Inhibition of Endothelial Interleukin-8 Production and Neutrophil Transmigration by *Staphylococcus aureus* Beta-Hemolysin

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Neutrophils play a crucial role in the host response to infection with *Staphylococcus aureus*, which is a major human pathogen capable of causing life-threatening disease. Interleukin-8 (IL-8) is a potent chemoattractant and activator of neutrophils. We previously reported that *S. aureus* secretes a factor that suppresses IL-8 production by human endothelial cells. Here we isolated an inhibitor of IL-8 production from the supernatant and identified it as staphylococcal beta-hemolysin. Beta-hemolysin reduced IL-8 production without cytotoxicity to endothelial cells. Pretreatment with beta-hemolysin decreased the expression of both IL-8 mRNA and protein induced by tumor necrosis factor alpha (TNF-α). Migration of neutrophils across TNF-α-activated endothelium was also inhibited by beta-hemolysin. In contrast, beta-hemolysin had no effect on intercellular adhesive molecule 1 expression in activated endothelial cells. These results showed that beta-hemolysin produced by *S. aureus* interferes with inflammatory signaling in endothelial cells and may help *S. aureus* evade the host immune response.

*Staphylococcus aureus* is an important human pathogen that causes a wide range of diseases from wound infections to life-threatening infections, such as endocarditis, osteomyelitis, and septic shock (14). This organism is also a major cause of nosocomial infections, and frequent colonization of the anterior nares is a known risk factor for invasive diseases (29). Treatment of such infections with antibiotics has become more difficult because of recent increases in *S. aureus* resistance to multiple antibiotics.

A primary immune defense mechanism against *S. aureus* infections is innate immunity, and neutrophils are a significant innate component that is involved in the control of *staphylococcal* infections (8). An immediate host immune response to a bacterial infection is leukocyte migration from the circulation to the infection site. Vascular endothelial cells play an important role in this process. Endothelial cells mediate leukocyte migration through the expression of chemokines, including interleukin-8 (IL-8), and adhesion molecules induced by proinflammatory mediators, such as tumor necrosis factor alpha (TNF-α). These endothelial cell-associated molecules facilitate the rolling, adhesion, and emigration of circulating leukocytes across the endothelial cell barrier to sites of inflammation by a multistep process (23).

It has been suggested that *S. aureus* is able to escape host defenses (3), but the mechanisms are not clear. Recently, several studies have shown that *S. aureus* bacterial factors compromise the host immune response (8). These factors include the extracellular adherence protein (Eap), which blocks neutrophil recruitment by binding to host adhesion protein intercellular adhesive molecule 1 (ICAM-1) (2), and the chemotaxis-inhibiting proteins of *S. aureus*, which inhibit neutrophil chemotaxis by blocking neutrophil receptors for C5a and formyl peptide (6). Several host escape mechanisms have been defined for *S. aureus*, but not all of the mechanisms have been defined.

IL-8 is a neutrophil chemoattractant that is synthesized by endothelial cells and mediates neutrophil transmigration through activated endothelial cells (11, 19, 20). Inhibition of IL-8 production in endothelial cells results in a decrease in neutrophil migration across the endothelium (21). In a previous report, we showed that a factor secreted by *S. aureus* reduced IL-8 production in endothelial cells (24). Therefore, attenuation of IL-8 expression by *S. aureus* may delay neutrophil recruitment and contribute to bacterial persistence and growth in the host.

In this study, we isolated the secreted factor and identified it as beta-hemolysin, which inhibits TNF-α-induced IL-8 secretion in endothelial cells and neutrophil transendothelial migration. Our results showed that beta-hemolysin produced by *S. aureus* interferes with inflammatory signaling in endothelial cells and may weaken the immune response in the host.

**MATERIALS AND METHODS**

**Reagents and antibodies.** Recombinant human TNF-α was obtained from R&D Systems (Wiesbaden, Germany). Monoclonal antibodies against ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1) were purchased from BD PharIming (San Diego, CA). Mouse immunoglobulin G1 (IgG1), peroxidase-conjugated goat anti-mouse antibodies, and C2 ceramide were purchased from Sigma Aldrich (Tokyo, Japan). Rabbit antibodies against total and phosphorylated forms of ERK1/2 (p42/p44) and p38 were purchased from Cell Signaling Technology (Ozyme, St. Quentin Yveline, France).

