mechanism[1]

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α-Defensins are peptides secreted by polymorphonuclear cells and provide antimicrobial protection mediated by disruption of the integrity of bacterial cell walls. Staphylokinase is an exoprotein produced by *Staphylococcus aureus*, which activates host plasminogen. In this study, we analyzed the impact of interaction between α-defensins and staphylokinase on staphylococcal growth. We observed that staphylokinase induced extracellular release of α-defensins from polymorphonuclear cells. Moreover, a direct binding between α-defensins and staphylokinase was shown to result in a complex formation. The biological consequence of this interaction was an almost complete inhibition of the bactericidal effect of α-defensins. Notably, staphylokinase with blocked plasminogen binding site still retained its ability to neutralize the bactericidal effect of α-defensins. In contrast, a single mutation of a staphylokinase molecule at position 74, substituting lysine for alanine, resulted in a 50% reduction of its α-defensin-neutralizing properties. The bactericidal properties of α-defensins were tested in 19 *S. aureus* strains in vitro and in a murine model of *S. aureus* arthritis. Staphylococcal strains producing staphylokinase were protected against the bactericidal effect of α-defensins. When staphylokinase was added to staphylokinase-negative *S. aureus* cultures, it almost totally abrogated the effect of α-defensins. Finally, human neutrophil peptide 2 injected intra-articularly along with bacteria alleviated joint destruction. In this study, we report a new property of staphylokinase, its ability to induce secretion of defensins, to complex bind them and to neutralize their bactericidal effect. Staphylokinase production may therefore be responsible in vivo for defensin resistance during *S. aureus* infections. *The Journal of Immunology*, 2004, 172: 1169–1176.

The α-defensins are a group of bactericidal peptides characterized by a cationic charge and six invariant cysteine residues that form three disulfide bonds. Human neutrophil peptides (human neutrophil peptide (HNP) 1 and HNP-2) are originating from the same protein, being inactive in its original conformation (1). HNPs are broadly expressed by epithelial and hemopoietic cells, being especially abundant in azurophilic granules of polymorphonuclear leukocytes. Defensins are an essential part of the host innate immune system responsible for the first line of defense against pathogenic microorganisms (1). In addition, defensins also play a role in adaptive antimicrobial immunity by enhancing specific Ab responses and promoting maturation of dendritic cells (2).

In vitro, HNPs display cytolytic effects directed against bacteria, fungi, and viruses (3, 4). HNP-1 and HNP-2 are similar with respect to their antimicrobial properties. Cytolysis is mediated by interactions with anionic phospholipids on the bacterial surface leading to the permeabilization of microbial membranes by pore formation (5). It was recently shown that modification of lipids of the *Staphylococcus aureus* cytoplasmic membrane attenuated susceptibility of bacteria to host defensins (6). The membranes of host cells are protected from the hazardous effects of α-defensins by their high content of cholesterol and neutral phospholipids on their surface (7).

S. aureus is a bacterial species frequently encountered both in the hospital setting and as community infections. It gives rise to a diverse spectrum of diseases ranging from minor cutaneous and wound infections to life-threatening conditions such as sepsis, endocarditis, septic arthritis, and osteomyelitis (8, 9). Staphylokinase (SAK) is a 136-aa protein produced by lysogenic strains of *S. aureus*. The gene for SAK is carried by certain prophages and its synthesis is positively regulated by the accessory gene regulator (10) and negatively regulated by staphylococcal accessory regulator (11). SAK facilitates activation of plasminogen, the precursor of the fibrinolytic protease plasmin (12). Structurally, SAK resembles other plasminogen activators, containing a plasminogen-binding site and a serine protease domain (13). However, SAK is not an enzyme. It forms a 1:1 stoichiometric complex with plasmin(ogen) that converts other plasminogen molecules to plasmin, a potent enzyme that degrades proteins of the extracellular matrix. The high affinity of the SAK-plasminogen complex for fibrin makes it a promising thrombolytic agent (12, 14), a property that is exploited in treatment of acute myocardial infarctions and arterial thromboses. There is only limited knowledge of the role of SAK in staphylococcal infection. SAK might facilitate *S. aureus* to bind host plasminogen through bacterial cell surface receptors (15) and thereby to promote invasion of host tissues (16).

In the present study, we demonstrate that staphylokinase directly interacts with the host innate immune system. Indeed, SAK abrogates the bactericidal effect of α-defensins. We also show that

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[3] Abbreviations used in this paper: HNP, human neutrophil peptide; SAK, staphylokinase; THB, Todd-Hewitt broth; PPACK, H-D-Pro-Phe-Arg-chloromethylketone.

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bacterial SAK production leading to neutralization of host defensins is a new evasion mechanism facilitating *S. aureus* survival.

Materials and Methods

Reagents

Todd-Hewitt broth (THB) and horse blood agar were obtained from Difco (Boule Nordic, Huddinge, Sweden). Human Glu-plasminogen was obtained from Biopool (Umea, Sweden). HNP (HNP-1, 3442 g/mol, and HNP-2, 3371 g/mol) as well as H-D-Pro-Phe-Arg-chloromethylketone (PPACK) were purchased from Bachem (Feinchemikalien AG, Switzerland). Recombinant SAK (sakSTAR, 16,000 g/mol) and SAK variant K74A containing a single substitution of lysine for alanine at position 74 (17) were a kind gift from Dr. Y. Laroche and Prof. D. Collen (Center for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute of Biotechnology, Leuven, Belgium). Endotoxin content in the SAK preparation was 36 endotoxin units/ml (0.36 ng/1 μg of SAK) as assessed by the *Limulus* amebocyte lysate test (Charles River Endosafe, Charleston, SC). Staphylococcal peptidoglycans were a kind gift from Prof. S. J. Foster (Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, U.K.). The plasmin-specific synthetic substrate H-D-Val-Leu-Lys-Paranitroanilide (S-2251) was purchased from Chromogenix (Mölndal, Sweden). BSA fraction V and DMSO were purchased from Sigma-Aldrich (St. Louis, MO).

