Delta-Toxin from *Staphylococcus aureus* as a Costimulator of Human Neutrophil Oxidative Burst

Franz-Josef Schmitz, Karin-Ellen Veldkamp, Kok P. M. Van Kessel, Jan Verhoef, and Jos A. G. Van Strijp

Delta-toxin from *Staphylococcus aureus* is responsible for various pathophysiologic effects. By studying different cell types in binding of delta-toxin in low, noncytotoxic concentrations, a specific binding of fluorescein-labeled delta-toxin to neutrophils and monocytes was found. Studying direct effects of delta-toxin on neutrophils, a dose-dependent up-regulation of complement receptor 3 expression was found. Oxygen radical production, as determined by Luminol-enhanced chemiluminescence, was not directly induced by delta-toxin, and this toxin was also unable to prime neutrophils for an enhanced response to FMLP or complement-opsonized zymosan. However, the priming response induced by lipopolysaccharide or tumor necrosis factor-α (TNF-α) was significantly further enhanced in the presence of delta-toxin. Furthermore, as a direct effect on human monocytes, delta-toxin induced TNF-α production. These data provide evidence that delta-toxin has direct and indirect effects on the activity of neutrophils and monocytes with regard to its proinflammatory capacity.

During the last decade, gram-positive bacterial sepsis has increased steadily and is now as common as bacteremia caused by gram-negative bacteria. Staphylococci, including *Staphylococcus aureus*, are a major source of morbidity and mortality in medical facilities. The reasons for this may be bacteria-related, such as increased virulence or antibiotic resistance, or host-related, such as the increased use of foreign materials and immunosuppressive therapy.

A cascade of inflammatory mediators, including cytokines, leads to the development of gram-positive sepsis and septic shock. *S. aureus* synthesizes a large number of extracellular proteins that are potent inducers of sepsis [1]. In *S. aureus*, as in most other bacteria, pathogenicity is multifactorial, and the genes that encode the virulence factors are often subject to coordinate regulation. Temporal expression of many of the proteins involved in virulence has been shown to be under the control of the *agr* locus [2]. The delta-toxin gene (*hld*) is a part of the *agr* gene cluster in *S. aureus*. The production of delta-toxin is responsible for various pathophysiologic effects during infection with *S. aureus* [3–7]. Bacterial toxins, released into the microenvironment or remaining cell-associated on the bacterial surface, affect various cellular functions, such as the release of inflammatory mediators and the activation of T cells via the major histocompatibility complex class II molecules.

*S. aureus* delta-toxin, a 26–amino acid polypeptide, is produced in vitro by >97% of *S. aureus* isolates [1]. Delta-toxin possesses the structural characteristics of a typical surface-active protein and can readily insert itself into hydrophobic membrane structures [8, 9]. Delta-toxin is distinguished from other staphylococcal hemolysins by its heat stability and the pattern of its activity on erythrocytes of various species [1]. A variety of activities has been attributed to delta-toxin [5–7, 10–19]. It can damage a variety of cell types as a result of its action on cell membranes. It causes dose-dependent damage to the bowels of guinea pigs, leading to speculation that delta-toxin is a mediator of staphylococcal membranous enterocolitis. Delta-toxin increases vascular permeability in guinea pig skin, inhibits water absorption, and activates adenylate cyclase in the ileum. In addition, it has many other effects on various cell systems, such as activation of membrane phospholipase A₂, stimulation of prostaglandin synthesis, inhibition of the binding of epidermal growth factor to cell surface receptors, release of lysozyme and β-glucuronidase from neutrophil granules, and the activation of the acetyltransferase, leading to the formation of platelet-activating factor (PAF). Furthermore, the protein has been shown to stimulate cyclic adenosine monophosphate accumulation and arachidonic acid release. Some of these effects were reported to occur under conditions in which gross cell damage could not account for the cell response.

