INFECTION AND IMMUNITY, June 1994, p. 2529–2535
0019-9567/94/$04.00+0

Vol. 62, No. 6

Leukotriene B_4 Generation and DNA Fragmentation Induced by Leukocidin from *Staphylococcus aureus*: Protective Role of Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and G-CSF for Human Neutrophils

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Received 29 October 1993/Returned for modification 3 March 1994/Accepted 24 March 1994

We studied the effect of leukocidin from *Staphylococcus aureus* V8 strains (Luk-PV) on the generation of Leukotriene B_4 (LTB_4) and its metabolites from human polymorphonuclear neutrophils (PMNs). Significant amounts of LTB_4 were generated by PMNs after leukocidin exposure in a time- and dose-dependent manner, as shown by reversed-phase high-performance liquid chromatography analysis. In this regard, the S and F components of leukocidin acted synergistically. The calcium ionophore A23187 induced LTB_4 generation, and the metabolism of exogenously added LTB_4 into biologically less active omega-oxidated compounds was significantly decreased after leukocidin exposure. Priming of PMNs with granulocyte-macrophage colony-stimulating factor (GM-CSF) or G-CSF prior to leukocidin exposure substantially increased toxin- and calcium ionophore A23187-induced LTB_4 formation. The inhibitory effects of leukocidin on mediator release were accompanied by membrane damage and DNA fragmentation, which were both restored after pretreatment with GM-CSF. The data suggest that the presence of costimulatory priming factors such as GM-CSF or G-CSF in the microenvironment of an inflammatory focus determines the pathophysiological effects induced by *S. aureus* leukocidin.

Leukocidin from *Staphylococcus aureus* V8 strains (Luk-PV) is known to be important for the pathogenicity of certain staphylococcal diseases (7, 11, 22, 32). The toxin consists of two protein components, S (38 kDa) and F (32 kDa), that act synergistically to induce cytotoxic changes in monocytes, macrophages, and polymorphonuclear neutrophils (PMNs) (31–33). Morphological changes such as cellular swelling, appearance of rounded nuclei, and loss of granules are associated with the formation of plasma membrane pores (10, 11, 33).

The word leukocidin represents different staphylococcal leukotoxins. Noda et al. (22–24) described a leukocidin, also produced by strain V8, whose component F and S molecular masses (31 and 32 kDa, respectively) were different from those described by Woodin (31, 32) and whose nucleotide sequence was 91% homologous to that of gamma-hemolysin. The gamma-hemolysin had 74% homology, on the nucleotide level, as well as in its peptide sequence, to Luk-PV (26).

We analyzed the effects of Luk-PV on leukotriene (LT) B_4 (LTB_4) generation and metabolism by human PMNs. In 1982, Noda et al. reported that the S component of leukocidin stimulated high levels of phospholipase A_2 activity in cell membranes from rabbit PMNs with concomitant synthesis and release of prostaglandins (24). LTs are potent phospholipase A_2-derived lipid mediators which induce inflammatory and allergic reactions (4, 16, 17). In this regard, LTB_4 plays a crucial role as an inflammatory chemotactic factor for neutrophils and eosinophils (4). It exerts autocrine effects on PMNs (20) and induces neutrophils to degranulate, generate superoxide, and adhere to the vascular endothelium (16, 17). However, human PMNs are also capable of deactivating LTB_4 via specific hydroxylation at position C-20 (omega oxidation), resulting in the formation of 20-OH-LTB_4 and 20-COOH-LTB_4. These compounds have diminished biological activities compared with LTB_4 (6, 12).

An increasingly complex role for cytokines in the modulation of PMN functions has been described. Specific colony-stimulating factors (CSFs), e.g., granulocyte-macrophage (GM)-CSF, G-CSF, or interleukin 3 (IL-3) (multi-CSF), are known to interact with mature PMNs via ligand-receptor binding and are thus responsible for the priming of inflammatory cells (13, 18, 21). Previous studies have shown that priming of mature PMNs with GM-CSF enhances neutrophil functions such as cytotoxicity, superoxide production, phagocytosis, and granule release in response to particulate stimuli (19). Cytokines may also upregulate or modulate the cellular response of PMNs to bacterial toxins.

It was the purpose of the present studies to analyze the effects of GM-CSF, G-CSF, and IL-3 on (i) leukocidin-induced LTB_4 formation, (ii) the cellular activation of leukocidin-treated PMNs by the calcium ionophore A23187, and (iii) the LTB_4 metabolism of leukocidin-treated PMNs. We also assessed whether the presence of CSFs exerts protective effects against the leukotoxic action of *S. aureus* leukocidin as assessed by trypan blue staining and DNA fragmentation studies.