Isolation and characterization of S. aureus *strains*

Nasal carriage isolates were obtained from the anterior nares of healthy Swedish volunteers (40 males, 58 females; age, 19–91 years, mean, 36 years) by rotating a sterile Dacron fiber-tipped swab within both nostrils. Nasal secrete was spread on casein-gelatin agar plates containing NaCl (7.5%) to select for staphylococci. Colonies were later transferred to horse blood agar plates and tested for coagulase production using rabbit EDTA plasma (BD Microbiology Systems, Sparks, MD). Ninety-seven coagulase-positive *S. aureus* isolates were found and subsequently evaluated for the SAK production.

This study was approved by the Ethic Committee of Sahlgrenska Hospital and animal experimentation guidelines were followed.

In addition, the following *S. aureus* strains were used: Newman, RN6390 (18), LS-1 (19), P1 (20), 1061 (21), MRSA67–0 (a kind gift from Dr. J. M. Patti, Inhibitex Inc., Alpharetta, GA). *Escherichia coli K-type*, *Streptococcus bovis*, and *Staphylococcus epidermidis* strains were obtained from the Laboratory of Microbiology, Sahlgrenska University Hospital (Göteborg, Sweden).

Staphylococcal strains were cultured on blood agar plates for 24 h, harvested, and kept frozen at −20°C in PBS containing 5% BSA and 10% DMSO. Before experiments, the bacterial solution was thawed, washed in PBS, and adjusted to the concentration required.

Genetic manipulation of sak

Oligonucleotide primers complementary to the *sak* gene and *spa* promoter nucleotide sequences (Table I) were designed according to the *S. aureus* NCTC 8325 genome sequence (http:www.genome.ou.edu/staph.html). Restriction sites for *Bam*HI, *Nco*I, or *Eco*RI were incorporated at the 5′ end of each primer to facilitate creation of an in-frame fusion between the *spa* and *sak* initiation codons, as well as directional cloning into vectors. Genomic DNA was isolated from a 2-ml overnight culture of *S. aureus* using the AGTC bacterial genomic DNA purification kit (Edge BioSystems, Gaithersburg, MD). PCR amplifications were conducted in a DNA thermal cycler (PCR Sprint; Thermo Hybaid, Middlesex, U. K.) in a total volume of 100 μl containing 100 ng of bacterial genomic DNA, 100 pM primers, 250 μM dNTPs, 10 mM $MgCl_2$, and 5 U of Pfu polymerase in standard Promega Pfu reaction buffer (Madison, WI). Amplified DNA products were analyzed by agarose gel electrophoresis and purified using the PCR product purification kit (Roche Diagnostics, Mannheim, Germany) and then cleaved with appropriate restriction enzymes.

Two SAK expression systems were constructed: 1) a 1322-bp PCR fragment containing the *sak* gene with 5′ and 3′ flanking sequences, including the putative promoter, was cloned into the single copy integrating plasmid pCL84 (22). (ii) The 415-bp PCR-amplified *spa* promoter fragment and the 897-bp PCR-amplified *sak* gene were cloned into pCU1 (23), forming pCU1*sak*. The *sak* gene was linked in-frame to the *spa* initiation codon which had been converted by PCR from TTG to ATG. The resulting plasmid was used as a template for cloning the *spa-sak* fusion into pCL84. Chimeric plasmids were transformed by electroporation into *S. aureus* CYL316 where the pCL84 derivatives integrated into the lipase (*geh*) gene directed by the phage L54a integrase. The integrated plasmid was subsequently transduced into strain LS-1 using phage 85 (24).

Assessment of HNP influence on S. aureus *growth*

A standard number of bacteria (10^3/ml in THB) was incubated with increasing concentrations (range, 0–10 μg/ml) of HNP-1 or HNP-2. At specific time intervals, samples of the bacterial mixtures (0.1 ml) were spread on horse blood agar. After incubation for 18 h at 37°C, colonies were counted. The number of CFU in the intact and HNP-treated staphylococcal cultures were assessed as a percentage of viable bacteria in comparison to intact cultures. The latter was referred to as the bactericidal effect of α-defensins. The effect of exogenous SAK on the bactericidal properties of α-defensins was evaluated by comparing staphylococcal survival in the presence of SAK/HNP mixtures and HNP alone. Results were recorded as the reduction of the bactericidal effect of HNPs.

Culturing of S. aureus *strains for SAK production*

S. aureus isolates were cultured on horse blood agar and a single colony was transferred into 2 ml of THB and incubated for 18 h at 37°C. Bacterial cells were harvested by centrifugation (4000 × *g*, 10 min, 4°C). The supernatant was tested for the amount of SAK released. For the evaluation of surface-bound SAK activity, bacterial cells were resuspended in THB at 10^9 CFU/ml and incubated for 4 h at 37°C with or without human Glu-plasminogen (2×10^{-7} M). Excess plasminogen was removed by two washes with PBS. Bacterial cells were resuspended in Tris-HCl buffer (50 mM, pH 7.4) containing the plasmin substrate (S-2251, see below) and incubated for another 30 min to allow SAK-dependent activation of plasminogen attached to the bacterial surface.

