Effect of *Staphylococcus aureus* Delta-Toxin on Human Granulocyte Functions and Platelet-Activating-Factor Metabolism

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The production of delta-toxin is supposed to be responsible for various pathophysiological effects during infection with *Staphylococcus aureus*. We compared the effects of delta-toxin with the structurally related bee venom toxin melittin on granulocyte functions and inflammatory mediator release. Delta-toxin and melittin induced a rapid Ca²⁺ influx, as shown by fluorescence detection. Furthermore, oxygen radical production, as determined by luminol-enhanced chemiluminescence, was triggered by delta-toxin (0.15 to 15 μg/ml), whereas melittin showed only marginal effects. Release of lysozyme and β-glucuronidase was observed only at high concentrations of 15 μg of melittin and delta-toxin per ml. Preincubation (15 min) of neutrophils with both toxins resulted in the formation of [3H]-platelet-activating factor ([3H]-PAF) from [3H]-lyso-PAF. After 5 min of incubation, the exogenously added lyso-PAF was converted to PAF (delta-toxin, 80 ± 2%; melittin, 27 ± 12% of total radioactivity; n = 3, mean ± standard error of the mean) and 1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine (alkyl-acyl-GPC) (corresponding values, 20 ± 3% and 51 ± 14% of total radioactivity). The newly generated PAF was rapidly metabolized to lyso-PAF and alkyl-acyl-GPC during the subsequent incubation period of 60 min. In the absence of any toxin, no formation of PAF from lyso-PAF was observed. Further studies indicated that the metabolism of PAF into lyso-PAF and alkyl-acyl-GPC was inhibited in the presence of delta-toxin. Melittin had no significant effects on PAF metabolism. Neither delta-toxin nor melittin modulated the uptake of PAF and lyso-PAF significantly. Our data provide evidence that delta-toxin has an effect on the activity of neutrophil granulocytes with regard to its proinflammatory capacity.

Human polymorphonuclear leukocytes (PMNs) have important functions in the host defense against invading microorganisms (15). Commonly, they are activated as a result of an interaction between ligands and membrane receptors on the cell surface. This interaction is followed by processes of transmembrane signaling such as an increase in the intracellular Ca²⁺ concentration, translocation of protein kinase C, and phosphoinosite turnover (2). These events result in the activation of a variety of enzymes that initiate the cell-specific response, e.g., oxygen radical production and the generation of lipid mediators from membranous phospholipids, such as arachidonic acid metabolites and platelet-activating factor (PAF).

PAF is a unique phospholipid with the chemical structure 1-O-alkyl-2-acetyl-sn-glycerol-3-phosphocholine, derived from 1-O-alkyl-2-arachidonyl-phosphatidylcholine. PAF is generated after cleavage of the fatty acid at position C-2 of the carbon backbone (phospholipase [PLA₂]) and subsequent transfer of an acetyl residue by an acetyltransferase (9, 10, 25, 26). It is inactivated by the removal of the acetyl group (acetylhydrolase), resulting in the production of lyso-PAF and transfer of a long-chain fatty acid (acyltransferase) into 1-O-alkyl-2-acyl-glycerophosphocholine (alkyl-acyl-GPC). In addition, alkyl-acyl-GPC and lyso-PAF may serve as a potent source for the generation of PAF. According to Lee and Snyder (20), this activation-inactivation cycle is proposed to be the major pathway for the generation of PAF in neutrophils. PAF exhibits potent biological activities toward various cell types and tissues, such as aggregation of PMNs, chemotaxis of eosinophils, and immunomodulation (6, 17, 28, 34, 35), whereas lyso-PAF and alkyl-acyl-GPC have no biological activity. In the past, evidence was presented that various bacterial exotoxins or exoenzymes (e.g., staphylococcal alpha-toxin and lipase, alveolysin, and streptolysin O) induced arachidonic acid transformation into various proinflammatory lipid mediators (7, 18, 30). In this regard, delta-toxin from *Staphylococcus aureus* has pronounced effects on various cell types. In addition to its hemolytic effects, it activates PLA₂ and leads to prostaglandin synthesis in cells (3). Amino acid sequence analysis revealed evidence for structural homology between delta-toxin and the bee venom toxin melittin, with which it shares physicochemical and biological properties (4, 12, 14); moreover, comparable to delta-toxin, melittin activates PLA₂ and the formation of lipid mediators in various cell types (19).