**Bacterial strain and growth condition.** *S. aureus* strain A17 (a clinical isolate from an atopic patient) was cultured overnight at 37°C in brain heart infusion broth, diluted 60-fold in fresh brain heart infusion broth, and cultured with constant agitation for 4 h. Bacterial supernatants were passed through a 0.22-μm-pore-size filter and used for purification of the IL-8-inhibiting factor.

**Endothelial cell cultures.** Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex (Walkersville, MD) and were grown in 25-cm tissue culture flasks coated with 0.2% gelatin (Sigma) in HEC-C1 medium, which consisted of medium MCD107, acid fibroblast growth factor, and heparin (IFP, Yamagata, Japan) containing 10% fetal bovine serum and supplemented with...
100 U/ml penicillin and 100 µg/ml streptomycin. Cells were cultured at 37°C in the presence of 5% CO₂ and used between passages 3 and 6.

**Chromatography.** Solid ammonium sulfate was added to bacterial culture supernatants with gentle stirring to obtain 50% saturation, and the mixture was centrifuged at 13,000 × g for 15 min. After removal of the precipitate, the resulting supernatant was brought to 100% ammonium sulfate saturation, left overnight at 4°C, and then centrifuged at 13,000 × g for 30 min. The precipitated proteins were dissolved in 25 mM Tris-HCl buffer (pH 8.0), dialyzed against the same buffer, and applied to a Vivapure anion-exchange spin columns (Vivascience AG, Hannover, Germany). The flowthrough was applied to Vivapure cation-exchange spin columns at pH 5.4 and eluted with increasing concentrations of sodium chloride (0 to 1 M). The fractions were then applied to cation-exchange columns at pH 7.0 and eluted with 0 to 0.5 M NaCl. Fractions were screened for inhibition of IL-8 production. Active fractions were analyzed by 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (12) and by isoelectric focusing electrophoresis, followed by Coomassie brilliant blue staining. To perform isoelectric focusing electrophoresis, a sample was applied to amphotolyte polyacrylamide gels with a pH gradient from 3 to 10 (Technical Frontier Co.), which were then electrophoresed at 100 V for 1 h, at 200 V for 1 h, and finally at 500 V for 30 min according to the manufacturer's instructions.

**Assay for IL-8 and MCP-1.** IL-8 and monocyte chemotractant protein 1 (MCP-1) in the supernatant of endothelial cells were quantified by using an enzyme-linked immunosorbent assay (ELISA) kit (BioSource International, Inc., California) according to the manufacturer's instructions.

**N-terminal sequence determination.** The purified protein was blotted onto a polyvinylidene difluoride membrane and stained with Coomassie brilliant blue R-250. The band of interest was cut out, and the N-terminal amino acid sequence was determined as described by Smyth et al. (22). Briefly, 1% (vol/vol) sheep erythrocytes suspended in TBS buffer containing 150 mM NaCl, 0.02 M Tris (pH 7.0), and 2 mM MgCl₂, was added to a dilution of protein or beta-hemolysin of 0.1% (Sigma). After 30 min of incubation at 37°C, micelles were cooled in an ice-water bath, and the absorbance at 540 nm of the hemoglobin in supernatants was determined.

**LDH release assay.** Cytotoxicity was evaluated by determining the release of lactate dehydrogenase (LDH) into cell culture supernatants. HUVEC were seeded on gelatin-coated 96-well plates (4 × 10³ cells/well), cultured for 48 h, and treated with several concentrations of beta-hemolysin for 24 h. The release of intracellular LDH was determined using a cytotoxicity detection kit purchased from Roche Applied Science (Mannheim, Germany) according to the manufacturer's instructions. The optical density at 492 nm for each condition was determined by using a reference wavelength of 620 nm. The LDH activity was expressed as the percentage of the total cellular LDH activity, which was determined by lysis of cells with the lysis buffer in the kit.