Determination of SAK production and SAK-dependent plasmin generation

SAK activity was determined by hydrolysis of a specific plasmin substrate in the presence of plasminogen. Human Glu-plasminogen (2×10^{-7} M in Tris-HCl buffer, 50 mM, pH 7.4) was incubated with culture supernatants or bacterial cells at 37°C for 10 min to allow plasmin formation. Plasmin-dependent hydrolysis of the chromogenic substrate H-D-Val-Leu-Lys-paranitroanilide (S-2251, 4×10^{-4}M) was measured at 405 nm. The amount of SAK was estimated by reference to dilutions of recombinant SAK (sakSTAR). The sensitivity of the assays was set to 0.3 μg/ml. Samples with absorbance values below 0.3 μg/ml were considered as negative.

To evaluate the effect of α-defensins on SAK-dependent generation of plasmin, human Glu-plasminogen (1×10^{-7} M in 50 mM Tris-HCl buffer, pH 7.4) was incubated with SAK (5 μg/ml) or SAK/HNP mixture (5 + 5

Table I. *Oligonucletide primers used for amplification of the* sak *gene and* spa *promoter*

Primer	Primer sequence	Plasmid
SAKflankF	CGCGGATCCGGTCAAAAGGTGCTTATTTTAATG	pCL84
SAKflankR	CCGGAATTCTATATACTCTCTATCATAAGAAAAAAC	pCL84
Spa*Hin*dF	CCCAAGCTTCTATTACGCAAGTGTGCTGT	pCU1/Spa
Spa*Nco*1R	CGCGGATCCCCATGGATTAATACCCCCTGT	pCU1/Spa
SAK*Nco*1F	CATGCCATGGCACTCAAAAGAAGTTTATTATTTTTAACT	pCU1
SAK*Eco*RR	CCGGAATTCTATATACTCTCTATCATAAGAAAAAAC	pCU1
Spa*Bam*HF	CGCGGATCCCTATTACGCAAGTGTGCTGTATT	pCL84/spaSAK
SAK*Eco*RR	CCGGAATTCTATATACTCTCTATCATAAG	pCL84/spaSAK
SAK*Nco*1F	CATGCCATGGCACTCAAAAGAAGTTTATTATTTTTAACT	SAK
SAKstopR	CCGGAATTCTTATTTCTTTTCTATAATAACCTTTGT	

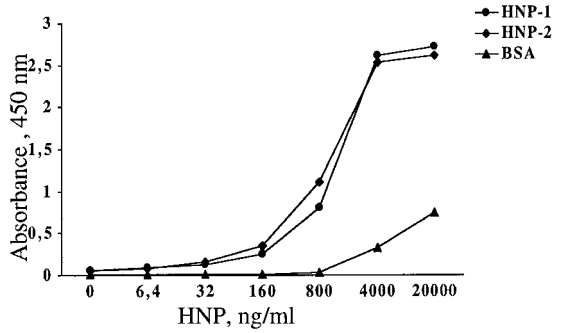

FIGURE 1. Complex formation between SAK and α-defensins. ELISA wells were coated with rSAK, 10 μg/ml, or an equimolar amount of BSA. HNP-1 or HNP-2 was added at different concentrations as indicated on the *x*-axis followed by incubation with HNP-specific Abs. The *y*-axis represents the absorbance values obtained at the given HNP concentration in the SAK and the BSA-coated wells.

μg/ml) at 37°C for 10 min to allow plasmin formation. Plasmin-dependent hydrolysis of S-2251 (4×10^{-4} M) was registered spectrophotometrically at 405 nm. Control experiments were performed using SAK preincubated with an excess of PPACK (10^{-4} M).

Preparation of SAK-defensin complexes

To allow the reaction between SAK and HNPs, 5 μg/ml rSAK was incubated with increasing amounts of HNP-1 and HNP-2 (0–30 μg/ml) or with an excess of PPACK (10^{-4} M) in 50 mM Tris-HCl buffer (pH 7.4) for 30 min at 37°C. The ability of the SAK/HNP and SAK/PPACK mixtures, and each of the components separately, to hydrolyze S-2251 (4×10^{-4} M) was tested in the presence of plasminogen (2×10^{-7} M). The absorbance values were registered at 405 nm and the results were evaluated with respect to standard dilutions of Glu-plasminogen.

Analysis of SAK-HNP complex formation

The 96-well flat-bottom polyester ELISA plate (Nunc, Roskilde, Denmark) was coated with rSAK (10 μg/ml) in carbonate buffer (pH 9.6) overnight. The control wells were coated with equimolar amounts of BSA. The plates were washed with PBS/0.05% Tween 20 and blocked with 1% Tris-BSA (pH 7.4) at 37°C for 1 h. After washing, different amounts of HNPs (6.4–20,000 ng/ml) were added and incubated for 1 h at 37°C. After washing, SAK-immobilized HNP was detected by addition of biotinylated anti-human HNP1–3 Abs (Hycult Biotechnology, Enskede, Sweden) for 1 h at room temperature, followed by avidin-HRP and enzyme substrate.