It was the purpose of this investigation to study the influence of delta-toxin on human polymorphonuclear cell functions such as Ca²⁺ influx, oxygen radical production, enzyme release, and PAF generation as well as metabolism. The structurally related toxin melittin was used as a control stimulus.

(This study was carried out by S. Kasimir in partial fulfillment of the requirements for a Ph.D. degree.)

**MATERIALS AND METHODS**

**Materials.** Reagents were purchased from Pharmacia (Ficoll 400, Uppsala, Sweden), Knoll (6% [wt/vol] Macrodex; Ludwigshafen, Federal Republic of Germany), and Nyegaard (75% [wt/vol] sodium metrizoate solution; Oslo, Norway). All other fine chemicals were obtained from Sigma (Munich, Federal Republic of Germany) and Merck AG (Darmstadt, Federal Republic of Germany). Methanol and chloroform (analytical grade) were from Riedel de Haën.

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(Seelze, Federal Republic of Germany). \(^1H\)-PAF [alkyl-2-acetyl-sn-glycerol-3-phosphorylcholine, 1-O(alkyl-1,2-\(^3\)H)]; specific activity, 1.11 to 2.22 TBq/mmol] and \(^3H\)-lyso-PAF [alkyl-sn-glycerol-3-phosphorylcholine, 1-O(alkyl-1,2-\(^3\)H)]; specific activity, 1.11 to 2.22 TBq/mmol] were supplied by Dupont, NEN Research Products (Dreieich, Federal Republic of Germany).

Phosphate-buffered saline (PBS) was used throughout cell isolation and incubation and consisted of 0.137 M NaCl, 8 mM Na\(_2\)HPO\(_4\), 2.7 mM KH\(_2\)PO\(_4\), and 2.7 mM KCl (pH 7.4). For studies of PAF and lyso-PAF metabolism, 0.2% bovine serum albumin (Boehringer GmbH, Mannheim, Federal Republic of Germany) was added. HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer consisted of 1654 KASIMIR ET AL.

Preparation of the cells. Human PMNs were isolated from 200 ml of heparinized blood (15 U/ml) from healthy donors and separated on a Ficoll-metrizoate gradient followed by dextran sedimentation as described previously (5, 30). The erythrocytes were lysed by exposing the cell suspension to hypotonic conditions. This method revealed more than 97% pure PMNs. Less than 1% of the total cells were platelets. The PMNs were suspended to a final concentration of 2 \times 10\(^7\) cells per ml in PBS.

Toxins. The synthetic delta-toxin was prepared as described previously (1). Delta-toxin and melittin (Sigma) were dissolved in 0.01 M Tris hydrochloride (pH 7.4) at a concentration of 1 mg/ml. The experiments were carried out at three different concentrations (0.15, 1.5, and 15 \(\mu\)g/ml). At these concentrations the release of lactate dehydrogenase from stimulated cells was determined as described previously (1). Delta-toxin and melittin (Sigma) were added and incubated over the indicated time periods in the presence of physiological concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) at 37°C and the various toxins. The reactions were stopped after the addition of one volume of ice-cold buffer and subsequent centrifugation (9,600 \(\times\) g, 30 s). The pellets were washed three times in 1 ml of PBS buffer, suspended in 0.5 ml of PBS buffer, and counted in a liquid scintillation counter.

Statistical analysis. The experiments described here were performed four times with cells from different donors. Student's \(t\) test for independent means was used to provide a statistical analysis (P < 0.05 was considered significant).

RESULTS

Ca\(^{2+}\) influx. Figure 1 shows the results of a representative Ca\(^{2+}\) influx experiment. Delta-toxin (0.15 to 15 \(\mu\)g/ml) led to a rapid increase of free Ca\(^{2+}\) in the cytosol of PMNs. This cytosolic increase was detected exclusively in the presence of oxazene only added Ca\(^{2+}\) (1 mM), whereas in the absence of extracellular Ca\(^{2+}\), no change in fluorescence intensity was observed. Within 30 s a maximum was reached, which declined to the background level at later times. Melittin provoked a sustained rise in cytosolic Ca\(^{2+}\) levels at a concentration of 15 \(\mu\)g/ml; within the concentration range from 0.15 to 1.5 \(\mu\)g/ml, no increase in free calcium was observed.