MATERIALS AND METHODS

Materials. (i) Cell separation. Ficoll 400 was obtained from Pharmacia, Uppsala, Sweden. Macrodex (6%, wt/vol) was from

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Knoll, Ludwigshafen, Germany. Sodium metrizoate solution (75%, wt/vol) was from Nycomed, Oslo, Norway.

(ii) Cell stimulation. Calcium ionophore A23187 was purchased from Sigma, Munich, Germany.

(iii) CSFs. GM-CSF, G-CSF, and IL-3 were kindly provided by Sandoz AG, Nürnberg, Germany.

(iv) HPLC procedure. Acetonitrile (high-performance liquid chromatography [HPLC] grade) was supplied by Baker Chemicals (Gross-Gerau, Germany), and methanol, dipotassium hydrogen phosphate, and phosphoric acid were from Riedel de Haën (Seelze, Germany). All other chemicals were from Merck, Darmstadt, Germany.

(v) Buffer. The buffer used throughout all of the experiments consisted of 0.137 M NaCl, 8 mM Na_2HPO_4, 2.7 mM KH_2PO_4, and 2.7 mM KCl (pH 7.4) (modified Dulbecco phosphate-buffered saline [PBS]).

Leukocidin from *S. aureus*. The leukotoxin used was Panton-Valentine leukocidin PVL (EMBL/GenBank accession no. 72700; 26). The F and S components were purified to homogeneity by G. Prévost (Faculte de Médecine, Université Louis Pasteur, Strasbourg, France) as described before (10). Contamination of the leukocidin preparation with endotoxin was ruled out with the *Limulus* test. Contamination with other staphylococcal products was ruled out by enzyme-linked immunosorbent assay (25a). The preparations were also devoid of detectable hemolytic activity on sheep, human, and rabbit erythrocytes, indicating absence of contamination by hemolytic toxins. The S and F components of leukocidin were applied to the cell suspensions in a ratio of 1:1 (wt/wt) at the concentrations stated in Results.

Purification of human neutrophils. Human PMNs were isolated from heparinized (15 U/ml) peripheral blood of healthy donors by using a Ficoll-metrizoate gradient and subsequent dextran sedimentation as described by Böyum (3).

Incubation conditions. PMNs (10^7/500 μl of PBS) were primed for 60 min at 37°C either with PBS as a control or with GM-CSF (10 ng/10^7 PMNs), G-CSF (10 ng/10^7 PMNs), or IL-3 (4 ng/10^7 PMNs). Subsequently, leukocidin components F and S were added to the cell suspensions in the presence of calcium (2 mM) and magnesium (1 mM) and incubation proceeded for an additional 30 min (except for the kinetic studies). For calcium ionophore A23187 stimulation, the cells were washed and resuspended in 500 μl of PBS. Subsequently, calcium ionophore A23187 (6.3 μM) was added to the cell suspensions in the presence of calcium (2 mM) and magnesium (1 mM) and incubation proceeded for a further 20 min at 37°C.

Cell viability. Lactate dehydrogenase (LDH), lysozyme, and β-glucuronidase release was assessed as described before (5). Cytoplasmic LDH release after treatment with leukocidin was 1.8% ± 3.1% of the total cellular LDH content when 500 ng of leukocidin per 500 μl/10^7 human PMNs was applied during a typical incubation period of 30 min. Release of granular constituents was 47.3% ± 25.2% for lysozyme and 43.2% ± 13.4% for β-glucuronidase.

Analysis of LT generation. After the incubation period, 2 ml of methanol-acetonitrile (50:50, vol/vol) was added to the complete incubation mixture and the total generation of LTB_4 and its metabolites was measured. The vials were overlaid with argon and frozen at −70°C for 12 h. After centrifugation at 1,900 × *g* for 10 min (Cryofuge 6-4; Heraeus Christ, Osterode, Germany), the supernatants were removed and evaporated to dryness by lyophilization (EF 4 Modulyo; Edwards-Kniese, Marburg, Germany). The precipitate was dissolved in 600 μl of methanol-water (30:70, vol/vol), and centrifugation was performed at 9,600 × *g* for 4 min for further purification. Aliquots of 200 μl were subjected to reversed-phase HPLC analysis. The HPLC equipment consisted of a CM 4000 pump and an SM 4000 detector (Laboratory Data Control/Milton Roy, Hasselroth, Germany) and a WISP 710B automatic sample injector (Waters, Eschborn, Germany). The reversed-phase column (4.6 by 250 mm) was packed with Nucleosil 5 C_{18} (pore size, 5 μm; Macherey-Nagel, Düren, Germany). Isocratic elution for LT analysis was done with a solvent system consisting of water-acetonitrile-methanol (50:30:20, vol/vol/vol) and including 0.04% EDTA and 0.15% K_2HPO_4. The pH was adjusted to 5.0 by addition of phosphoric acid. The flow rate was maintained at 0.9 ml/min. All solvents were degassed before use and constantly stirred during HPLC analysis. The A_{280} of the column effluent was measured. Quantification and identification of LTs were performed with synthetic standard solutions. LTB_4 generation was calculated as the combined amounts of LTB_4 and the LTB_4 omega-oxidation products (20-OH-LTB_4 and 20-COOH-LTB_4), except for the studies on LTB_4 metabolism.