In the present study, we investigated the influence of delta-toxin in low (≤30 μg/mL), noncytotoxic concentrations on human phagocyte function. One purpose of the present study was to assess whether delta-toxin is able to bind specifically to human neutrophils or monocytes; another purpose was to analyze direct effects of delta-toxin on neutrophils and monocytes as well as the effect of delta-toxin on neutrophil oxidative burst.

Received 31 March 1997; revised 16 June 1997.
Informed consent was obtained for blood draws.
Reprints or correspondence: Dr. Franz-Josef Schmitz, Eijkmans-Winkler Institute for Medical Microbiology, Utrecht University, Netherlands; Institute for Medical Microbiology and Virology, Heinrich-Heine Universität Düsseldorf, Düsseldorf, Germany.

The Journal of Infectious Diseases 1997;176:1531–7
© 1997 by The University of Chicago. All rights reserved.
0022–1899/97/7606–0017$02.00
Materials and Methods

Delta-toxin was synthesized according to the published amino acid sequence from S. aureus, and purity was controlled by mass spectrometry (gift of K. Köhler, Biologisch-Medizinisches Forschungszentrum, Universität Düsseldorf, Düsseldorf, Germany).

Delta-toxin was dissolved in 6 M urea, and after dialysis overnight against PBS, stock solutions were made and stored at −20°C until use. Before use, the stock solution was sonicated in a water bath for 5 min. Delta-toxin was labeled with fluorescein isothiocyanate (FITC) for use in flow cytometry. A solution of 0.1 mg/mL FITC (FITC isomer I; Sigma, St. Louis) in 0.1 M carbonate buffer of pH 9.6 was mixed 1:1 with 1 mg/mL delta-toxin in PBS and incubated for 60 min at room temperature, protected from light while rotating. After dialysis against PBS overnight, the optical densities at 280 and 495 nm were measured to determine the labeling efficiency, which was 85%. FITC-labeled delta-toxin was stored at −20°C at a concentration of 0.8 mg/mL.

Lipopolysaccharide (LPS) isolated from Salmonella minnesota Re 595 was purchased from Sigma. Stock solutions of 1 mg/mL in pyrogen-free water were stored at −20°C until use. Before use, the stock solution was sonicated in a water bath for 5 min. Recombinant tumor necrosis factor-α (TNF-α) and polymyxin B were purchased from Sigma.

C3bi-opsonized zymosan (2.5–3 × 10⁹ particles/μL) and PAF (10⁻⁵ M)–coated chemiluminescence tubes were purchased from ExOxEmis (San Antonio, TX). C3bi-opsonized zymosan (C3bi-zym) was stored at −70°C until use, whereas PAF-coated tubes were stored at 4°C.

Hybridoma cells secreting monoclonal antibody 44a (anti-CD11b, isotype IgG1) were obtained from American Type Culture Collection (Rockville, MD). Monoclonal antibody in culture supernatant was purified over a protein G column (Pharmacia, Uppsala, Sweden) and labeled with FITC for use in flow cytometry.

Blood and serum. Blood was drawn from healthy human volunteers and heparinized. Normal human serum was obtained after pooling the serum of 10 donors and was stored at −70°C.

Neutrophils were isolated from peripheral blood. Heparinized blood (LPS-free Vacutainer 9-mL tubes; Greiner, Frickenhausen, Germany) was diluted 1:1 with PBS (pH 7.4) and loaded on a ficoll (Pharmacia) and Histopaque (density 1.119; Sigma) gradient. After centrifugation for 20 min at 320 g at room temperature, the neutrophil fraction was collected and washed with RPMI (pH 7.4) containing 0.05% human serum albumin (CLB, Amsterdam). The remaining erythrocytes were lysed for 45 s with distilled water, after which concentrated PBS was added to reestablish an isotonic condition. After washing, cells were counted, resuspended in RPMI at 10⁷ neutrophils/mL, and checked for viability and purity. The viability always exceeded 95% by either trypan blue or propidium iodide exclusion. Purity was checked by microscopic evaluation of cytospin slides and was always >98%.