Oxygen radical production. Figure 2A indicates that delta-toxin triggers oxygen radical production in human PMNs in a dose-dependent manner, with an early maximum after 2 min of incubation followed by a sharp decline within the subsequent time period. At a concentration of 0.15 \(\mu\)g/ml no differences, as compared with the buffer control, were obtained. In contrast, melittin (0.15 to 15 \(\mu\)g/ml; Fig. 2B) induced only a significant chemiluminescence response at 1.5 \(\mu\)g/ml compared with that induced by the buffer control; in general, the chemiluminescence response was fivefold less compared with the response of delta-toxin. At 0.15 \(\mu\)g/ml, oxygen radical production was about the same as that by the buffer control, whereas at 15 \(\mu\)g/ml, decreased oxygen radical production was obtained.

Enzyme release. The release of lysozyme from and azurophilic granules as well as \(\beta\)-glucuronidase from azurophilic granules by delta-toxin and melittin was studied at concentrations of 15, 1.5, and 0.15 \(\mu\)g/ml. The effects were seen after 5 min of incubation (Table 1); prolonged incubation (30 min) gave no significant differences from those seen after 5 min (data not shown). A significant increase of \(\beta\)-glucuronidase release was observed only at concentrations of 15 \(\mu\)g of delta-toxin and melittin per ml. Melittin showed a stronger effect than that of delta-toxin (Table 1). Lysozyme was released significantly only by melittin. At all three concentrations tested, no correlation between the amount of
FIG. 1. Induction of Ca\(^{2+}\) influx by fMLP (2.5 \times 10^{-4} \text{ mM}), delta-toxin (0.15 to 15 \mu g/ml), and melittin (0.15 to 15 \mu g/ml). Results of one of three typical experiments are shown. The axes indicate fluorescence (in arbitrary units) versus time, which increased from right to left. fMLP, Formylmethionyl-leucyl-phenylalanine.

lysozyme as well as \(\beta\)-glucuronidase and the release of lactate dehydrogenase could be established. The amount of lactate dehydrogenase released never exceeded the amounts of lysozyme and \(\beta\)-glucuronidase in the cell supernatant.

Metabolism of PAF. Figure 3 shows the effects of delta-toxin and melittin on the metabolism of PAF by human granulocytes. The cells were prelabeled with \(^3\)H-PAF (2 min at 4°C), and PAF metabolism was monitored. About 50\% of \(^3\)H-PAF was metabolized into alkyl-acyl-GPC within 15 min in the absence of any stimulus. Incubation with delta-toxin resulted in a significantly decreased metabolism of PAF. After 15 min only 25\% was metabolized into alkyl-acyl-GPC. Melittin showed a slightly delayed metabolism; however, no significant differences compared with the buffer control were obtained. Lyso-PAF was not detected during the monitoring period.

The next experiments addressed the question of whether PMNs preincubated with the toxins influenced PAF metabolism. Therefore, PMNs were pretreated with delta-toxin, melittin, and PBS, and subsequently, \(^3\)H-PAF was added. Under these conditions no significant differences were observed between the toxin-treated cells and the buffer control (PBS). A time-dependent decrease of PAF was combined with an increase of alkyl-acyl-GPC, whereas the concentrations of lyso-PAF were below the detection limit (data not shown).

Metabolism of lyso-PAF. In subsequent studies we investigated the effect of delta-toxin and melittin on the activity of the acetyltransferase enzyme which converts lyso-PAF into PAF. PMNs were incubated with \(^3\)H-lyso-PAF (0.74 kBq) for 2 min; subsequently, delta-toxin and melittin were added to the cells. In the absence of any toxin (buffer control), lyso-PAF was converted into alkyl-acyl-GPC without any formation of PAF (Fig. 4A). A different pattern was obtained with melittin and delta-toxin. Within 5 min about 50\% of \(^3\)H-lyso-PAF was converted into PAF (40\%) and alkyl-acyl-GPC (10\%). Lower amounts of PAF were generated by melittin as compared with that generated by delta-toxin. During the subsequent incubation period, the newly generated PAF was again metabolized into lyso-PAF and alkyl-acyl-GPC (Fig. 4B and C).

