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GTP-Binding Proteins Are Involved in the Modulated Activity of Human Neutrophils Treated with the Panton-Valentine Leukocidin from *Staphylococcus aureus*

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Significant amounts of leukotriene B_4 (LTB_4) are generated by human polymorphonuclear neutrophils (PMNs) after incubation with the Panton-Valentine leukocidin (Luk-PV) from *Staphylococcus aureus* V8 strains. We showed that GTP-binding proteins (G proteins) are involved in the Luk-PV-activated signal transduction of PMNs. ADP-ribosylation of heterotrimeric G proteins by cholera and pertussis toxins decreased the Luk-PV-induced LTB_4-generation. In contrast, ADP-ribosylation of the low-molecular-weight G proteins rho and rac by *Clostridium botulinum* exoenzyme C3 increased the Luk-PV-induced LTB_4 synthesis. The subsequent stimulation of Luk-PV-treated PMNs by either calcium ionophore A23187, sodium fluoride, or formylmethionyl-leucyl-phenylalanine was significantly inhibited. This decrease was paralleled by a loss of G-protein functions, including GTPase activity and GTP-binding capacity. An increase of G-protein functions was obtained with low amounts of Luk-PV. In addition to the modulated G-protein functions, ADP-ribosylation of 24-, 40-, and 45-kDa proteins by Luk-PV was detected. As shown in control experiments, the ADP-ribosylated 24-kDa proteins were not substrates for *C. botulinum* exoenzyme C3. Introduction of ras p21 into digitonin-permeabilized PMNs was without effect on subsequent Luk-PV stimulation. In addition, the translocation of ras p21, ras GAP, and 5-lipoxygenase into the membrane of Luk-PV-treated PMNs, as well as the expression of chemotactic membrane receptors for LTB_4 and formylmethionyl leucyl phenylalanine, was significantly diminished.

The Panton-Valentine leukocidin (Luk-PV) from *Staphylococcus aureus* V8 strains is important for the pathogenicity of certain staphylococcal diseases (6, 9, 25, 40). The toxin, first described by Woodin et al., 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) (39–41). Morphological changes such as cellular swelling, appearance of rounded nuclei, and loss of granules are associated with the formation of plasma membrane pores (7, 9, 41). The word leukocidin represents different staphylococcal leukotoxins. The nucleotide sequence of Luk-PV has been established. In contrast to Luk-PV, Noda et al. (25–27) described a leukocidin also produced by strain V8 whose molecular masses (F and S, 31 and 32 kDa, respectively) were different from those described by Woodin (39, 40) 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 (31a).

We analyzed the effects of Luk-PV on leukotriene B_4 (LTB_4) generation and the involvement of different elements in signal transduction. 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 release of free arachidonic acid and synthesis of prostaglandins (27). Via the 5-lipoxygenase (5-LO) pathway, free arachidonic acid can be converted into leukotrienes, which induce inflammatory and allergic reactions (3, 19, 20). In this regard, we demonstrated that Luk-PV induced PMNs to generate LTB_4 (12), which plays a crucial role as an inflammatory chemotactic factor for neutrophils and eosinophils (3). LTB_4 exerts autocrine effects on PMNs (23) and induces neutrophils to degranulate, generate superoxide, and adhere to the vascular endothelium (19, 20).

GTP-binding proteins (G proteins) serve as transducers for signal processing, linking extracellularly oriented receptors to membrane-bound effector systems (8). Many of the cellular effects of the toxins generated by *Bordetella pertussis* (pertussis toxin [PT]) and *Vibrio cholerae* (cholera toxin [CT]) strains result from their abilities to affect G proteins by ADP-ribosylation of the G-protein alpha subunits (14, 28, 37). Sensitivity towards *Clostridium botulinum* exoenzyme C3 has often been used as a valuable probe for rho and rac involvement in signal transduction (24). The physiological function of ras and ras-related proteins is not known, but their biochemical and structural similarities to the alpha chain of the heterotrimeric G proteins suggest that they function as regulatory elements in signal transduction pathways that control cellular proliferation and differentiation. In this context, ras p21 is not the only member of the ras family of low-molecular-weight (LMW) G proteins in cell activation. There are others, e.g., rho, rac, and rab, which commonly regulate vital functions in actin polymerization, respiratory burst, phagocytosis, motility, and secretion. Thus, ras p21 was assayed as a major representative of the LMW G proteins, since its activity is linked to important steps in signal transduction (10). The critical events for ras protein functions are interaction with guanine nucle-

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otides (GTP and GDP), intrinsic GTPase activity, which is stimulated by a GTPase-activating protein (ras GAP), and association with the plasma membrane.