Binding experiments with FITC-labeled delta-toxin. A total of 100 μL of heparinized blood was incubated with 10 μL of FITC-labeled delta-toxin (serially 3 log–diluted) for 30 min at 4°C. Erythrocytes were lysed by addition of 2 mL of lysis buffer (0.1 mM EDTA in 170 mM NH₄Cl, 10 mM KHCO₃, pH 7.3). Cells were washed once with PBS and subsequently analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Neutrophils, monocytes, and lymphocytes were identified on the basis of their forward and sideward scatter characteristic.

In an additional experiment, erythrocytes were not lysed and separately analyzed for their binding properties.

Complement receptor 3 (CR3) and FMLP receptor expression on neutrophils. A sample of 50 μL of neutrophils (10⁷/mL) was incubated with 50 μL of serially 3 log–diluted delta-toxin for 30 min at 37°C. Afterwards, 5 μL of FITC-labeled 44a monoclonal antibody (50 μg/mL) or 10 μL of Bodipy-labeled FMLP (Molecular Probes, Eugene, OR; 50 μg/mL) was added and incubated at 4°C for another 30 min. Cells were washed twice with PBS, and the mean fluorescence of 10,000 neutrophils was subsequently determined with a FACScan.

Chemiluminescence experiments. For analysis of direct chemiluminescence, neutrophils were diluted in Hanks’ balanced salt solution (HBSS) with 2% human serum albumin with a final concentration of 10⁵ neutrophils/mL. A total of 100 μL of serially 3 log–diluted delta-toxin and 100 μL of neutrophils were transferred to a test tube and placed in the luminometer (Autolumat LB 953; Berthold, Wildbad, Germany). By use of automated injection, 600 μL of 150 μM Luminol–balanced salt solution (LBSS) was added, resulting in a total volume of 800 μL for each sample. Chemiluminescence response was measured during a 30-min interval for analyzing direct chemiluminescence. Data were calculated with the AXIS software package. Absolute counts were obtained by calculating the area under the curve of the chemiluminescence for 10 min.

For analysis of priming for an enhanced response to FMLP, neutrophils were diluted in HBSS with 2% human serum albumin and primed for 30 min at 37°C with serially 3 log–diluted delta-toxin in combination with 3 ng/mL TNF-α, 2.5 ng/mL LPS, or buffer control. Incubation with LPS was done in the presence of 1% normal human serum to ensure the presence of LPS-binding protein. The total volume of a sample was 300 μL, with a final concentration of 10⁵ neutrophils/mL. Subsequently, 100 μL was transferred to a test tube (in duplicate or in triplicate) and placed in the luminometer. Using automated injection, cells were stimulated with 100 μL of FMLP (10⁻⁶ M final concentration) in the presence of 600 μL of LBSS, resulting in a total volume of 800 μL for each sample. Chemiluminescence response was measured automatically at least eight times during a 10-min time interval.

In an additional experiment, C3bi-opsonized zymosan instead of FMLP was used as a stimulator of primed neutrophils. As an additional control, cells in one tube were primed with PAF (10⁻⁵ M).

Induction of TNF-α. The ability of delta-toxin to induce TNF-α was tested by use of heparinized blood from healthy volunteers. In sterile tubes, 100 μL of blood was mixed with 100 μL of serially 3 log–diluted delta-toxin and incubated in a humid atmosphere at 37°C with 5% CO₂. Supernatants were then harvested after 6 h and analyzed by use of a TNF-α–specific ELISA (Pelikine Compact human TNF-α ELISA kit; CLB).