Preincubation of the cells with melittin and delta-toxin revealed differences with regard to PAF generation from lyso-PAF. Pretreatment of the cells with delta-toxin for 15 min strongly enhanced the conversion of the added lyso-PAF into PAF (Fig. 5B). The highest amounts of PAF were observed after 5 min of incubation. During the subsequent incubation period, the PAF that was generated was converted into lyso-PAF (45\%) and alkyl-acyl-GPC (40\%). In contrast to these data, preincubation with melittin did not enhance PAF formation compared with the enhancement by the delta-toxin (Fig. 5C).

\(^3\)H-lyso-PAF and \(^3\)H-PAF uptake in human granulocytes. Our previous data indicated that melittin and delta-toxin have strong effects on the activity of the enzymes that are involved in PAF and lyso-PAF metabolism. Experiments were then directed to analyze whether the toxins modulate the uptake of PAF and lyso-PAF into the cells. Incubation of 0.37 kBq of \(^3\)H-PAF for 2, 5, 10, 20, 30, and 60 min showed a time-dependent uptake into the cells. After 60 min, about 55 \pm 10\% (mean \pm standard error of the mean) of the total activity was associated within the cells. In the presence of delta-toxin or melittin, no differences compared with the PBS control were seen during the 30-min time period of
monitoring. Studies on the uptake of lyso-PAF showed a similar pattern, although lyso-PAF was incorporated to a lower degree (over 60 min, 45 ± 4%). During the time period of monitoring, either 3H-PAF or 3H-lyso-PAF and alkylacyl-GPC were retained within the cells.

**DISCUSSION**

In the past it became evident that bacterial toxins that are released into the microenvironment or that remain 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 (8, 13). These results were obtained in experiments in which the respective target cells (e.g., PMNs, monocytes, or lymphocytes) were treated with the toxin in vitro below cytolytic concentrations. For S. aureus, different exotoxins (alpha-, beta-, gamma-, and delta-toxins) have been described.

Our data provide evidence that delta-toxin exerts proinflammatory effects because of its capacity to increase (i) the intracellular concentration of free calcium, (ii) the generation of oxygen radicals, (iii) the release of enzymes, and (iv) the activation of the acetyltransferase, leading to the formation of PAF.

The capacity of delta-toxin to form pores within the membrane (2, 3) because of the amphiphilic structure of delta-toxin may be responsible for the observed influx of Ca2+ into the cell, which is one mechanism for the activation of neutrophils (29). In this regard, enzymatic activity, e.g., of PLAs2, which catalyzes the cleavage of arachidonic acid from phospholipids and initiates the generation of leukotrienes and PAF, is strongly dependent on Ca2+. Therefore, this suggests that the effects of delta-toxin on mediator production are regulated in part by the availability of free cytosolic Ca2+.

The pronounced oxygen radical production from PMNs after treatment with delta-toxin suggests that delta-toxin plays an important role in bacterial killing and in the potential damage of the microenvironment, e.g., by inactivation of protease inhibitors. In addition, delta-toxin was capable of releasing lysozyme and β-glucuronidase to a limited degree from PMN granules. However, only at the highest concentrations of delta-toxin used was enzyme release observed. Thus, it appears that the induction of granular enzyme release has only limited significance for the biological role of delta-toxin in vivo.

Delta-toxin activates the acetyltransferase which generates PAF from lyso-PAF. In this regard, Sisson et al. (31) described a correlation between PAF production and leukotriene B4 (LTB4) release in ionophore-stimulated PMNs, which demonstrated the coordinate production of the two biologically active lipids. These results were confirmed, in part, by results of our studies. We recently demonstrated that delta-toxin is capable of modulating leukotriene generation and metabolism induced by the Ca ionophore and opsonized zymosan (M. Raulf, J. Alouf, and W. König, submitted for publication). Although delta-toxin, by itself, was not sufficient to induce leukotriene generation, pretreatment with delta-toxin led to an increased production of

**TABLE 1. Release of lysozyme and β-glucuronidase**

<table>
<thead>
<tr>
<th>Toxin</th>
<th>% Release of total amount</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Lysozyme</td>
</tr>
<tr>
<td>Delta-toxin (15 μg/ml)</td>
<td>28.5 ± 9.5</td>
</tr>
<tr>
<td>Melittin (15 μg/ml)</td>
<td>73.7 ± 9.5*</td>
</tr>
<tr>
<td>Buffer control</td>
<td>14.5 ± 5.1</td>
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</tbody>
</table>

* Values are means ± standard errors of the mean of three independent experiments.