Metabolism of exogenously added LTB_4. PMNs (10^7/500 μl of PBS) were incubated as described above, in the paragraph on incubation conditions. Afterwards, the cells were washed and resuspended in 500 μl of PBS and 100 ng of exogenous LTB_4 was added to the samples. Incubation then proceeded in the presence of calcium (2 mM) and magnesium (1 mM) for an additional 20 min at 37°C. Samples receiving PBS instead of CSFs and leukocidin served as controls. The reaction was stopped by addition of methanol-acetonitrile (50:50, vol/vol). The metabolites were detected as described in the previous paragraph, and the distribution of LTB_4, OH-LTB_4, and COOH-LTB_4 was determined as percentages of the recovered LTB_4.

DNA fragmentation. PMNs (10^7/500 μl of PBS) were incubated as already described in the paragraph on incubation conditions, and cellular integrity was checked by the trypan blue exclusion assay. Afterwards, cellular DNA was isolated as described in reference 26a. The PMNs were resuspended in 250 μl of 10 mM Tris/HCl containing 1 mM EDTA and lysed after addition of 250 μl of 100 mM NaCl, 50 mM EDTA, and 1% sodium dodecyl sulfate. The mixture was incubated for 5 min at room temperature. Thereafter, 300 μl of a 3 M potassium acetate solution and 400 μl of phenol-chloroform (50:50) were added, mixed, and centrifuged for 5 min at 8,000 rpm. The viscous supernatant was extracted once more with chloroform-isoamyl alcohol (24:1) and then precipitated with isopropanol-ethanol (50:50). The precipitate was washed once in 70% ethanol and resuspended in 10 mM Tris/HCl–1 mM EDTA–20 μg of RNase A per ml. Electrophoresis was carried out in a 0.7% agarose gel for 3 h at 70 V. The agarose gel was stained with ethidium bromide and visualized with UV light.

Statistics. If not stated otherwise, data from at least three independent experiments with different donor cells were combined and expressed as means ± the standard deviations. Student's *t* test for independent means was used for statistical analysis ($P > 0.05$ was considered not significant).

RESULTS

Leukocidin-induced LTB_4 generation. In a first set of experiments, human PMNs were incubated with leukocidin at various concentrations (10 to 1,000 ng/500 μl of incubation buffer) for 30 min at 37°C. As is apparent from Fig. 1A, enhanced amounts of LTB_4 and omega-oxidated products, representing the results of de novo synthesis, were detected when PMNs were treated with leukocidin concentrations ranging from 100 to 500 ng. A further increase of the toxin concentration to up to 1,000 ng did not lead to additional

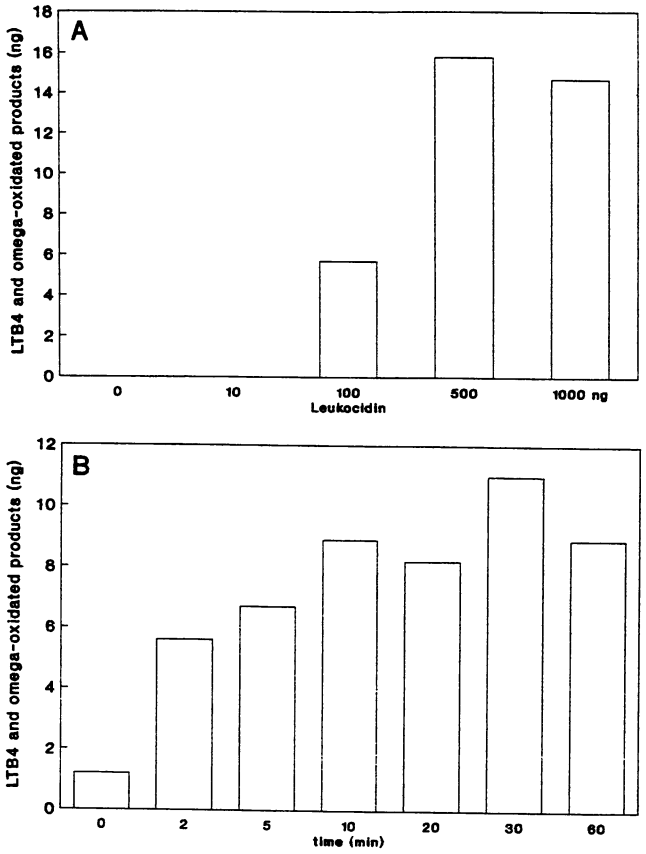

FIG. 1. (A) Generation of total LTB_4 (20-COOH-LTB_4, 20-OH-LTB_4, and LTB_4) from PMNs treated with different concentrations of leukocidin for 30 min at 37°C. (B) Kinetic studies with 500 ng of leukocidin per 10^7 PMNs. The values shown are from typical experiments. Additional experiments showed the same pattern.