Effect of SAK on the course of S. aureus *arthritis*

Female 5- to 6-wk-old NMRI mice were purchased from ALAB (Stockholm, Sweden) and maintained in the animal facility of the Department of Rheumatology and Inflammation Research, University of Göteborg. They were housed 10–11 animals per cage under standard conditions of temperature and light and fed standard laboratory chow and water ad libitum. *S. aureus* strain LS-1 (19) was used to induce septic arthritis. An arthritogenic dose of *S. aureus* (8×10^4 CFU/knee) was injected in the left knee joint of mice either alone ($n = 8$) or along with HNP-2 ($n = 8$), SAK ($n = 10$), or SAK/HNP-2 mixture ($5 + 5$ μg/knee; $n = 9$) in a 20-μl volume. Intra-articular injections of the increasing amounts of SAK or HNP (0.3–30 μg/knee) were also performed to exclude an arthritogenic effect of these substances. Control experiments were performed using intra-articular injections of purified *S. aureus* peptidoglycans alone (50 μg/knee; $n = 9$) or in combination with HNP-2 ($50 + 5$ μg/knee; $n = 9$). The arthritogenic dose of peptidoglycans was established in a previous study (25). Mice were sacrificed on day 4 after injection and the knee joints were submitted to morphological evaluation. Histological examination of joints was done after routine fixation, decalcification, and paraffin embedding of the tissue. Tissue sections of the knee joints were cut and stained with H&E. All of the slides were coded and evaluated blindly by two investigators with respect to the inflammatory cell infiltration of synovia, pannus formation, and cartilage and subchondral bone destruction. Intensity of inflammatory cell infiltration of synovia and severity of bone destruction were graded arbitrarily from 0 to 3.

To assess bacterial viability in the injected joint, healthy mice were injected with an arthritogenic dose of *S. aureus* (8×10^4 CFU/knee) alone ($n = 9$), simultaneously with HNP-2 (5 μg/knee; $n = 9$), or with SAK/HNP-2 ($5 + 5$ μg/knee; $n = 9$). Four days after injection, mice were sacrificed and samples from the joints were spread on casein-gelatin agar plates containing NaCl (7.5%) to select *S. aureus.*

Isolation and stimulation of human neutrophils

Peripheral blood neutrophils from healthy individuals were isolated from buffy coats using dextran sedimentation and Ficoll-Paque gradient centrifugation (26). The cells were washed and resuspended in Krebs-Ringer phosphate buffer (KRB, pH 7.3) containing glucose (10 mM), Ca^{2+} (1 mM), and Mg^{2+} (1.5 mM) and stored on ice until used.

Neutrophils (5×10^5/ml) were incubated along with various amounts of SAK in KRB (0.1–10 μg/ml) or KRB as a control for the time period of 1–30 min at 37°C. Neutrophil suspension was than centrifuged at $500 \times g$ for 5 min and supernatants were collected. The collected supernatants were further analyzed for concentration of defensins by ELISA using biotinylated anti-human HNP1–3 Abs (Hycult Biotechnology) as described above.

Statistical analyses

The difference between groups with respect to SAK production and HNPs was analyzed by the Mann-Whitney *U* test. The difference between treatment groups were analyzed by the Wilcoxon signed rank test. The data are presented as mean ± SEM. A value of $p < 0.05$ was considered to be statistically significant.

Results

Complex formation between SAK and HNP molecules

To assess possible physical interaction between the HNP and SAK molecules, increasing amounts of HNPs (6.4–20,000 ng/ml) were incubated with rSAK that was immobilized in polystyrene wells. Subsequent addition of HNP-specific Abs led to a dose-dependent increase in absorbance of the wells containing SAK but not in those containing control protein (BSA) (Fig. 1). Incubation with HNP-1 and HNP-2 showed almost identical binding patterns. These results indicate that both HNP isotypes display dose-dependent binding to SAK.

The bactericidal effect of α-defensin

The influence of α-defensins on bacterial survival was assessed using *S. aureus* strain LS-1. The bactericidal properties of α-defensins were evaluated with respect to their concentration and incubation time (Fig. 2). In the concentration range (1–10 μg/ml), the bactericidal effect of defensins was directly related to their

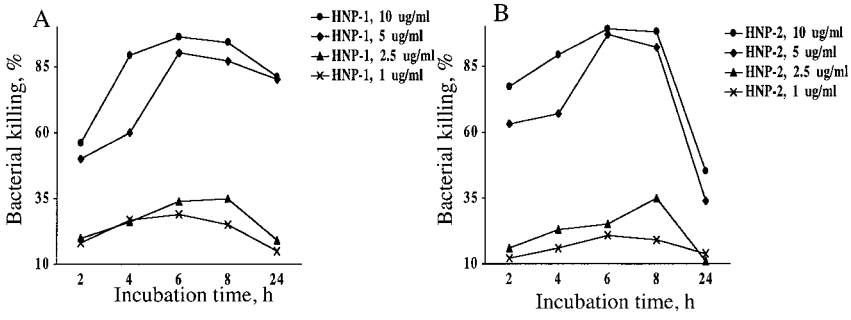

FIGURE 2. Bactericidal effect of α-defensins is time limited. *S. aureus* strain LS-1, 10^3 CFU/ml, was incubated either with HNP-1 (*A*) or HNP-2 (*B*) in vitro. Bacterial growth was monitored at time intervals indicated on the *x*-axis.

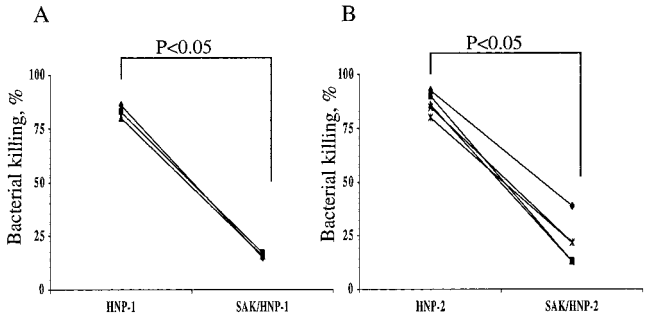

FIGURE 3. Survival of non-SAK-secreting *S. aureus* strains incubated with α-defensins or a mixture of SAK and α-defensins. *S. aureus* strains (10^3 CFU/ml) were incubated either with HNP-1 or HNP-2 (5 μg/ml) alone or in combination with SAK (5 plus 5 μg/ml).

concentration, resulting in >50% bacterial killing in the samples treated with 5 μg/ml and approaching 90% in the samples treated with 10 μg/ml HNPs following 4 h of incubation. With respect to the incubation time, the bactericidal effect of HNPs increased during the first 6–8 h of incubation and then leveled off in the case of HNP-1, whereas it decreased in the case of HNP-2 (Fig. 2). In all subsequent experiments, 10^3 CFU/ml bacteria were incubated with HNP-1 or HNP-2 at 5 μg/ml for 6 h.