**Cell viability assay.** Cell viability was assessed by the WST-8 [2-(2-methoxy-4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (Kishida Chemical, Japan) colorimetric assay. This assay is based on the cleavage of the tetrazolium salt WST-8 to formazan by mitochondrial dehydrogenases in viable cells. The amount of formazan dye produced is proportional to the number of metabolically active cells and is quantified by its absorbance at 450 nm. HUVEC were seeded on gelatin-coated 96-well plates (4 × 10³ cells/well), cultured for 48 h, and treated with several concentrations of beta-hemolysin for 24 h. The wells were pulsed with WST-8 for 1 h, and the optical density at 450 nm was determined with a microplate reader to determine cell viability.

**Annexin V-PI staining.** Dual staining with fluorescein isothiocyanate (FITC)-labeled annexin V (FITC-annexin V) and propidium iodide (PI) was performed to determine the proportion of cells undergoing apoptosis and necrosis. For this purpose, an FITC-annexin V kit (Beckman Coulter, Inc., Fullerton, CA) was employed, using the manufacturer's directions. Briefly, HUVEC were collected by trypsination, washed, and incubated with FITC-annexin V and PI as directed by the manufacturer. The stained cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences Clontech, Palo Alto, CA) to determine the percentage of annexin V-positive PI-negative cells (apoptosis) or the percentage of annexin V-negative PI-positive cells (necrosis). Data acquisition (10⁵ events per sample) and analysis were carried out using CellQuest software (BD Biosciences). RT-PCR. HUVEC cultured in a 35-mm dish were treated with beta-hemolysin (100 ng/ml) for 3 h and then treated or not treated with TNF-α. Total RNA was isolated using TRI-REAGENT (Molecular Research Center, Inc., Cincinnati, OH) and treated with RNase-free DNase (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. DNase-treated total RNA was reverse transcribed at 50°C for 1 h using a One-Step reverse transcriptase (RT)-PCR kit (BD Biosciences Clontech). The RT reaction products were subsequently PCR amplified with a thermal cycler (Techgene; TECHNE, Cambridge, United Kingdom) using the following primers: IL-8 forward (5'-ATGACTTCC AAGCTGCGCTTGCT-3') and IL-8 reverse (5'-TCTAGCTTCCTCCTAATT AACTTCTC-3'). The PCR conditions were as follows: initial denaturation at 94°C for 5 min, 25 cycles of 94°C for 30 s, annealing at 65°C for 30 s, and 1 min at 66°C for, and final extension at 68°C for 2 min. The PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

**Isolation of neutrophils.** Neutrophils were prepared from human donor blood. Heparinized blood was sedimented with dextran to remove the majority of the red blood cells, and the buffy coat was separated by Ficoll-Hypaque (GE Health-care UK Ltd., Buckinghamshire, United Kingdom) gradient centrifugation, followed by hypotonic lysis of the remaining red blood cells. The preparation contained >95% neutrophils, as judged by morphological examination.

**Neutrophil transmigration assay.** Endothelial cells were seeded on gelatin-coated inserts (diameter, 6.5 mm; pore size, 3 µm; Becton Dickinson Labware, Franklin Lakes, NJ) at a concentration of 2.5 × 10⁵ cells/insert in 300 µl of HEC-Cl medium. The inserts were then placed in a 24-well plate, in which each well contained 700 µl medium, and cultured until the monolayers were confluent. HUVEC were either not treated or were treated with beta-hemolysin for 3 h and stimulated with TNF-α for 4 h before the assay. HUVEC were washed with medium, and neutrophils (5 × 10⁶ cells in 100 µl medium) were added to each well and allowed to migrate for 3 h. After incubation, the neutrophils that migrated to the lower chamber were collected for counting with a hemocytometer, and the percentage of cells that migrated was determined.

**Cell surface ELISA.** The expression of VCAM-1 and the expression of VCAM-1 monoclonal antibody (1 µg/ml) overnight at 4°C, washed again with PBS, and then incubated with peroxidase-conjugated goat anti-mouse IgG1 (1:8,000) for 1 h. The cells were washed again with PBS and exposed to o-phenylenediamine, and the optical density at 490 nm was determined using a microplate reader. The value obtained with mouse IgG1 was subtracted from the value obtained for expression of each adhesion molecule.