In the present studies, we investigated the biochemical mechanisms by which Luk-PV affects leukotriene synthesis by PMNs by studying (i) the expression of chemotactic receptors, (ii) heterotrimeric and LMW G-protein functions, and (iii) the translocation of 5-LO.

MATERIALS AND METHODS

Materials. (i) Cell separation. Ficoll 400 was obtained from Pharmacia, Uppsala, Sweden; Macrodex (6%, wt/vol) was from Knoll, Ludwigshafen, Germany. Sodium metrizoate solution (75%, wt/vol) was from Nycomed, Oslo, Norway.

(ii) Cell stimulation. Calcium ionophore A23187, sodium fluoride (NaF), and formylmethionyl-leucyl-phenylalanine (fMLP) were purchased from Sigma, Munich, Germany.

(iii) 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. Leukotriene standards (20-COOH-LTB_4, 20-OH-LTB_4, and LTB_4) were generously provided by Merck Frosst, Pointe Claire-Dorval, Quebec, Canada.

(iv) 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]).

(v) Bacterial toxins. The leukotoxin used was Luk-PV PVL (EMBL/GenBank accession number 72700 [31a]). The F and S components were purified to homogeneity by G. Prévost, Université Louis Pasteur, Strasbourg, France, as described before (7). 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 (31). 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. PT and CT were purchased from List Biological Laboratories, Inc., Campbell, Calif. Stock solutions were prepared by the reconstitution of lyophilized powder in a sterile buffer containing 0.1 M sodium phosphate and 0.5 M NaCl (pH 7.0). The stock solutions were stored at 4°C and diluted with PBS. *C. botulinum* exoenzyme C3 was kindly provided by K. Aktories (Institut für Pharmakologie und Toxikologie der Universität Homburg/Saar, Homburg/Saar, Germany). The stock solution was stored at −20°C. Ras p21 and the corresponding control peptide (Ala-Ser-Ser-Asp-Ser-Gly-Asn-Thr-Glu-OH) were obtained from S. Jung (Neurologische Universitäts-klinik und Poliklinik, Würzburg, Germany).

(vi) Radioactivity. [^{3}H]fMLP (specific activity, 1.5 to 2.2 TBq/mmol), [^{3}H]LTB_4 (specific activity, 1.1 to 2.2 TBq/mmol), [^{3}H]Gpp(NH)p (specific activity, 666 GBq/mmol), [γ-^{32}P]GTP (specific activity, 222 TBq/mmol), ^{125}I-protein A (specific activity, 2.6 to 3.7 MBq/μg), and [^{32}P]NAD (specific activity, 29.6 TBq/mmol) were supplied by New England Nuclear, Dreieich, Germany.

(vii) Antibodies. The monoclonal antibody directed against ras p21 (M90) was kindly provided by E. G. Lapetina (Burroughs Wellcome, Research Triangle Park, N.C.). Rabbit polyclonal antibody against ras GAP (RH6-2A) was generously provided by K. Koths (Cetus Corp., Emeryville, Calif.). Specific rabbit antisera against 5-LO were kindly given by J. Evans (Merck Frosst).

Purification of human neutrophils. Human PMNs were isolated from heparinized (15 U/ml) peripheral blood of healthy donors, using a Ficoll-metrizoate gradient and subsequent dextran sedimentation as described by Böyum (2). The erythrocytes were lysed by exposing the cell suspension to hypotonic conditions. The purified cell fraction contained more than 95% pure and intact PMNs.

Incubation conditions. If not stated otherwise, PMNs (10^7/500 μl of PBS) were stimulated with 1 μg of Luk-PV per ml in the presence of calcium (2 mM) and magnesium (1 mM) for 30 min. If PMNs were pretreated with specific ADP-ribosylating toxins, cells were incubated for 2 h at 37°C with either 1 μg of PT or 10 μg of CT per ml or for 30 min with 1 μg of *C. botulinum* exoenzyme C3 per ml. Dose-response studies with CT, PT, and C3 were carried out in our previous investigations (11). As a control for toxin activity, we determined either their effects on fMLP-induced LTB_4 generation or their ADP-ribosylative capacity. In the case of pretreatment with ras p21, PMNs were permeabilized with 10 μM digitonin, as described by Prentki et al. (30), and were subsequently incubated for 30 min at 37°C with either ras p21 or the same amounts of a control peptide. Afterwards, LTB_4 synthesis was induced with either fMLP (7 μM) or Luk-PV (1 μg/ml). In order to investigate the secondary stimulation of Luk-PV-pretreated PMNs, cells were washed after Luk-PV incubation, resuspended in 500 μl of PBS, and subsequently stimulated with calcium ionophore A23187 (6.3 μM), fMLP (7 μM) or NaF (9.3 mM) in the presence of calcium (2 mM)-magnesium (1 mM) for 20 min at 37°C.