enhancement of LTB_4 formation. Induction of LTB_4 generation by leukocidin was dependent on the presence of both toxin components S and F (synergistic effects). Kinetic experiments were then performed with 500 ng of leukocidin at intervals ranging from 2 to 60 min. As shown in Fig. 1B, maximal LTB_4 generation was obtained after 30 min of incubation. A slight variation of the total amount of LTB_4 was obtained after 60 min of incubation, while conversion of LTB_4 into its omega-oxidated products was significantly decreased (Table 1). Thus, in subsequent experiments, incubation for 30 minutes was chosen for further analysis. Under these conditions, most of the LTB_4 was detected in its omega-oxidated state (65.2% ± 6.1% OH-LTB_4 and 34.8% ± 6.1% COOH-LTB_4).

Influence of CSFs on leukocidin-induced LTB_4 generation. We and others have previously demonstrated that GM-CSF-pretreated PMNs reveal enhanced LTB_4 formation after stimulation with the bacterial chemotaxin fMLP (8). To determine whether CSFs may influence the biological activity of leukocidin, human PMNs were pretreated with either GM-CSF (10 ng/10^7 PMNs), G-CSF (10 ng/10^7 PMNs), or IL-3 (4 ng/10^7 PMNs) for 60 min. The primed PMNs were subsequently stimulated with leukocidin (500 ng/500 μl of incubation buffer) for a further 30 min, and LTB_4 generation by the cells was assessed by reversed-phase HPLC. As shown in Fig. 2, GM-CSF-primed PMNs revealed enhanced release of LTB_4 up to 176.9 ± 79.6 ng, compared with 8.4 ± 1.9 ng in the absence of

TABLE 1. Conversion of exogenous LTB_4 by leukocidin-treated human PMNs

Primer and stimulus[a] (amt [ng])	Mean % of recovered LTB_4[b] ± SD		
	COOH-LTB_4	OH-LTB_4	LTB_4
PBS			
PBS	69.7 ± 3.2	22.9 ± 0.8	7.5 ± 2.4
Leu (5)	65.5 ± 3.4	24.8 ± 0.5	9.8 ± 2.9
Leu (500)	12.3 ± 0.4	49.3 ± 1.6	38.5 ± 1.3
GM-CSF			
PBS	57.9 ± 7.2	34.0 ± 3.8	8.1 ± 3.4
Leu (5)	61.8 ± 0.4	29.6 ± 0.5	8.7 ± 0.2
Leu (500)	10.8 ± 4.5	59.7 ± 0.9	29.5 ± 3.6
G-CSF			
PBS	61.3 ± 9.9	28.4 ± 4.8	10.4 ± 5.2
Leu (5)	61.2 ± 7.3	31.7 ± 4.4	7.1 ± 2.9
Leu (500)	10.1 ± 0.9	49.7 ± 4.4	40.3 ± 3.5
IL-3			
PBS	67.9 ± 6.8	28.3 ± 3.1	3.8 ± 3.7
Leu (5)	63.4 ± 3.7	30.2 ± 1.8	6.4 ± 2.0
Leu (500)	8.0 ± 1.9	48.1 ± 2.3	43.9 ± 2.8

[a] Leu, leukocidin.
[b] At least three experiments were done under each set of conditions, and 100 ng of exogenous LTB_4 was applied.

GM-CSF. G-CSF-primed PMNs were less responsive in terms of LTB_4 formation (77.6 ± 7.5 ng), while IL-3 had no effect (8.7 ± 4.3 ng) compared with the control values. In addition, we used a suboptimal amount of leukocidin (5 ng) for cell stimulation. In these experiments, upregulation of LTB_4 generation was observed only for GM-CSF-primed PMNs (from 1.5 to 8 ng).