**Nuclear extract and EMSA.** Nuclear protein was prepared with a nuclear NE-PER nuclear and cytoplasmic extraction reagent (Pierce, Rockford, IL) used according to the manufacturer's instructions and was stored at −80°C until use. An electrophoretic mobility shift assay (EMSA) was performed using a digoxigenin (DIG) gel shift assay kit (Roche) as recommended by the manufacturer. Briefly, nuclear extract was incubated with a 150 ng/labeled probe labeled with DIG using terminal transferase, and the labeled probe was incubated with the nuclear protein extract (10 µg) in a binding buffer at room temperature for 20 min. For competition experiments, a 100-fold excess of unlabeled double-stranded oligo-nucleotide was added to the reaction mixture. After incubation, the protein-DNA complexes were electrophoresed on a 5% nondenaturing polyacrylamide gel using 0.5× Tris-borate-EDTA buffer, transferred to a nylon membrane by electroblotting, and detected using a DIG detection kit.

**Cell lysis and Western blot analysis.** HUVEC were starved for 16 h and then treated with TNF-α and beta-hemolysin. To obtain total-cellular-protein extracts, the cells were washed with ice-cold PBS and lysed with SDS sample buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin) and a phosphatase inhibitor (1 mM sodium orthovanadate) and boiled for 5 min. The extracts were subjected to 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking with 5% nonfat milk for 1 h, the membranes were incubated with anti-phospho-p44/42 (ERK1/2, Thr202/Tyr204) antibody or anti-phospho-p38 (Thr180/Tyr182) mitogen-activated protein kinase (MAPK) antibody overnight at 4°C (1:1,000 dilution). Antibodies against ERK1/2 or p38 MAPK were used as a control. The immunoreactive bands were detected using a secondary antibody (horse-radish peroxidase-conjugated anti-rabbit immunoglobulin) and the ECL Plus Western blotting detection system (Amersham Pharmacia Biotech).

**Statistical analyses.** Results were expressed as means ± standard deviations. The significance of differences between the mean results for the different groups was determined by Student’s t-test. A P value of <0.05 was considered statistically significant.
sequence encoded by the *S. aureus* *hbl* gene, known as beta-hemolysin (beta-toxin, sphingomyelinase C). According to previously published data, beta-hemolysin is 35-kDa protein, is extremely basic, and has an pI greater than 9 (7, 17). This protein has been identified as an enzyme that specifically cleaves sphingomyelin and has been shown to be highly hemolytic with sheep erythrocytes (hot-cold hemolysis). The purified protein exhibited the same hemolytic activity as the beta-hemolysin from Sigma with sheep erythrocytes (Fig. 1C). Taken together, these results indicated that the IL-8-inhibiting factor was beta-hemolysin.

Inhibition of IL-8 production by beta-hemolysin is not a result of a reduction in cell viability. HUVEC were incubated with various concentrations of beta-hemolysin for 3 h, and IL-8 production was analyzed (Fig. 2A). IL-8 secretion was reduced when HUVEC were incubated with 1 to ~1,000 ng/ml of beta-hemolysin, and maximum inhibition was observed with 100 ng/ml of beta-hemolysin. This inhibitory effect was detected at 3 h, and it persisted for 24 h (Fig. 2B). The culture supernatants obtained from beta-hemolysin-producing *S. aureus* strains inhibited IL-8 production, whereas the culture supernatants obtained from non-producing strains did not (data not shown).

To determine whether the reduction in IL-8 production resulted from cell damage, we examined the toxicity of beta-hemolysin. HUVEC were incubated with beta-hemolysin for 24 h, and then cytotoxicity was measured by using the LDH release assay and cell viability was assessed by using the WST-8 assay. Over 24 h, exposure to 1 to ~1,000 ng/ml of beta-hemolysin did not result in an increase in the release of LDH into the cell culture medium (Fig. 2C). Data from the WST-8 assay indicated that the viability of HUVEC was not decreased by beta-hemolysin treatment. By contrast, Triton X-100-treated cells showed a decrease in viability (Fig. 2D). Additionally, to determine whether apoptosis contributes to the reduction in IL-8 production, we examined apoptosis by using annexin staining. Cycloheximide and TNF-α treatment (positive control) induced apoptosis in HUVEC, whereas beta-hemolysin did not. Similarly, there was no difference in the percentage of necrotic cells (positive for both annexin and PI) between control and beta-hemolysin-treated HUVEC (Fig. 2E). In our experiments, obvious cytotoxic effects were not observed for HUVEC treated with beta-hemolysin. These results indicate that the inhibition of IL-8 production in beta-hemolysin-treated HUVEC was not a result of cell damage.