Cell viability. Lactate dehydrogenase, lysozyme, and β-glucuronidase release was assessed as described before (4). Cytoplasmic lactate dehydrogenase release after treatment with leukocidin was 1.8% ± 3.1% of the total cellular lactate dehydrogenase content when 500 ng of leukocidin per 10^7 human PMNs per 500 μl of PBS 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 leukotriene generation. After the incubation period, 2 ml of methanol-acetonitrile (50:50, vol/vol) was added to the complete incubation mixture, and the total amount 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 710 B 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 leukotriene 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 the 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 leukotrienes 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).

fMLP and LTB_4 receptor assays. PMNs (10^7/500 μl of PBS) were incubated for 30 min in the presence or absence of Luk-PV (1 μg/ml) at 37°C. Afterwards, the cells were washed and resuspended in PBS. The fMLP binding studies were carried out using 96-well filtration plates with 5-μm-pore-size polyvinylidene fluoride membranes (Millipore, Eschborn, Germany). Each well contained 4×10^6 PMNs, 20 nM [^{3}H]fMLP, and 37.5 μg of bovine serum albumin. The nonspecific binding was assessed in the presence of 10 μM unlabelled fMLP. After 45 min of incubation at 4°C, the unbound [^{3}H]fMLP was removed by rapid filtration, using a Millititer vacuum holder. Subsequently, the filters were washed with PBS and transferred to scintillation vials; methanol (0.5 ml) and Rotiszint 2211 (4 ml) were added, and the radioactivity was measured by liquid scintillation counting (Rack beta 1209; LKB, Turku, Finland). Specific binding was expressed as total binding minus nonspecific binding. Total and nonspecific binding values were calculated from triplicate determinations.

LTB_4 binding studies were carried out as described for fMLP. Each well contained 2.3 nM [^{3}H]LTB_4 (0.9 KBq). For determination of nonspecific binding, 220 nM unlabelled LTB_4 was added.

Specific binding of guanylylimidodiphosphate. PMNs (10^7/500 μl of PBS) were incubated for 30 min in the presence or absence of Luk-PV (1 μg/ml) at 37°C. Afterwards, the cell suspensions were washed and suspended in Tris (0.05 M)-sucrose (0.34 M) buffer (pH 7.5), and EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; 1 mM], dithiothreitol (1 mM), and leupeptin (100 μg/ml) were added. Cell disruption was carried out by sonication (Sonifier 250; Branson Power Co., Danbury, Conn.). The sonicates were centrifuged at $300 \times g$ for 10 min, and the resulting postnuclear supernatant was further differentiated by centrifugation at $10{,}000 \times g$ for 20 min (J2-21 centrifuge with a JA-20 rotor; Beckman, Palo Alto, Calif.) and at $100{,}000 \times g$ for 60 min (L8-70 ultracentrifuge, using an SW 60Ti rotor; Beckman). The $100{,}000 \times g$ pellet was resuspended in PBS buffer, assayed for protein content, and stored at −70°C.

The binding of [^{3}H]Gpp(NH)p was determined by the modified procedure of Matsumoto et al. (22). The membrane fraction (10 μg of protein in the reaction mixture) was incubated in 20 mM Tris buffer (pH 7.5) containing 150 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 1.14 mM ATP, 0.5 mM App(NH)p, 0.25 mM ouabain, and 1 μM [^{3}H]Gpp(NH)p. Nonspecific binding was defined as the amount of [^{3}H]Gpp(NH)p bound in the presence of 10 μM nonlabelled Gpp(NH)p. Incubations were terminated after 60 min at room temperature by rapid filtration through cellulose ester membranes with a pore size of 0.45 μm (Millititer HA filtration plates; Millipore). In previous experiments we established the experimental conditions and also checked the remainder material (5). The filters were washed four times with 20 mM Tris buffer (pH 7.5) containing 0.25 mM $MgCl_2$ and 50 μM EGTA. No bound radioactivity was detected on filters of control samples without PMN membranes. The dried filters were measured for radioactivity by liquid scintillation counting. The amount of specific binding of [^{3}H]Gpp(NH)p was calculated as the total binding minus nonspecific binding.