P < 0.05 compared with the buffer control by Student's t test.
FIG. 4. Metabolism of lyso-PAF. Cells were prelabeled with 
$^3$H-lyso-PAF; 90% of the total radioactivity was incorporated into
the cells. (A) PBS control; (B) delta-toxin (1.5 μg/ml); (C) melittin
(1.5 μg/ml). Values are means ± standard errors of the mean (n = 3).
Symbols: ●, PAF; ○, lyso-PAF; □, alkyl-acyl-GPC.

LTB$_4$, provided that the cells were subsequently stimulated
with the Ca ionophore.

Moreover, delta-toxin-treated cells revealed a decreased
capacity to inactivate exogenously added PAF. This effect
did not depend on a reduced uptake of PAF, since the
incorporation of PAF was not influenced by delta-toxin. It is
more likely that the endogenous production of PAF from
preexisting pools may be responsible, in part, for this
phenomenon. This assumption is confirmed by the fact that
the inhibition of PAF metabolism into lyso-PAF and alkyl-
acyl-GPC was observed during the first few minutes after the
addition of the toxins, when PAF production from lyso-PAF
was at a maximum, whereas no effect on PAF metabolism
was seen after an incubation period of 15 min.

The results suggest a differential role of delta-toxin in lipid
mediator generation compared with the roles of other toxins.
While the delta-toxin of S. aureus, unlike alpha-toxin, does
not induce LTB$_4$ formation by itself, it has pronounced
activity on the generation of PAF. PAF exerts a broad
spectrum of proinflammatory and immunomodulatory ef-
fects; however, the conditions under which human PMNs
release PAF into the cell supernatant have been discussed
but are controversial. Several reports indicated that the
phospholipid is released into the fluid phase (9, 17, 22),
whereas it was also observed that PAF is largely retained by
stimulated PMNs (23, 24, 31, 35). In our experiments,
the majority of PAF from delta-toxin-treated neutrophils
remained cell associated and was not secreted into the
medium. Therefore, it appears likely that PAF also has an
intracellular role with regard to PMN functions which does
not require its release into the fluid phase (31).

Parallel to delta-toxin, melittin was tested as a control
stimulus in the various assays; it is of interest whether the
slight differences in the biochemical structures of melittin
and delta-toxin have a significant impact on granulocyte
functions. The results indicate that melittin and delta-toxin
differ significantly, especially with regard to oxygen radical production and PAF generation. Bernheimer and Rudy (3) proposed that melittin is unable to penetrate completely into the cellular membrane because of its short alpha-helical structure, which prevents complete permeabilization of the cells. This may explain the differences in the Ca²⁺ influx obtained by melittin and delta-toxin, which is one of the central mechanisms of signal transduction and cellular activation of PMNs.

In contrast to Tomita et al. (33), we observed only a less pronounced chemiluminescence response induced by melittin as compared with those by other agonists, e.g., formylmethionyl-leucyl-phenylalanine, the Ca ionophore (data not shown), and delta-toxin, although the cells of the same donors responded well to melittin when the Ca²⁺ influx was studied. Moreover, Somerfield et al. (32) previously observed an inhibitory effect of melittin on oxygen radical production, as was the case at the highest doses tested in our experiments. Additional experiments are required to determine what these effects are on the NADPH-oxidase system.

Results of this study suggest that delta-toxin is an important pathogenic factor in S. aureus infections and may lead to increased vasopermeability and damage of the microenvironment, e.g., by oxygen radicals. A comparison of the various toxins, e.g., delta-toxin compared with the alpha-toxin of S. aureus and with E. coli alpha-hemolysin, emphasizes the potent and differential role of microbial toxins during bacterial infections (15, 18). Additional studies are under way to elucidate the precise mechanisms of action of delta-toxin on the signal transduction cascades in PMNs.

ACKNOWLEDGMENT

This study was supported by grant K6 427/74 from the Deutsche Forschungsgemeinschaft.

LITERATURE CITED


EFFECT OF S. AUREUS DELTA-TOXIN