**RESULTS**

**Isolation and identification of the IL-8-inhibiting protein.** *S. aureus* strain A17 secreted the factor that attenuated IL-8 production in HUVEC (24). The inhibitory activity in the bacterial culture supernatant increased with bacterial growth and reached a maximum level at 4 h (late log phase). To obtain the IL-8-inhibiting factor, the supernatant of *S. aureus* strain A17 was purified by ammonium sulfate precipitation and anion- and cation-exchange chromatography. To identify the IL-8-inhibiting factor, *S. aureus* supernatant fractions were added to HUVEC cultures for 3 h, and then the culture supernatants were screened by ELISA for IL-8 production. As shown in Fig. 1A, the inhibitory activity was isolated in fractions 14 to 16, which were obtained from cation-exchange chromatography. The SDS-PAGE profile of the fraction with the highest inhibitory activity is shown in Fig. 1B, and a ~35-kDa protein was detected. The protein band at ~35 kDa was obtained with active fractions but not with inactive fractions. The purified protein exhibited the same activity as the supernatant. Isoelectric focusing electrophoresis analysis showed that the isoelectric point (pI) was greater than 9. The N-terminal sequence of the 35-kDa protein that was obtained was ESKKDGDLDKLVSHN. A search of the genome database of *S. aureus* revealed that this sequence matched an amino acid
hemolysin on the expression of MCP-1, which is a chemoattractant for monocytes. Beta-hemolysin reduced TNF-α-induced MCP-1 production (6,518.4 ± 81.8 pg/ml for TNF-α alone versus 5,806 ± 10.8 pg/ml for TNF-α plus beta-hemolysin; P < 0.01; n = 4).

**Beta-hemolysin suppressed transendothelial migration of neutrophils.** To investigate whether beta-hemolysin inhibited transendothelial migration of neutrophils, we tested neutrophil transmigration through TNF-α-activated endothelium pretreated with beta-hemolysin and through TNF-α-activated endothelium not pretreated with beta-hemolysin. HUVEC monolayers cultured in transwells were incubated with beta-hemolysin and stimulated with TNF-α and then washed before addition of neutrophils. After 3 h, the neutrophils that had transmigrated were counted. TNF-α increased the transmigration rate of neutrophils so that it was three times that of the
control, and beta-hemolysin reduced the TNF-α-induced transmigration of neutrophils 66% (Fig. 4).

Beta-hemolysin suppresses expression of VCAM-1 but not expression of ICAM-1 in endothelial cells. To determine whether the inhibitory effects of beta-hemolysin were specific to chemokine production, we examined the expression of leukocyte adhesion molecules in HUVEC. HUVEC monolayers pretreated with beta-hemolysin were stimulated with TNF-α for 6 h, and the expression of ICAM-1 and expression of VCAM-1 were measured by the cell surface ELISA. As shown in Fig. 5, TNF-α treatment significantly induced the expression of both adhesion molecules. Beta-hemolysin inhibited the up-regulation of VCAM-1 in TNF-α-stimulated HUVEC, but it did not affect the ICAM-1 expression.

Beta-hemolysin does not alter NF-κB–DNA binding activity induced by TNF-α. To investigate the mechanism by which beta-hemolysin inhibited IL-8 synthesis, we examined the effect of beta-hemolysin on NF-κB activation in TNF-α-stimulated HUVEC. The NF-κB transcription factor is a major regulator of expression of proinflammatory genes, including genes encoding adhesion molecules and chemokines in TNF-α-stimulated endothelial cells (4, 10). Nuclear extracts were prepared from TNF-α-activated HUVEC pretreated with beta-hemolysin and from TNF-α-activated HUVEC not pretreated with beta-hemolysin and were assayed to determine the NF-κB activity by EMSA (Fig. 6). NF-κB binding activity was not detected in HUVEC pretreated with beta-hemolysin or in HUVEC not pretreated with beta-hemolysin, but it was dramatically increased following incubation with TNF-α. Pretreatment with beta-hemolysin did not attenuate the TNF-α-induced NF-κB activation. The DNA-protein complex formed in TNF-α-activated cells was specific, since formation of this complex was completely inhibited by a 100-fold excess an unlabeled NF-κB consensus oligomer.