GTPase activity. PMNs (10^7/500 μl of PBS) were incubated for 30 min in the presence or absence of Luk-PV (1 μg/ml) at 37°C. The membrane fraction was prepared as described above. GTPase activity was determined by measuring the liberation of ^{32}P from [γ-^{32}P]GTP according to the modified procedure of Matsumoto et al. (22). The final assay mixture contained 10 μg of protein, 150 mM KCl, 20 mM Tris buffer (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EGTA, 1.14 mM ATP, 0.5 mM App(NH)p, 0.25 mM ouabain, 0.375 μM GTP, and 0.125 μM [γ-^{32}P]-GTP. The reaction proceeded for 60 min at 37°C and was terminated by adding 0.5 ml of a 5% charcoal mixture containing 0.1% dextran and 0.5% bovine serum albumin in 20 mM phosphate buffer (pH 7.5). The tubes were then centrifuged to sediment the charcoal. The supernatants were removed, and free ^{32}P was determined by liquid scintillation.

Western blotting (immunoblotting). After cell rupture by sonication, equal protein concentrations (10 μg) of the cell membrane supplemented with protease inhibitors (1 mM EDTA, 1 mM leupeptin, and 1 μM phenylmethylsulfonyl fluoride) were suspended with an equal volume of Laemmli's sample buffer containing dithiothreitol (0.1 M; up to a final volume of 50 μl [21]) and resolved on a sodium dodecyl sulfate (SDS)-polyacrylamide (10%) gel in a vertical slab unit (Mighty Small II; Atlanta, Heidelberg, Germany). Afterwards, proteins were blotted on cellulose nitrate for the detection of ras p21, ras GAP, and 5-LO. The bound monoclonal antibodies were detected with rabbit anti-mouse immunoglobulin G antibody (RAM-IgG-7S; Nordic Immunology, Tilburg, The Netherlands) and subsequently with ^{125}I-labeled protein A. Autoradiography was carried out as described previously (17).

ADP-ribosylation. ADP-ribosylation was determined by the method of Okajima and Ui (28). PMNs, 2×10^7, were resuspended in 200 μl of PBS containing 5 mM $MgCl_2$ and 0.5 mM phenylmethylsulfonyl fluoride. The samples were sonicated (three 10-s periods on ice; energy output, 40 W) with a Branson Sonifier 250 W. Granules, nuclei, cell debris, and intact cells were separated at $10{,}000 \times g$ (20 min at 4°C). The postnuclear supernatant, 50 μl, was added to 70 μl of an incubation buffer consisting of 0.1 M dithiothreitol, 5 μCi of ^{32}P-NAD, 10 μl of Luk-PV (1 μg/ml) or PBS (for control), 0.2 M thymidine, 0.01 M ATP, 0.01 M EDTA, and 0.001 M GTP. Incubation proceeded for 60 min at 30°C and was stopped by the addition of 30% trichloroacetic acid. Subsequently, the samples were washed in PBS, resuspended in 100 μl of Laemmli's sample buffer (19), and resolved on an SDS-polyacrylamide (12%) gel in a vertical slab unit. The dried gels were exposed to Kodak XAR-5 films. The molecular weights of the ADP-ribosylated proteins were assessed by molecular weight markers.

Statistics. If not stated otherwise, data from at least three independent experiments with different donor cells were combined and expressed as means ± 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 (Luk-PV) at various concentrations (10 to 1,000 ng per 500 μl of incubation buffer) for 30 min at 37°C. Enhanced amounts of LTB_4 and omega-oxidated products, representing the results of de novo synthesis, were detected when PMNs were treated with Luk-PV concentrations ranging from 100 to 500 ng (Fig. 1A). A further increase of the toxin concentration up to 1,000 ng did not lead to additional enhancement of LTB_4 formation. Induction of LTB_4 generation by Luk-PV was dependent on the presence of both toxin components S and F (synergistic