Exogenous SAK abrogates the bactericidal effect of α-defensins

The influence of exogenous SAK on the bactericidal effect of HNPs was studied using five *S. aureus* strains that did not produce SAK. Bacterial survival of each strain was compared following 6 h of incubation with SAK-HNP complexes or one of the components, respectively. Viable counts of bacteria exposed to HNPs or SAK-HNP complexes were compared with bacteria incubated with SAK. Incubation of *S. aureus* with HNP-1 reduced bacterial viability from 100 to 18% and with HNP-2 from 100 to 8% (Fig. 3). In contrast, bacterial viability was considerably higher in samples incubated with SAK/HNP mixtures, demonstrating neutralization of HNPs bacteriolytic properties following incubation with SAK (both $p < 0.05$, Fig. 3). SAK was equally effective at neutralizing both HNP-1 and HNP-2 peptides. To assess whether the neutralizing effects of SAK regarding α-defensins were also valid for other microorganisms, the growth of *E. coli*, *S. bovis*, and *S. epidermidis* was tested after incubation with HNP-2 and SAK-HNP-2 complexes. Even here the bactericidal effect of HNPs was eliminated by SAK in each case (98, 75, and 87%, respectively).

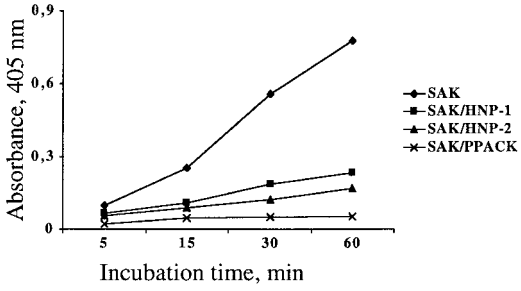

FIGURE 4. Defensins abrogate the plasminogen-activating properties of SAK. SAK-dependent activation of plasminogen to plasmin was assessed by cleavage of plasmin-specific chromogenic substrate S-2251 changes and was monitored at 405 nm. SAK-HNP complexes were prepared by incubation of 5 μg/ml HNP-1 or HNP-2 with 5 μg/ml SAK for 30 min at 37°C. Mixture of SAK/PPACK (5 μg/ml plus 0.5 mg/ml) was used as a positive control.

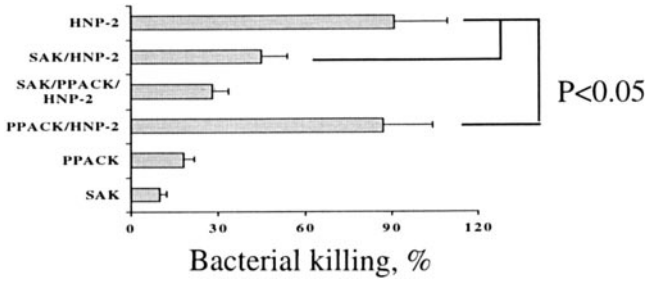

FIGURE 5. Inhibition of α-defensins by SAK is independent on its ability to activate plasminogen. Bacterial growth was monitored when *S. aureus* strain LS-1, 10^3 CFU/ml, was incubated either with HNP-2 (5 μg/ml) alone, HNP-2 (5 μg/ml) in combination with SAK (5 μg/ml), or with the SAK/PPACK mixture (5 μg/ml plus 0.5 mg/ml). Results presented are cumulative for two experiments, each including three *S. aureus* strains unable to produce SAK.

These observations allowed us to conclude that complex formation between SAK and HNPs resulted in almost total abrogation of the bactericidal effect of α-defensins. Moreover, SAK neutralized α-defensin-mediated killing of both Gram-positive and Gram-negative bacteria.

Abrogation of SAK-dependent plasminogen activation by α-defensins

Plasminogen activation was previously seen as the only biological property of SAK. To evaluate whether α-defensins influence the plasminogen-activating capacity of SAK, we tested SAK/HNP-1, SAK/HNP-2, and SAK/PPACK mixtures for their ability to facilitate plasminogen cleavage to plasmin. Plasmin generation was monitored colorimetrically following cleavage of plasmin-sensitive substrate. Plasminogen generation in the presence of SAK/HNP mixtures was significantly less efficient compared with SAK alone (Fig. 4). Plasmin generated by SAK/HNPs mixtures comprised only 37% (31 ± 18%) of plasmin generated by SAK alone ($p < 0.05$). Incubation of SAK with an excess of the synthetic proteinase inhibitor (PPACK) totally prevented SAK-dependent activation of plasminogen.