Statistics. Data from four different experiments with different donor cells were combined and reported as mean ± SE. Student’s t test for independent means was used to provide a statistical analysis (P = .05 was considered significant).
A statistically significant up-regulation of CR3 expression was observed with delta-toxin concentrations $\geq 0.1 \mu\text{g/mL}$. To exclude endotoxin contamination, we used polymyxin B to specifically block the response of neutrophils to LPS. Polymyxin B did not inhibit the up-regulation of CR3 expression induced by delta-toxin, whereas the up-regulation of CR3 expression in response to Re LPS (10 ng/mL) was completely abolished by polymyxin B (data not shown). From our results, we concluded that the observed effect concerning up-regulation of CR3 expression in response to delta-toxin is not due to contamination by endotoxin. An isotype-specific control antibody (IgG1: 18.116 [anti-herpes simplex virus glycoprotein B]) was used to ensure that increased Fc binding of the antibody to the Fc receptor was not occurring in the presence or absence of delta-toxin (data not shown). In contrast to up-regulation of CR3 expression, no effect of delta-toxin on FMLP receptor expression could be observed (figure 2). These data provide evidence for specific direct effects of delta-toxin to neutrophils and, therefore, we performed the following functional experiments.

**Chemiluminescence experiments.** Delta-toxin by itself did not elicit a metabolic burst in neutrophils, as analyzed by chemiluminescence up to concentrations of 30 $\mu\text{g/mL}$ (data not shown).

Furthermore, neutrophils incubated with delta-toxin and subsequently stimulated with FMLP or C3bi-zym in the presence of Luminol showed no increased indirect chemiluminescence response (figures 3 and 4), illustrating that delta-toxin alone was unable to prime neutrophils for enhanced response to FMLP or C3bi-zym.

**Results**

**Cytotoxicity of delta-toxin.** Delta-toxin was serially 3 log-diluted in PBS, added to isolated neutrophils or monocytes, and incubated for 60 min at 37°C. The viability of the cells always exceeded 95% by use of trypan blue exclusion for delta-toxin concentrations up to 100 $\mu\text{g/mL}$. Cytotoxic effects could be observed with delta-toxin concentrations $\geq 100 \mu\text{g/mL}$ (data not shown). This is in line with observations by Raulf et al. [5] and König et al. [20], as determined by the release of lactate dehydrogenase.

**Binding of FITC-labeled delta-toxin to different cells.** The interaction between FITC-labeled delta-toxin and neutrophils, monocytes, and lymphocytes at 4°C was studied with serially 3 log-diluted FITC-labeled delta-toxin concentration. Figure 1 shows a representative fluorescence histogram, illustrating binding specificity of FITC-labeled delta-toxin at 3 $\mu\text{g/mL}$. The binding of FITC-labeled delta-toxin at 3 $\mu\text{g/mL}$ to neutrophils was more pronounced than the binding to monocytes. The lowest fluorescence signal was observed with erythrocytes. At a delta-toxin concentration of 3 $\mu\text{g/mL}$, binding of labeled delta-toxin to neutrophils was 7-fold more pronounced than binding to monocytes and 30-fold higher than binding to lymphocytes.

These data indicate binding specificity of delta-toxin to neutrophils and monocytes and, therefore, direct effects of delta-toxin on these cells were studied.

**Direct effects of delta-toxin on neutrophils.** Up-regulation of CD11b/CD18 (CR3) as well as the FMLP receptor are rapid parameters indicating neutrophil activation. As shown in figure 2, there was a dose-dependent up-regulation of CR3 expression.
Enhancement of lipopolysaccharide (LPS)- and tumor necrosis factor (TNF)-mediated priming for FMLP response by delta-toxin. Neutrophils were incubated with delta-toxin (▲) or delta-toxin with additional LPS at 2.5 ng/mL (●) (A) or delta-toxin with additional TNF-α at 3 ng/mL (■) (B) for 30 min at 37°C. Samples were subsequently stimulated with FMLP, and luminescence response was measured for 10 min in presence of Luminol. 10-min integral was calculated as measure for luminescence. Data represent means of 4 independent experiments ± SE. * Enhancement of LPS (A)- or TNF (B)-mediated priming was statistically significant (P ≤ .05) by 2-tailed Student’s t test.