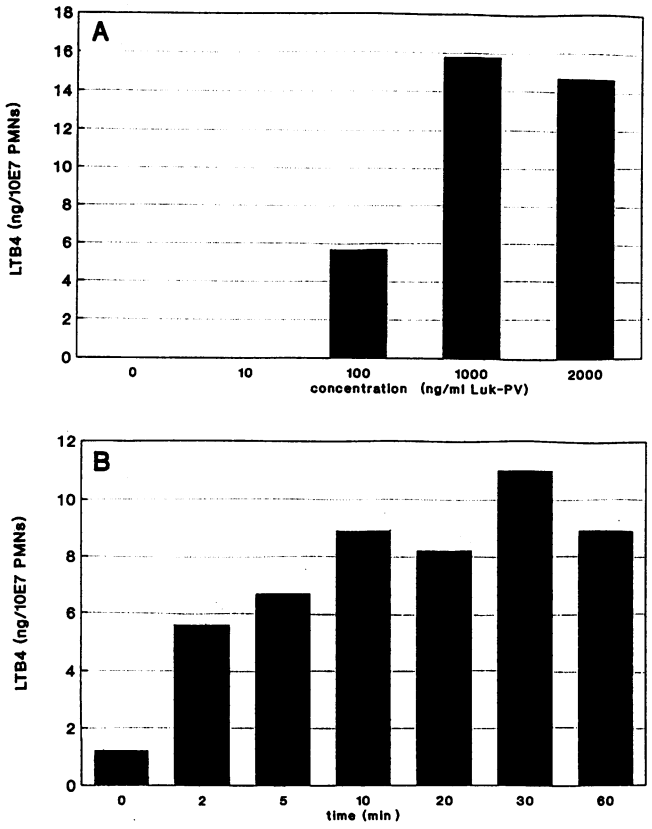

FIG. 1. (A) Generation of total LTB_4 (20-COOH-LTB_4, 20-OH-LTB_4, and LTB_4) from PMNs treated with different concentrations of leukocidin (Luk-PV) for 30 min at 37°C. (B) Kinetic studies with 1 μg of leukocidin per ml per 10^7 PMNs. The values shown are from typical experiments. Additional experiments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

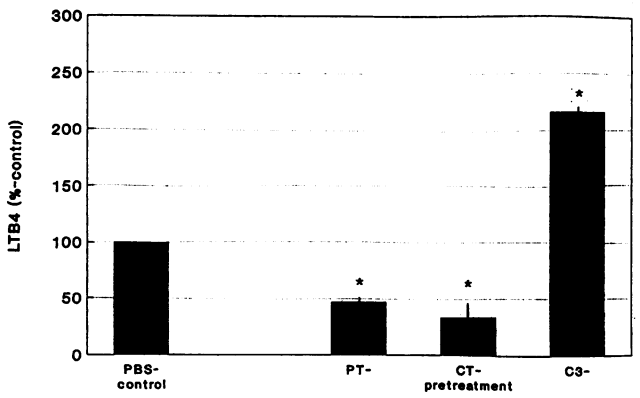

FIG. 2. Effect of PT, CT, or *C. botulinum* exoenzyme C3 on LTB_4 generation by PMNs induced by 1 μg of leukocidin per ml. The data are expressed as means ± standard deviations ($n = 3$) obtained with different donor cells (*, $P < 0.01$).

effects). Kinetic experiments were then performed with 500 ng of Luk-PV at intervals ranging from 2 to 60 min. Maximal LTB_4 generation was obtained after 30 min of incubation (Fig. 1B). Thus, in subsequent experiments, incubation for 30 min 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).

Effect of ADP-ribosyltransferases on leukocidin-induced LTB_4 formation. Pretreatment of PMNs with the ADP-ribosyltransferase CT, PT, or *C. botulinum* exoenzyme C3 revealed evidence of the involvement of both G-protein subtypes, heterotrimeric (CT and PT) and LMW (C3), in the signal transduction of Luk-PV-stimulated PMNs. For this purpose, 10^7 PMNs per 500 μl of incubation buffer were incubated for 2 h with 1 μg of PT or 10 μg of CT per ml or for 30 min with 1 μg of C3 per ml. Afterwards, LTB_4 synthesis was stimulated for 30 min with 1 μg of Luk-PV per ml. Luk-PV-induced LTB_4 generation was significantly inhibited after CT and PT pretreatment (CT, 33.4%; PT, 47.3% [Fig. 2]). In contrast, incubation of PMNs with C3 increased the Luk-PV-stimulated LTB_4 synthesis (C3, 215.9%). These results indicate the involvement of heterotrimeric and LMW G proteins in the signal transduction of Luk-PV-activated PMNs.

Effect of ras p21 on leukocidin-stimulated LTB_4 formation. In order to study the role of LMW G proteins in Luk-PV-induced LTB_4 formation, digitonin-permeabilized PMNs (10 μM; 10^7 PMNs per 500 μl of PBS) were incubated for 30 min with different concentrations of ras p21 and were subsequently stimulated for 30 min with 1 μg of Luk-PV per ml (Table 1). The effects of intracellularly increased ras p21 levels after Luk-PV stimulation were compared with those obtained after fMLP stimulation (7 μM). As a control, ras p21 was replaced by a control peptide (Ala-Ser-Ser-Asp-Ser-Gly-Asn-Thr-Glu-OH). Incubation of permeabilized PMNs with ras p21 did not stimulate LTB_4 synthesis in PMNs per se. The fMLP-stimulated LTB_4 formation of ras p21-treated PMNs was increased in a concentration-dependent manner. In contrast, Luk-PV-stimulated LTB_4 formation was not affected after incubation with ras p21. Additionally, the incubation of permeabilized PMNs with the control peptide was without effect for either stimulus. These results show that ras p21 may be involved in fMLP-, but not Luk-PV-, stimulated LTB_4 synthesis.