Structural requirements of SAK to neutralize α-defensins

To assess whether proteinase activity of SAK was essential for its neutralization of α-defensins, SAK inactivated with PPACK was further incubated with HNP, and prepared thrimeric complexes were introduced to bacterial culture. Survival of *S. aureus* strain LS-1 in the presence of the preformed trimeric complex SAK/PPACK/HNP-2 and HNP-2, SAK/HNP-2, respectively, was assessed (Fig. 5). Both SAK and the SAK-PPACK complex abrogated HNP-2-promoted bacterial killing, reducing it from 90 to 45% and 23%, respectively. In contrast, PPACK alone did not

Table II. *Consequence of a single amino acid substitution (K74A) within the SAK molecule on the bactericidal effect and plasminogen-activating properties of the SAK-HNP complex*

	Bactericidal Effect (%)	Plasminogen Activation (%)
HNP-2 + SAK	35	35
HNP-2 alone	93	0
HNP-2 + SAK M8 (K74A)	70	15
HNP-2 + SAK SY46 (K35A E65D K74Q E80A D82A K130T K135R)	77	36
SAK alone	0	100
SAK (K74A) alone	0	89

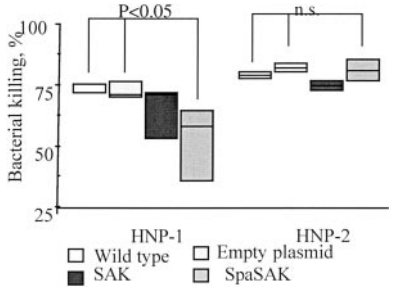

FIGURE 6. Bactericidal effect of α-defensins on *S. aureus* strains isogenic for SAK production. Impact of α-defensins on bacterial growth of four *S. aureus* LS-1 strains isogenic for SAK expression was studied in vitro. The growth of staphylococci (10^3 CFU/ml) was monitored in the presence or absence of HNP-1 or HNP-2 (5 μg/ml). Mean of four independent experiments is provided.. Boxes represent SD whereas horizontal lines within each box represent median values. LS-1 wild type, not producing SAK; LS-1 carrying empty plasmid, not producing SAK; LS-1*sak*, transgenic strain carrying *sak* gene and producing SAK; LS-1*spa*SAK, strain transduced with *spa* promoter and *sak* gene, producing high amounts of SAK.

affect the bactericidal activity of HNP-2. Control experiments performed with PPACK and SAK alone showed that neither molecule had any significant bactericidal effect. These results showed that SAK deprived of its ability to activate plasminogen still could reduce the bactericidal effect of α-defensins.

The region of SAK molecule located around the K74 position has been shown of importance for its interaction with plasminogen (28). SAK variant M8, carrying K74A substitution, was used for complex formation with HNP-2 to assess the role of this region in neutralization of α-defensins. Bacterial survival and the plasminogen-activating capacity of the M8-HNP complex was tested using *S. aureus* LS-1 and compared with HNP-2 or SAK/HNP-2 mixtures (Table II). We observed that a single amino acid substitution at position 74 resulted in a 50% reduction of SAK-neutralizing properties (bactericidal effect of HNP-2 increased from 35 to 70%) and the ability of SAK to facilitate plasminogen activation. These findings indicate a central role of K74 residue in the interaction between SAK and HNPs.

Sensitivity of S. aureus *strains to α-defensin-mediated killing depends on their SAK production*

The sensitivity of *S. aureus* to α-defensins was assessed using isogenic variants of *S. aureus* LS-1. Wild-type LS-1, which is unable to produce SAK, and LS-1 carrying a single copy of *sak* in the integrating vector pCL84 were assayed for α-defensin-triggered killing. LS-1 pCL84*sak* exhibited significant resistance to the HNP-1-dependent bactericidal effect (72 and 51% killing for LS-1 and LS-1 SAK^+, respectively, $p = 0.03$), while bacterial killing by HNP-2 did not change significantly (Fig. 6).

Evaluation of secreted SAK in the culture medium and on the bacterial cell surface revealed high levels of SAK production by 10 of 19 tested strains (3 laboratory and 7 nasal isolates), while the remaining strains (3 laboratory and 6 nasal isolates) did not produce SAK at all (Table III). SAK-producing strains were significantly less sensitive to the bactericidal effect of HNPs than SAK-nonproducing strains (Fig. 7). Decreased HNP-mediated bacterial killing of SAK-producing strains was observed with both HNP-1 ($p = 0.034$) and HNP-2 ($p = 0.016$). Addition of exogenous SAK (20 μg/ml) to the bacterial medium had no influence on staphylococcal growth (data not shown).

Additional experiments were performed using 19 *S. aureus* strains with known SAK phenotype to assess their sensitivity to α-defensin peptides HNP-1 and HNP-2 (Table III). In these strains, the bactericidal effect of HNP-2 was significantly more pronounced compared with HNP-1 ($p < 0.001$). This difference could be observed both in laboratory strains and in nasal isolates and resulted in the bacterial killing of 76% in the case of HNP-1

Table III. *Characteristics of* S. aureus *strains with respect to their production of SAK and sensitivity to α-defensins*

	SAK Production		Bactericidal Effect (%)	
	Released (μg/ml)	Surface, Abs (405 nm)	HNP-1	HNP-2
Newman	5.0	NA[a]	0	62
RN6390	6.0	NA	9	72
MRSA 67-0	1.0	NA	69	72
Nas1	11.9	0.357	85	86
Nas2	9.0	0.335	33	76
Nas3	9.9	0.341	33	83
Nas4	1.1	0.335	37	70
Nas5	3.8	0.400	23	90
Nas6	7.3	0.064	58	92
Nas7	1.0	0.335	81	93
Mean ± SEM	5.5 ± 3.2	0.310 ± 0.127	43 ± 9	80 ± 3
LS-1	0	NA	86	93
P1	0	NA	80	86
1061	0	NA	83	90
Nas8	0	0.060	62	94
Nas9	0	0.052	41	85
Nas10	0	0.051	52	80
Nas11	0	0.054	64	94
Nas12	0	0.062	72	96
Nas13	0	0.319	91	98
Mean ± SEM	<0.3	0.098 ± 0.044	70 ± 6[b]	91 ± 2[c]

[a] NA, Not assessed.
[b] Bactericidal effect of HNP-1 on SAK-producing vs nonproducing *S. aureus* strains, $p = 0.034$.
[c] Bactericidal effect of HNP-2 on SAK-producing vs nonproducing *S. aureus* strains, $p = 0.016$.