However, when neutrophils were primed with Re LPS or TNF-α in the presence of delta-toxin and subsequently stimulated with FMLP or C3bi-zym, the luminescence response (figures 3 and 4) was significantly increased. This increased response was observed with neutrophils that were first preincubated with Re LPS or TNF-α for 15 min before delta-toxin was added. Preincubation of neutrophils with delta-toxin for 15 min followed by Re LPS or TNF-α for an additional 15 min gave comparable results. The stimuli could act after each other, and the sequence of addition was not important for the enhancement of LPS or TNF-α priming for FMLP and C3bi-zym response in the presence of delta-toxin. In addition, washing the cells once after the first stimulus followed by the second stimulus (Re LPS, TNF, or delta-toxin) did not abolish the enhanced priming (data not shown).

This enhancement of LPS and TNF-α priming for FMLP and C3bi-zym response in the presence of delta-toxin had a maximum of 3 μg/mL delta-toxin, with a 2- to 3-fold increase of FMLP and C3bi-zym response.
PAF was used as an additional control for priming of neutrophils in the experiment with C3bi-zym, indicating that priming of neutrophils was possible and that stimulation via C3bi-zym took place in the primed cells (figure 4).

The enhancement of LPS and TNF-α priming for FMLP and C3bi-zym response in the presence of delta-toxin was not caused by endotoxin contamination of delta-toxin, since delta-toxin alone was unable to prime neutrophils for FMLP or C3bi-zym responses. To exclude the possibility of neutrophil damage by delta-toxin incubation or interference of delta-toxin with the oxidative burst, neutrophils preincubated with delta-toxin were stimulated with the receptor-independent agonist phorbol myristate acetate (PMA). The response to PMA was not changed in neutrophils preincubated with delta-toxin compared with buffer control (data not shown).

These data provide evidence for a costimulatory effect of delta-toxin on neutrophil oxidative burst.

**Induction of TNF-α.** Whole blood was stimulated with serially diluted delta-toxin. Supernatants of these incubations were tested for the presence of TNF-α by a specific ELISA. An increase in TNF-α production could be observed, with a maximum at 0.3 μg/mL delta-toxin as stimulus (figure 5).

Polymyxin B did not inhibit TNF-α release induced by delta-toxin, whereas the induction of TNF-α release in response to Re LPS (10 ng/mL) was completely abolished by polymyxin B (data not shown). In contrast to the costimulatory effect of delta-toxin in combination with LPS or TNF-α on neutrophil oxidative burst, no increase in TNF-α release from monocytes could be observed if serially diluted delta-toxin combined with LPS (2.5 ng/mL) was used as stimulus (data not shown). To be assured that the TNF-α formed by whole blood in response to delta-toxin comes from monocytes, isolated monocytes were exposed to delta-toxin, and TNF-α in monocyte supernatants was measured. More than 90% of the produced TNF-α in response to delta-toxin came from monocytes (data not shown).

**Discussion**

In the present study, experiments with delta-toxin in low, noncytolytic concentrations are described. Our data provide evidence that delta-toxin exerts proinflammatory effects because of its binding specificity to neutrophils and monocytes, the up-regulation of CR3 expression, the enhancement of LPS- or TNF-α induced priming of neutrophils for FMLP and C3bi-zym response, and the stimulation of TNF-α production in monocytes. To our knowledge, it is the first molecule to be shown to do so.

The binding of FITC-labeled delta-toxin to neutrophils is more pronounced than the binding to monocytes, lymphocytes, or erythrocytes. This indicates that the interaction is not the result of simple insertion of the amphipathic molecule into a membrane but points to a specific interaction, especially with

![Figure 5](https://academic.oup.com/jid/article-abstract/176/6/1531/807353) Delta-toxin induces tumor necrosis factor (TNF) production in monocytes. Heparinized blood was incubated with delta-toxin for 6 h at 37°C with 5% CO2. Harvested supernatants were analyzed by TNF-α–specific ELISA. Each data point represents mean of 4 independent experiments using heparinized blood from different donors ± SE. * TNF induction was statistically significant (P ≤ .05) by 2-tailed Student’s t test.
Incubation of neutrophils with delta-toxin and LPS or TNF-α led to an enhanced priming of neutrophils for FMLP and C3bi-zym response. In this regard, delta-toxin acts as a co-stimulator for neutrophil priming and oxidative burst.