Effect of leukocidin on calcium ionophore A23187-, NaF-, and fMLP-stimulated LTB_4 formation. Experiments to study the modulatory effect of Luk-PV on a secondary cell activation were performed. For cellular activation, we used stimuli which trigger cells via well-defined cell biological mechanisms. Calcium ionophore A23187 bypasses receptor-mediated cell activation and directly activates cells via an enhanced calcium influx. Luk-PV-pretreated PMNs (1 μg/ml; 30 min) were washed with PBS and subsequently stimulated with the calcium

TABLE 1. Effect of ras p21 on fMLP- or Luk-PV-induced LTB_4 generation[a]

Primer and amt (ng)	LTB_4 (ng) induced by:		
	PBS	fMLP	Luk-PV
Control peptide			
0	—[b]	10.7	17.5
12	—	10.8	17.5
120	—	10.7	14.2
1,200	—	11.1	17.6
ras p21			
0	—	10.7	17.5
12	—	11.4	17.3
120	—	11.4	14.4
1,200	—	12.7	17.7

[a] Values are from one typical experiment. Two identical experiments showed the same pattern.

[b] Values below 0.5 μg, i.e., detection limit of LTB_4.

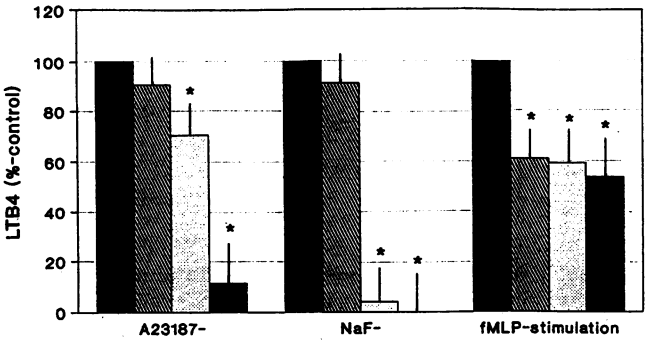

FIG. 3. Effect of leukocidin on LTB_4 generation by PMNs induced by different stimuli. PMNs, 10^7, were pretreated with 0 (■), 10 (▧), 100 (▤), or 1,000 (▨) ng of leukocidin per ml and were subsequently stimulated by the calcium inophore A23187 (6.3 μM), NaF (9.3 mM), or fMLP (7 μM). The 100% value for A23187-stimulated PMNs was 378.3 ± 83.2 ng, that for NaF-stimulated PMNs was 49.0 ± 3.3 ng, and that for fMLP-stimulated PMNs was 11.4 ± 3.2 ng. The data are expressed as means ± standard deviations (n = 3) obtained with different donor cells (*, $P < 0.01$).

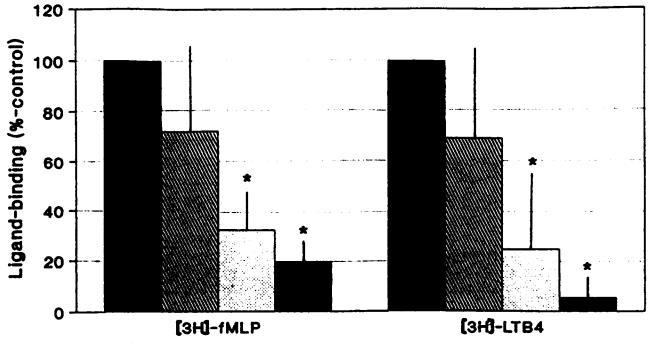

FIG. 4. Effect of leukocidin on specific binding of [^{3}H]fMLP and [^{3}H]LTB_4 to PMNs. PMNs, 10^7, were incubated with 0 (■), 10 (▧), 100 (▤), or 1,000 (▨) ng of leukocidin per ml. The binding capacity of control incubations was defined as 100%. The 100% values for the specific binding of [^{3}H]fMLP and [^{3}H]LTB_4 were 176.0 ± 14.5 and 134.0 ± 29.5 cpm, respectively. The data are expressed as means ± standard deviations (n = 3) obtained with different donor cells (*, $P <$ 0.01).

ionophore A23187 (6.3 μM) for an additional 20 min. Pretreatment of PMNs with Luk-PV at low concentrations (100 ng/ml) already reduced the amounts of LTB_4 generated after ionophore stimulation by 29% (Fig. 3). With 1 μg of Luk-PV per ml, the reduction was 89% compared with a control.