Effect of leukocidin on LTB_4 conversion. Conversion of exogenous LTB_4 into its biologically less active compounds 20-OH-LTB_4 and 20-COOH-LTB_4 is one important cellular parameter for the regulation of inflammatory processes (6). In our experiments, we studied the conversion of 100 ng of exogenous LTB_4 by leukocidin-treated PMNs compared with that of untreated cells. As shown in Table 1, 92.5% of the recovered LTB_4 was metabolized into omega-oxidated products by control cells during a 20-min incubation period. Preincubation of PMNs with 500 ng of leukocidin per 500 μl of

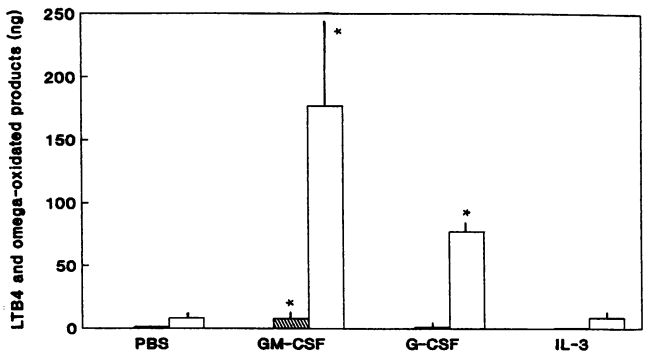

FIG. 2. Effect of GM-CSF, G-CSF, or IL-3 on LTB_4 generation by PMNs induced by 5 (▨) or 500 (□) ng of leukocidin. The data are expressed as mean values ± standard deviations ($n = 4$) obtained with different donor cells (*, $P < 0.01$).

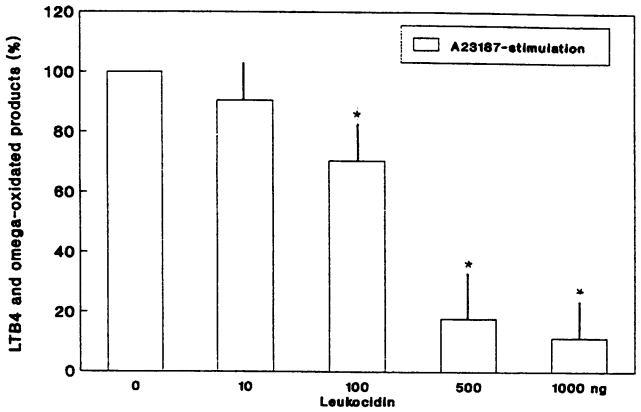

FIG. 3. Effect of leukocidin on LTB_4 generation by PMNs induced by the calcium ionophore A23187. The 100% value for A23187-stimulated PMNs was 378.3 ± 83.2 ng. The data are expressed as mean values ± standard deviations (n = 4) obtained with different donor cells (*, $P < 0.01$).

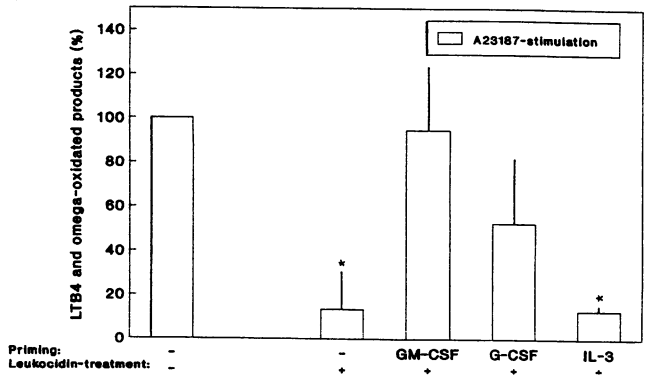

FIG. 4. Effect of GM-CSF, G-CSF, or IL-3 priming on the leukocidin treatment of PMNs and subsequent stimulation by the calcium ionophore A23187. The 100% value for A23187-stimulated PMNs was 378.3 ± 83.2 ng. The data are expressed as mean values ± standard deviations (n = 5) obtained with different donor cells (*, $P < 0.01$).

incubation buffer impaired the metabolism of LTB_4 (38.5% of the recovered LTB_4 was not metabolized). Similar decreases in LTB_4 metabolism were detected when GM-CSF (29.5%)-, G-CSF (40.3%)-, and IL-3 (43.9%)-primed PMNs were used for leukocidin treatment.

Influence of leukocidin on calcium ionophore A23187-induced LTB_4 generation. In comparison with the calcium ionophore A23187, which has been shown to be a potent activator of LTB_4 formation (378.3 ± 83.2 ng), leukocidin by itself is a weak stimulus (8.4 ± 1.9 ng). Therefore, we addressed the question of whether leukocidin-pretreated PMNs can be activated for LTB_4 formation by using the calcium ionophore A23187 as a subsequent stimulus. PMNs were pretreated with leukocidin at various concentrations (10 to 1,000 ng/500 μl of incubation buffer) for 30 min at 37°C. The PMNs were then stimulated with the calcium ionophore A23187 (6.3 μM) for an additional 20 min. As is apparent from Fig. 3, pretreatment of PMNs with a small amount of leukocidin (100 ng) already reduced the amounts of LTB_4 generated after ionophore stimulation by 30%. With 500 ng of leukocidin, the reduction amounted to 82% compared with the control, and the reduction was slightly increased to 88% when 1,000 ng of leukocidin was used. Studies were also performed to exclude the possibility that leukocidin binds LTB_4. When different amounts of leukocidin were incubated with exogenous LTB_4 (102 ng), similar amounts of LTB_4 were detected in the samples (5 ng of leukocidin, 102 ng of LTB_4; 50 ng of leukocidin, 104 ng of LTB_4; 500 ng of leukocidin, 104 ng of LTB_4).