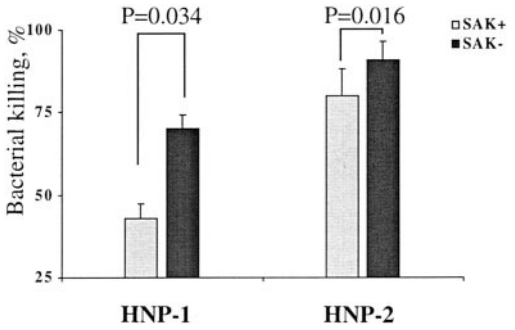

FIGURE 7. Bactericidal effect of α-defensins on *S. aureus* strains depends on their production of SAK. Bacterial growth of different *S. aureus* strains, 10^3 CFU/ml, was monitored with or without the addition of 5 μg/ml HNP-1 or HNP-2. Bacterial growth of *S. aureus* strains following 6 h of incubation with HNPs was monitored in SAK-producing ($n = 9$) and -nonproducing ($n = 10$) strains.

(range, 9–86%) and 81% in the case of HNP-2 (range, 23–98%) of bacteria, respectively.

Protective role of α-defensins and their neutralization by SAK in an animal model of S. aureus *arthritis*

To assess the role of HNP on the course of septic arthritis, intra-articular injections of *S. aureus* were performed in the knee joints of 30 healthy mice (Table IV). Eight mice were simultaneously injected with HNP-2 (5 μg/knee), seven mice received SAK (10 μg/knee), and eight mice received a mixture of SAK/HNP-2 (5 + 5 μg of each/knee). The reason for why the in vivo assay only HNP-2 was used is that it has somewhat more pronounced antimicrobial properties in vitro as compared with HNP-1 (see Table III). All of the mice (100%) injected with the LS-1 strain (4×10^3 CFU/knee) developed severe arthritis (arthritis index = 12.5), which in four of seven cases was associated with bone destruction and pannus formation. In contrast, only three (37%) of eight mice injected with *S. aureus* plus HNP-2 developed arthritis. In the latter cases, arthritis was also less severe (arthritis index = 4.0; $p < 0.05$), indicating a significant alleviation of *S. aureus*-induced inflammation by HNP-2. In the mice injected with *S. aureus* and the SAK/HNP-2 mixture, five of eight developed arthritis (62%, arthritis index = 3.6), indicating at least partial in vivo neutralization of HNP-2 by SAK. Control experiments were performed by injecting staphylococcal peptidoglycans (50 μg/knee) alone or in combination with HNP-2 (5 μg/knee). Severe destructive arthritis (arthritis index = 3.75) developed in 16 of 18 mice, equally often in both groups (89% vs 89%). Bacterial growth in the infected knee joints was assessed 3 days after the injection of *S. aureus.* Growth of *S. aureus* was registered in only two of nine joints injected with *S. aureus and* HNP-2 (viable count, 12–19; mean, 16), which was significantly lower than in the joints injected with *S. aureus* alone (9/9, $p < 0.025$). Both the incidence of bacteria in the infected joints (4/9) and the number of organisms detected (viable count, 4–108; mean, 71) increased when joints were injected with *S. aureus* and the HNP-2/SAK mixture.

Frequency of SAK-producing strains within nasal isolates of healthy individuals

The ability of *S. aureus* strains isolated from the anterior nares of 97 healthy individuals to produce SAK was assessed by measuring the plasminogen-activating capacity of the bacterial supernatants following overnight culture. The plasminogen-activating capacity of the supernatants was related to serial dilutions of rSAK and levels of SAK above detection level (0.3 μg/ml) were considered as positive. Of 97 *S. aureus* isolates, 66 (67%) secreted detectable levels of SAK (0.4–3.7 μg/ml, mean, 2.3 ± 0.7). These findings indicate a high frequency of SAK-producing strains within the upper respiratory tract of clinically healthy individuals.

Release of α-defensins from human neutrophils following stimulation with SAK

Neutrophil preparations (5×10^5/ml) from eight healthy individuals were incubated with increasing concentrations of rSAK (0.1–10 μg/ml). Supernatants from five of eight tested cell suspensions exhibited a significant increase of HNP concentrations upon stimulation with SAK compared with those stimulated with the buffer alone. HNP release was observed already with the lowest SAK concentration tested (0.1 μg/ml; Table V) and remained similar following stimulation with higher SAK concentrations. The remaining three neutrophil preparations displayed high HNP concentrations at the baseline due to spontaneous degranulation, which made difficult the interpretation of the SAK stimulation experiment.

Discussion

In the present study, we have demonstrated an ability of SAK, a serine protease-like molecule produced by *S. aureus*, to interact with and neutralize α-defensins, important effector molecules of the host innate and adaptive immune system. Complex formation between SAK and HNPs resulted in a large decrease of their bactericidal properties.