In another set of experiments, we investigated the effects of Luk-PV on receptor-mediated LTB_4 synthesis with the bacterial chemotaxin fMLP (7 μM; 20 min). Luk-PV inhibited LTB_4 formation by PMNs stimulated with fMLP in a concentration-dependent manner. Maximal inhibition was obtained with 1 μg of Luk-PV per ml (46%; Fig. 3).

To investigate the effect of Luk-PV on the direct activation of heterotrimeric G proteins with NaF, PMNs were incubated with Luk-PV for 30 min and subsequently stimulated with NaF (9.3 mM; 20 min). Incubation of PMNs with Luk-PV inhibited the generation of LTB_4 stimulated with NaF in a concentration-dependent manner. Maximal inhibition was obtained with 1 μg of Luk-PV per ml (0.01%; Fig. 3).

Effect of leukocidin on expression of chemotactic membrane receptors. We next investigated the effect of Luk-PV on the expression of chemotactic receptors for LTB_4 and fMLP. For this purpose, PMNs were incubated with various amounts of Luk-PV, and the binding of radiolabelled ligands was detected as described in Materials and Methods. Control incubations were performed in the absence of Luk-PV, and the specific binding of [^{3}H]fMLP or [^{3}H]LTB_4 was defined as 100%. Incubation of cells for 30 in with Luk-PV inhibited the specific binding of [^{3}H]fMLP in a concentration-dependent manner (Fig. 4). Similar inhibitions were seen for the specific binding of [^{3}H]-LTB_4. In parallel to the diminished receptor expression for LTB_4 and fMLP, a decreased capacity of Luk-PV-treated PMNs to respond a subsequent stimulus (e.g., fMLP) was observed.

Effect of leukocidin on 5-LO activity. Activation of 5-LO (78 kDa) is one crucial prerequisite for the generation of LTB_4 in stimulated PMNs. After cellular activation, 5-LO is translocated from the cytoplasm into the membrane, where it binds to the 18-kDa, 5-LO-activating protein FLAP (13, 32). As shown by Western blotting (Fig. 5), Luk-PV treatment inhibited translocation of 5-LO to the membrane fraction of PMNs (lane 2). In addition to the diminished receptor expression of PMNs, this fact may lead to a disturbed LTB_4 formation after Luk-PV treatment. Lane 1 shows control cells without Luk-PV treatment.

Effect of leukocidin on G-protein functions. To investigate the effect of Luk-PV on G-protein functions, the GTPase activity and [^{3}H]Gpp(NH)p binding of the membrane fraction of Luk-PV-treated PMNs were measured. Control incubations were performed in the absence of Luk-PV, and the GTPase activity and [^{3}H]Gpp(NH)p binding of these cells were defined as 100%. The GTPase activity of PMNs was increased by low Luk-PV concentrations (10 ng/ml, 116%; Fig. 6). A further increase in toxin concentration led to inhibitory effects (100 ng/ml, 80%; 1,000 ng/ml, 53%). A similar pattern was obtained for the [^{3}H]Gpp(NH)p binding. In this case, low Luk-PV concentrations increased the [^{3}H]Gpp(NH)p binding to the membrane fraction (10 ng/ml, 139%). A further increase of the toxin concentration led to inhibitory effects (100 ng/ml, 67%; 1,000 ng/ml, 44%).

ADP-ribosylation by leukocidin. To investigate the ADP-ribosylation pattern induced by Luk-PV, the postnuclear supernatant of PMNs was incubated with [^{32}P]NAD as described in Materials and Methods. Labelled proteins were separated

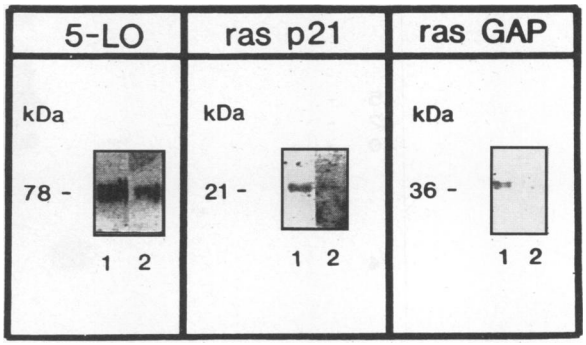

FIG. 5. Effect of leukocidin (1 μg/ml) on expression of 5-LO, ras p21, and ras GAP. After an incubation period of 30 min, the cellular membranes were prepared and blotted onto cellulose nitrate. Ras p21, ras GAP, and 5-LO were detected by monoclonal antibodies and ^{125}I-labelled protein A. The inserts represent autoradiographic lanes of leukocidin-treated PMNs (lanes 2) versus untreated control cells (lanes 1). The corresponding molecular sizes are given on the left.