Beta-hemolysin inhibited ERK activation but not p38 activation. In addition to NF-κB, TNF-α-induced IL-8 production is also regulated by members of the MAPK family. Next, we

FIG. 3. Effect of beta-hemolysin on TNF-α-induced IL-8 expression in HUVEC. HUVEC were incubated with or without 100 ng/ml of beta-hemolysin for 3 h and stimulated with TNF-α at the indicated concentrations. (A) IL-8 secretion into the cell culture supernatants collected after 6 h of incubation was analyzed by ELISA. The data are expressed as means and standard deviations of at least three separate experiments. *, P < 0.01 compared with the control. (B) After stimulation with TNF-α for 3 h, total RNA was extracted and IL-8 expression was analyzed by RT-PCR. The PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining. Oligonucleotides specific to β-actin were used as housekeeping genes.

FIG. 4. Effect of beta-hemolysin on neutrophil transendothelial migration. Confluent HUVEC monolayers plated on Transwells were treated or not treated with 100 ng/ml of beta-hemolysin for 3 h and then stimulated with TNF-α (1 ng/ml) for 4 h. As a control, HUVEC were treated with medium alone. Neutrophils (5 x 10⁵ cells/well) were added to the upper wells of the Transwells, and transmigration across endothelium was assayed. The data are expressed as means and standard deviations of at least three separate experiments. *, P < 0.01 compared with TNF-α treatment alone.

FIG. 5. Effect of beta-hemolysin on expression of adhesion molecules. HUVEC were pretreated or not pretreated with beta-hemolysin (100 ng/ml), and this was followed by treatment with TNF-α (1 ng/ml) for 6 h. As a control, HUVEC were treated with medium alone. Cell surface ICAM-1 and VCAM-1 levels were determined by cell surface ELISA. The data are expressed as means and standard deviations of at least three separate experiments. *, P < 0.01 compared with TNF-α treatment alone. OD₄₉₀, optical density at 490 nm.
examined the effects of beta-hemolysin on TNF-α-induced phosphorylation of ERK1/2 and p38 MAPK. To study the effect of beta-hemolysin on TNF-α-mediated p38 and ERK1/2 activation, starved cells were incubated with beta-hemolysin for 3 h and exposed to TNF-α for 15 min. TNF-α alone stimulated ERK1/2 and p38 activity compared to the control, and pretreatment with beta-hemolysin resulted in blockade of TNF-α-induced ERK1/2 but not p38 phosphorylation, but it had no effect on the total levels of these kinases (Fig. 7).

**Inhibition of IL-8 production appeared to be mediated by ceramide.** Beta-hemolysin has sphingomyelinase activity, which cleaves sphingomyelin and generates ceramide, a second messenger which regulates many signaling pathways (15). To determine whether the reduction in IL-8 production might be mediated by ceramide, HUVEC were incubated with membrane-permeable C2 ceramide and stimulated with TNF-α, and then IL-8 production was analyzed. As shown in Table 1, incubation of HUVEC with membrane-permeable C2 ceramide decreased basal and TNF-α-induced IL-8 production, similar to beta-hemolysin.

**DISCUSSION**

We report here how *S. aureus* inhibits IL-8 expression in endothelial cells. We have shown previously that *S. aureus* secretes a factor that suppresses IL-8 production in endothelial cells (24). In this study, we identified the factor that inhibits IL-8 production as staphylococcal beta-hemolysin.