Effect of CSFs on calcium ionophore-induced LTB_4 formation by leukocidin-pretreated PMNs. We then analyzed to what extent different CSFs may interfere with the inhibitory effects of leukocidin on the subsequent cell activation induced by the calcium ionophore A23187. PMNs were pretreated with GM-CSF (10 ng/10^7 PMNs), G-CSF (10 ng/10^7 PMNs), or IL-3 (4 ng/10^7 PMNs) for 60 min at 37°C and subsequently stimulated with 500 ng of leukocidin for 30 min. After this time period, the calcium ionophore was added for an additional 20 min. The controls were (i) cells exposed to buffer (PBS) for 60 min, subsequently treated with leukocidin, and then activated with the calcium ionophore and (ii) cells incubated in buffer, not treated with leukocidin, and then stimulated with the calcium ionophore. As is apparent from Fig. 4, leukocidin treatment and subsequent stimulation reduced the amount of LTB_4 formation from 100% (=378.3 ng) to 13.4%. After preincubation with GM-CSF, the amounts of LTB_4 generated after calcium ionophore stimulation of the cells were comparable to those generated in the absence of leukocidin (i.e., 95%). Priming of cells with G-CSF showed an upregulation similar to but less pronounced (i.e., 53%) than that obtained for GM-CSF, while IL-3 addition was ineffective.

Trypan blue staining and DNA fragmentation induced by leukocidin. Leukocidin as a leukotoxin induces cytolysis in monocytes, macrophages, and PMNs (31–33). To investigate to what extent the cytolytic properties of leukocidin may reduce the capacity of PMNs to generate LTB_4 in response to a subsequent stimulus, trypan blue staining and DNA fragmentation were investigated as parameters of membrane and cellular integrity. As is shown in Table 2, leukocidin treatment of PMNs led to dose-dependent trypan blue staining of the cells. With 500 ng of leukocidin, the trypan blue staining of

TABLE 2. Effect of leukocidin on trypan blue staining of human PMNs

Primer	Stimulus[a] (amt [ng])	Mean[b] % trypan blue staining ± SD
PBS	PBS	5.0 ± 1.0
PBS	Leu (5)	6.0 ± 2.0
PBS	Leu (50)	19.7 ± 4.9
PBS	Leu (500)	86.3 ± 7.2
GM-CSF	PBS	4.0 ± 1.0
GM-CSF	Leu (5)	5.7 ± 7.2
GM-CSF	Leu (50)	9.7 ± 1.5
GM-CSF	Leu (500)	55.0 ± 2.6
G-CSF	PBS	1.6 ± 1.5
G-CSF	Leu (5)	7.7 ± 2.9
G-CSF	Leu (50)	16.3 ± 3.2
G-CSF	Leu (500)	75.2 ± 9.7
IL-3	PBS	2.3 ± 2.1
IL-3	Leu (5)	5.0 ± 1.7
IL-3	Leu (50)	21.7 ± 4.0
IL-3	Leu (500)	85.0 ± 1.7

[a] Leu, leukocidin.
[b] At least three experiments were done under each set of conditions.

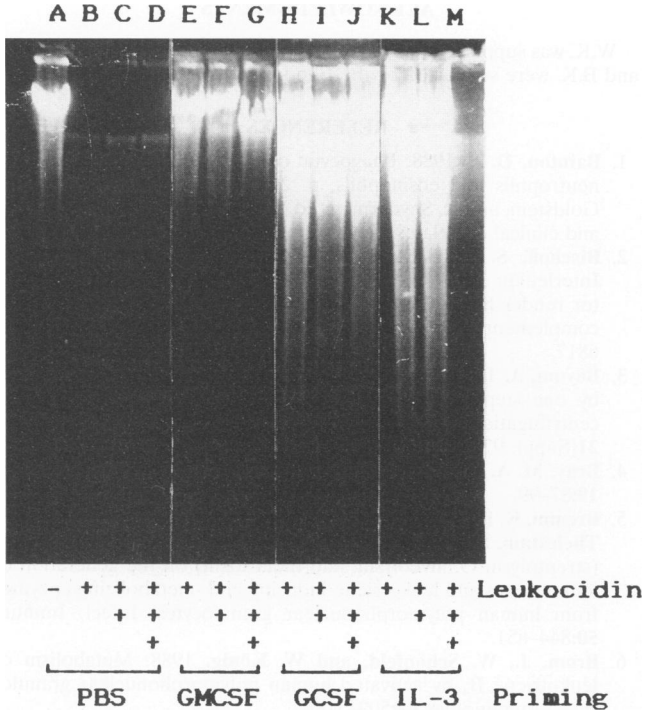

FIG. 5. Leukocidin-induced DNA fragmentation in human PMNs. Control incubations were performed without leukocidin (lane A). Amount of leukocidin (nanograms per 500 μl of incubation buffer): −, 0; +, 5; ++, 50; +++, 500.