Several aspects of the SAK-defensin interaction are of potential physiological relevance and should be discussed. We observed that SAK neutralizes defensins in >1:1 molar ratio. Indeed, only one of six of the molar SAK concentrations was required to neutralize the bactericidal effect of HNPs. Interaction between SAK and HNPs

Table IV. *Alleviation of* S. aureus- *induced arthritis by α-defensins*[a]

Group	No. of Mice	Frequency of Arthritis (%)	Frequency of Erosive Arthritis (%)	Bacterial Growth
S. aureus alone	7	100	57	6/6 (126 CFU/knee)
S. aureus + HNP-2	8	37[b]	25[c]	2/9 (16 CFU/knee)
S. aureus + SAK/HNP	8	62[c]	12	4/9 (71 CFU/knee)
Peptidoglycans	9	89	89	NA
Peptidoglycans + HNP-2	9	89	89	NA
HNP-2 alone	18	28	0	NA
SAK alone	14	43	0	NA

[a] *S. aureus* LS-1 (non-SAK producer) was injected intra-articularly at the concentration of 4×10^4/knee. Five micrograms of HNP-2 alone or along with 5 μg of SAK were coinjected intra-articularly. Fifty micrograms of peptidoglycans were injected intra-articularly either alone or along with HNP-2 (5 μg). NA, Not assessed.

[b] *S. aureus* + HNP-2 vs *S. aureus* alone, $p < 0.03$.

[c] *S. aureus* + SAK/HNP-2 vs *S. aureus* alone, NS.

Table V. *Release of α-defensins following stimulation of human neutrophils with different doses of SAK*

Sample	SAK, 0 μg/ml	SAK, 0.1 μg/ml	SAK, 1 μg/ml	SAK, 10 μg/ml
1	323[a]	1163	177	NA
2	5	360	187	340
3	0	NA	0	2407
4	228	326	NA	314
5	134	346	489	444
Mean ± SEM	138 ± 63	549 ± 205[b]	213 ± 101[c]	876 ± 511[d]

[a] Level of α-defensins is provided in picograms per milliliter. The impact of neutrophil incubation with SAK on release of defensins was calculated by the Mann-Whitney *U* test and compared with nonstimulated neutrophils (SAK, 0 μg/ml). Stimulation time was 5–15 min. NA, Not assessed.
[b] $p = 0.009$.
[c] *p*NS.
[d] $p = 0.027$.

occurs readily, with cell-free SAK resulting in complex formation. We found no evidence for a plasminogen requirement for this interaction. Indeed, SAK complexed with PPACK, which occupies the serine protease domain and thereby prevents the protein from binding and activating plasminogen, could still efficiently reduce the bactericidal properties of HNPs. These observations indicate the presence of several binding sites on the SAK molecule for HNPs, one of which is probably located in the vicinity of the plasminogen binding region of SAK. Furthermore, we observed that the complex formation between defensins and SAK results in a significant reduction of the plasminogen-activating capacity of SAK, supporting a direct binding of defensins to the serine protease-like domain of SAK responsible for its interaction with plasminogen. We also demonstrated that SAK mutated at residue K74 lost ~50% of its defensin-neutralizing capacity. In contrast, even multiple mutations in other SAK regions did not lead to any significant change of its neutralizing capacity. The flexible loop around K74 interacts sterically with the C terminus of the SAK molecule (27). This interaction is valuable for recognition of plasmin(ogen) (28) and potentially for recognition and complex formation with α-defensins.

We showed that SAK induces release of α-defensins by peripheral blood leukocytes. The amount of HNP released is not dose dependent but rather reflects the "all-or-nothing" type of response. Prompt release of HNPs occurring within a few minutes following peripheral blood stimulation corresponds to the release of peptides stored in the cellular granules rather than the result of de novo synthesis. When released, HNPs adhere to anionic phospholipid moieties of cellular membranes and structures of the extracellular matrix, resulting in a leakage of cellular contents (4, 5). One of the goals for bacterial evasion mechanisms is to prevent adhesion of HNPs to bacterial surface. The presence of multiple HNP binding sites on SAK provides a potent protective device for neutralizing bactericidal peptides and keeping them away from the bacterial surface. The interaction between SAK and defensins is a new mechanism by means of which *S. aureus* evades from killing by the host innate immune system.

The interaction between SAK and defensins is of obvious clinical relevance. Indeed, our studies using a murine model of *S. aureus* arthritis showed that defensins had a clear protective effect against joint inflammation and destruction. However, joint destruction and intra-articular bacterial growth were aggravated by preincubation of defensins with SAK. Since both defensins (29) and SAK may occur in the joint cavity of patients with septic arthritis, the interaction described above is likely to be present in the human setting. Our present study on *S. aureus* clinical isolates indicates that the great majority of staphylococcal isolates colonizing nasal mucosa produce SAK. In addition, the level of defensins in the nasal fluid of individuals colonized with *S. aureus* is significantly increased compared with noncarriers (30). Taken together, these observations favor the suggestion that SAK production supports staphylococcal survival in the host. Indeed, results from this study show that SAK 1) causes a significant release of defensins from human neutrophils and 2) once released defensins are efficiently neutralized by the SAK. Finally, we also demonstrate that the defensin-neutralizing effect of SAK is valid also for other Gram-positive and Gram-negative bacteria. Indeed, defensin-mediated killing of *E. coli*, *S. bovis*, and *S. epidermidis* was also abrogated by SAK. This finding is of importance keeping in mind that SAK is broadly used as a bolus infusion for thrombolysis in myocardial infarction and in the case of acute peripheral arterial occlusions. Patients receiving SAK infusion may be potentially submitted to an increased risk of development of infections, especially in a hospital setting.

Altogether our results demonstrate that SAK, a common product of *S. aureus*, interacts with and neutralizes an important part of the early innate immune system. A consequence of this interaction is impaired bacterial killing and increased susceptibility to bacterial colonization and to potentially life-threatening infections.

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