This costimulatory effect is due neither to indirect effects of delta-toxin on LPS or TNF nor to direct effects of delta-toxin on CD14- or TNF-receptor expression, since the sequence of addition did not change the enhanced priming of neutrophils for FMLP and C3bi-zym response. Even if neutrophils were washed after preincubation with delta-toxin, LPS, or TNF-α, the costimulatory effect of delta-toxin on neutrophil oxidative burst could be observed. This indicates that the substances can act independently after each other and that no CD14- or TNF-receptor up-regulation is involved.

If neutrophils were stimulated with the receptor-independent agonist PMA, the oxidative burst was not changed in neutrophils preincubated with delta-toxin compared with that in buffer control, indicating that delta-toxin does not directly interfere with the NADPH-oxidase system. The pronounced oxygen radical production from neutrophils induced by LPS and TNF-α priming after incubation with delta-toxin suggests that delta-toxin plays an important role in bacterial killing and in the potential damage of the microenvironment.

The observation that delta-toxin alone, in the absence of any additional stimulus, was not able to stimulate neutrophils is in line with observations by Raulf et al. [5] regarding leukotriene generation. It is well-established that human neutrophils, on stimulation by extracellular signals, release free arachidonic acid, which is metabolized to leukotrienes. The main product of neutrophils is leukotriene B4, which is known to stimulate chemotaxis, chemokinesis, and the release of granular enzymes. Delta-toxin alone was not able to induce leukotriene generation, but if neutrophils were incubated with delta-toxin and the Ca ionophore A23187 as an additional stimulus, leukotriene B4 formation was significantly enhanced. Further experiments with regard to the signal transduction cascade (e.g., protein phosphorylation, protein kinase C activity, and inositol turnover) are necessary to clarify the method by which delta-toxin costimulates the cells.

Because monocyes are the principal source of cytokines during sepsis, we determined the effect of delta-toxin on monocyte function. Delta-toxin induced TNF-α production in whole blood as well as in isolated monocytes, but in contrast to the costimulatory effect on oxidative burst in neutrophils, no additional TNF-α production could be observed if serially diluted delta-toxin combined with LPS was used as stimulus. The induction of TNF-α production was observed with relatively low delta-toxin concentrations.

Previous studies have demonstrated that other staphylococcal toxins, notably toxic shock syndrome toxin 1, and other components of gram-positive bacteria, such as peptidoglycans and lipoteichoic acid, are capable of inducing TNF-α release by monocytes. Nevertheless, in a staphylococcal infection, any of these constituents and toxins may act together, perhaps synergistically, to induce TNF and other cytokines. In a systemic staphylococcal infection, this sequence may cause the disastrous result of the induction of septic shock. Delta-toxin is able to induce TNF-α release, and furthermore, this TNF-α enhances priming of neutrophils for FMLP and C3bi-zym response, emphasizing the proinflammatory capacity of delta-toxin.

Results of the study suggest that delta-toxin could be an important pathogenic factor in S. aureus infections and emphasizes the potent and differential role of microbial toxins during bacterial infections. Delta-toxin is produced by >97% of S. aureus isolates. All supernatants of overnight cultures of S. aureus were able to lyse erythrocytes in an in vitro assay with sphingomyelinase-sensitized erythrocytes with a sensitivity of ≥1 µg/mL. Therefore, we conclude that in overnight cultures of S. aureus, ≥1 µg/mL of delta-toxin is produced.

The effects of delta-toxin described here may contribute to a better understanding of its biologic role and its implication in staphylococcal infections, as well as in mixed infections with gram-negative bacteria.

References