PMNs amounted to 86%. GM-CSF pretreatment (10 ng/10^7 PMNs) significantly reduced the trypan blue staining of leukocidin-treated PMNs (55% ± 2.6%). In contrast, G-CSF addition (10 ng/10^7 PMNs) and IL-3 addition (4 ng/10^7 PMNs) had no protective effects compared with the control (G-CSF, 75.2% ± 9.7%; IL-3, 85.0% ± 1.7%). The data fit the results obtained in the DNA fragmentation study. As is apparent from Fig. 5, leukocidin-treated PMNs showed DNA fragmentation after 30 min of incubation at concentrations of 5, 50, and 500 ng/500 μl of incubation buffer (lanes B, C, and D), unlike control cells (lane A). Pretreatment of PMNs with GM-CSF (lanes E, F, and G) prevented the DNA fragmentation induced by leukocidin. In contrast, G-CSF and IL-3 priming was ineffective and demonstrated DNA digestion under all of the conditions used.

DISCUSSION

Migration of inflammatory cells from human blood into tissue represents one of the most important components of the inflammatory response (1). In this regard, a variety of lipid mediators, e.g., LTB_4, which is highly chemotactic for neutrophils and eosinophils, are generated after cell activation (4, 16, 17). Major emphasis has been placed on the cytolytic properties inherent in many toxins, including staphylococcal leukocidin; it is increasingly evident that toxins at noncytolytic concentrations activate and modulate cellular functions. In previous publications, we demonstrated that noncytolytic toxin concentrations of streptolysin O, alveolysin, and *Escherichia coli* alpha-hemolysin lead to the generation of lipid mediators and oxygen radicals and modulate different effector functions of human PMNs (5, 28).

Luk-PV, which is suspected to be important in the pathogenicity of certain staphylococcal diseases (22, 32), is commonly referred to as a membrane-damaging cytolysin with high specificity for PMNs, monocytes, and macrophages (22, 33). Clinical investigations have shown that *S. aureus* strains obtained from patients with chronic furunculosis were very often leukocidin producers (7). To investigate the consequences of pore formation by leukocidin for the inflammatory response of PMNs, LTB_4 generation was analyzed. With regard to host immunity, it is evident that leukocidin activates the enzymes (5-lipoxygenase and 5-lipoxygenase-activating protein) required for LTB_4 formation. As we observed, staphylococcal leukocidin directly triggered LTB_4 release from human PMNs. Interestingly, even granulocytes permeabilized by digitonin (25) and then stimulated with leukocidin are potent LTB_4 producers (data not shown). These results suggest that in the host defense, severely damaged cells might still serve as potent targets and producers of chemotactically active LTB_4. Additionally, the LTB_4-metabolizing pathway to the biologically less active omega-oxidated compounds (20-COOH-LTB_4 and 20-OH-LTB_4) was significantly inhibited after toxin treatment. Additional experiments (unpublished data) revealed evidence of reduced expression of LTB_4 receptors. The downregulation of the LTB_4 receptor may be responsible for the reduced ability of leukocidin-treated PMNs to deactivate LTB_4 by omega oxidation. As a consequence for the microenvironment, one may suggest that the enhanced inflammatory mediator level may recruit additional inflammatory cells for acute and chronic inflammatory reactions, which may be responsible for the pathogenicity of leukocidin.

Our data support the results of previous investigations, which showed that the S component of leukocidin stimulated high levels of phospholipase A_2 activity in cell membranes from rabbit PMNs, with the concomitant synthesis and release of prostaglandins (22–24). Phospholipase A_2 activation is also a prerequisite for release of arachidonic acid, which is subsequently converted into LTB_4.