Staphylococcal beta-hemolysin is secreted into the culture medium as an exotoxin and is homologous to the Bacillus cereus sphingomyelinase at the amino acid sequence level (17). It is well known that beta-hemolysin is cytotoxic for erythrocytes through a neutral sphingomyelinase activity. Additional-
ally, selective cytotoxic activity of beta-hemolysin with human monocytes has been described, but no cytotoxic activity with leukocytes and fibroblasts has been described (26). Our results demonstrated that beta-hemolysin provoked neither apoptosis nor cell death, indicating that the inhibitory effect of beta-hemolysin on IL-8 expression was not the result of cytotoxicity for endothelial cells. Pretreatment of HUVEC with beta-hemolysin inhibited TNF-α-mediated upregulation of IL-8 expression, and this inhibition correlated with the downregulation of IL-8 mRNA expression. IL-8, the chemotactant for neutrophils, is produced by endothelial cells activated by TNF-α and IL-1, and it plays a major role in neutrophil transmigration through activated endothelial cells (11, 19, 20). The inhibition of IL-8 production in endothelial cells resulted in a decrease in neutrophil migration across the endothelium (21). In agreement with these findings, our results showed that beta-hemolysin also suppressed neutrophil transendothelial migration. Thus, we suggest that beta-hemolysin may play an important role as an anti-inflammatory factor. Recently, the factors secreted by *S. aureus* which inhibited leukocyte recruitment have been described (2, 6). The chemotaxis-inhibiting proteins of *S. aureus* inhibited neutrophil chemotaxis by blocking the neutrophil receptors for C5a and formyl peptide (2), and extracellular adherence protein (Eap) prevented leukocyte adhesion and extravasation by interacting with ICAM-1 on the surface of endothelial cells (6). Therefore, it is possible that *S. aureus* has several ways of avoiding leukocyte recruitment.

We found that pretreatment with beta-hemolysin did not affect TNF-α-induced expression of ICAM-1, which mediates leukocyte extravasation and is upregulated within hours after treatment with TNF-α (1). However, beta-hemolysin decreased TNF-α-induced VCAM-1 expression. These results indicate that beta-hemolysin can differentially modulate some gene expression but cannot globally inhibit expression. TNF-α has been shown to activate a variety of signaling pathways (27), and NF-κB signaling plays a key role in the transcription of many proinflammatory genes in endothelial cells, including adhesion molecules and chemokines (4, 10). We observed that TNF-α induced NF-κB activation, but beta-hemolysin did not affect this activation, suggesting that the NF-κB-dependent pathway is not involved in the inhibitory effect of beta-hemolysin. MAPK containing multiple subgroups, such as the ERK, JNK, and p38 kinases, were shown previously to be involved in TNF-α signaling (27). Previous reports showed that TNF-α-induced IL-8 expression was ERK dependent and that inhibition of ERK by an MEK-specific inhibitor (PD98059 or U0126) significantly reduced TNF-α-induced IL-8 production (13). Results of our study showed that beta-hemolysin inhibited IL-8 expression, and the inhibitory effect of beta-hemolysin appears at least in part to have occurred through the ERK pathway, since beta-hemolysin prevented TNF-α-induced ERK activation.

Beta-hemolysin has sphingomyelinase activity which leads to generation of ceramide, a potent second messenger. We observed that incubation of HUVEC with C2 ceramide decreased IL-8 production. Thus, it is likely that beta-hemolysin inhibited IL-8 production via hydrolysis of membrane sphingomyelin and the subsequent generation of ceramide. How ceramide inhibited IL-8 expression, however, is not clear. The available data indicate that endothelial cell activation induced by TNF-α is mediated through the generation of sphingosine 1-phosphate (SIP), and SIP is able to mimic the effect of TNF-α on endothelial cells (30). Because SIP-mediated IL-8 secretion relies on phospholipase D (PLD)-dependent ERK activation (5, 16, 28) and ceramide inhibits PLD activation (9, 25), it is possible that ceramide inhibits ERK phosphorylation and IL-8 secretion through PLD inhibition.

In conclusion, this study demonstrated that beta-hemolysin from *S. aureus* has anti-inflammatory properties in vitro. *S. aureus* suppresses TNF-α-induced IL-8 synthesis in endothelial cells and transmigration of neutrophils via beta-hemolysin. This effect could interfere with the ability of the host to induce an adequate inflammatory response in an *S. aureus* infection. Further studies must focus on investigating the signaling pathways responsible for the effects that we have described.

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