As we observed, the separated S and F components of leukocidin did not induce LTB_4 generation by themselves. Moreover, the two components acted synergistically. Similar synergistic effects of the S and F components of leukocidin on the intracellular Ca^{2+} concentration were recently described (10). It was shown that leukocidin apparently stimulates the opening of cation channels in the leukocyte membrane without affecting intracellular Ca^{2+} stores. Our data demonstrated increased trypan blue staining, depending on the leukocidin concentration, but no detectable LDH release. Release of the cytoplasmic marker LDH never exceeded 5% of the total cellular LDH content when 500 ng of leukocidin per 500 μl/10^7 human PMNs was used throughout a typical 30-min incubation period. Our results therefore indicate that molecules with the size of LDH (134 kDa) are not released. In contrast, Noda et al. reported that incubation with 12.5 ng of leukocidin per 500 μl/2.5×10^7 rabbit PMNs resulted in "degranulation, rupture of nuclei, appearance of intracellular vacuoles and complete cell lysis" within 10 min of incubation (23, 24). Such observations imply membrane damage sufficient to effect LDH release. These obvious differences may be dependent on the use of different leukotoxins. The leukotoxin described here was Panton-Valentine leukocidin PVL (EMBL/GenBank accession no. 72700). In contrast, Noda et al. used a leukocidin with a nucleotide sequence which was 91% homologous to that of gamma-hemolysin. The gamma-hemolysin itself possessed 74% homology on the nucleotide level, as well as in its peptide sequence, to Luk-PV, which was used in our experiments (26).

Recently, receptors for a number of CSFs have been demonstrated on PMNs (13, 18, 21). Several reports indicated that

IL-3, also referred to as multi-CSF, competes with GM-CSF for binding to the high-affinity site in human hemopoietic cells (2, 9, 21, 27). Therefore, IL-3, GM-CSF, and G-CSF were used for cellular priming to study the modulatory effects of CSFs on the leukocidin treatment of PMNs. Our results show that leukocidin-induced LTB_4 release was significantly enhanced after priming of the cells with GM-CSF or G-CSF but not after priming with IL-3. In this regard, Dahinden et al. reported increased LTB_4 production by human PMNs primed with recombinant human GM-CSF and subsequently stimulated with bacterial fMLP (8). Furthermore, an upregulation of cell surface receptors for bacterial and complement components after GM-CSF priming of PMNs has been shown (19). Current data also indicate an upregulation of 5-lipoxygenase and 5-lipoxygenase-activating protein gene expression in human PMNs after priming with GM-CSF (29). As was shown recently, the efficiency of cellular priming was inhibited by different tyrosine kinase inhibitors, e.g., lavendustin, tyrphostin, and genistein (unpublished data). It has also been shown that PMNs pretreated with cytokines, e.g., tumor necrosis factor α or GM-CSF, express intracellular heat shock proteins (14). Thus, multiple mechanisms are obviously responsible for the priming effects of cytokines, as well as for their protective effects against a cytolytic attack by leukocidin.

Previous investigations have shown that the reduced capacity to generate LTB_4 via a subsequent stimulus correlated with an outcome of shock and sepsis in severely burned patients (15). In this regard, we have demonstrated that leukocidin significantly inhibited the subsequent stimulation of PMNs with the calcium ionophore A23187 in a concentration-dependent manner. This may reflect preactivation of the cells by low concentrations of leukocidin, which is followed by cellular deactivation for a secondary signal. Since leukocidin induces an influx of calcium, one may suggest that a subsequent calcium signal, e.g., by the calcium ionophore, cannot trigger the cells. Obviously, the precise mechanisms for deactivation are not known. However, such a deactivation may paralyze immune effector cells, which then are unresponsive to microbial stimuli, thus favoring microbial invasion.

It has been shown that treatment of PMNs with leukocidin induces a significant discharge of protein from the cellular granules into the medium (33). We demonstrated a significant release of granular constituents from PMNs after incubation with leukocidin, as was measured for lysozyme and β-glucuronidase. The degranulation was paralleled by trypan blue staining and DNA fragmentation. However, the observations do not indicate that these parameters are correlated to each other. They may just reflect different cellular responses to leukocidin. GM-CSF-primed PMNs were protected from DNA fragmentation and from the pore-forming activity of leukocidin, as shown by reduced trypan blue staining. These results correlate with the restored capacity of GM-CSF- or G-CSF-primed PMNs to generate LTB_4 after leukocidin treatment and subsequent stimulation with the calcium ionophore A23187. In this regard, previous investigations support our data, indicating that specific growth factors rescue cells from apoptosis (2, 30, 34). Studies are presently directed toward analysis of whether the protective effects from the toxic action of leukocidin and the priming effects on the LTB_4 response are related or distinct events.

Our data therefore suggest that the relative toxicity of a microbial toxin has to be considered in relation to the overall conditions of an inflammatory environment. In this regard, ongoing GM-CSF or G-CSF production favors mediator generation, even at cytolytic concentrations of a microbial toxin, and thus supports the host defense.

ACKNOWLEDGMENTS

W.K. was supported by the Deutsche Forschungsgemeinschaft. T.H. and B.K. were supported by the Bundersamt für Zivilschutz.

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