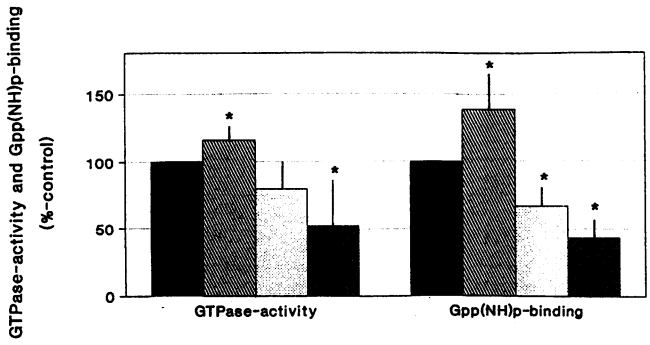

FIG. 6. Effect of leukocidin on GTPase activity and Gpp(NH)p binding of PMNs. PMNs, 10^7, were incubated with 0 (■), 10 (▧), 100 (▨), or 1,000 (▨) ng of leukocidin per ml. The GTPase activity and Gpp(NH)p binding capacities of control incubations were defined as 100%. The 100% value for GTPase activity was 103,892.3 ± 4,086.4 cpm, and that for specific Gpp(NH)p binding was 1,000.6 ± 157.7 cpm. The data are expressed as means ± standard deviations (n = 3) obtained with different donor cells (*, $P < 0.01$).

by gel electrophoresis and detected by autoradiography. No autoradiographic signals were obtained from untreated cells (PBS; Fig. 7A, lane 1). Control incubations with PT and CT (1 or 10 μg/ml) led to signals in the molecular size range of 40 to 42 kDa for PT-treated PMNs (lane 2) or 40 kDa for CT-treated PMNs (lane 3). Incubation with *C. botulinum* exoenzyme C3 (1 μg/ml) led to signals at a molecular size of 24 kDa (lane 4). After incubation with *C. botulinum* exotoxin C2, signals in the molecular size range of 40 to 42 kDa were detected (lane 5). Luk-PV treatment induced signals at molecular sizes of 24, 40, and 45 kDa. Figure 7B shows a control experiment in which PMNs were pretreated for 30 min with 1 μg of either C3 (lane 3) or Luk-PV (lane 4) per ml. Lane 2 shows untreated PMNs. Afterwards, the postnuclear supernatant of these PMNs was incubated with [^{32}P]NAD for 60 min in the presence of 1 μg of C3 per ml. As shown, the C3-induced ADP-ribosylation was diminished in the case of C3-pretreated PMNs (lane 3) but not in the case of Luk-PV-pretreated PMNs (lane 4). These results indicate that Luk-PV requires substrates for ADP-ribosylation other than C3. Lane 1 shows control cells without any toxin addition.

Effect of leukocidin on expression of ras p21 and ras GAP. It is well known that heterotrimeric G proteins are involved in the fMLP-induced LTB_4 generation of PMNs. Our investigations also suggest the involvement of heterotrimeric G proteins in Luk-PV-activated LTB_4 synthesis. But until now there has been little information about the regulatory role and participation of LMW G proteins in either signal transduction mechanism. Ras p21 was assayed as a major representative of the LMW G proteins, since its activity is linked to important steps in signal transduction (10). The level of active ras p21-GTP complexes is determined by the rate of exchange of bound GDP for cytosolic GTP. In this regard, the GTPase activity of ras p21 is controlled by a GTPase-activating protein (ras GAP). To determine the effect of Luk-PV on the expression of ras p21 and ras GAP in the membrane fraction of PMNs, cells were incubated with 1 μg of Luk-PV per ml. Expression of both ras p21 and ras GAP was diminished after 30 min of incubation with Luk-PV as assessed by Western blotting (Fig. 5). Lane 1 shows the PBS control, and lane 2 shows the membrane of Luk-PV-treated PMNs.

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, 19, 20). In the past, major emphasis has been placed on the cytolytic properties inherent in many toxins, including staphylococcal leukocidin. In this context Luk-PV from *S. aureus* V8,

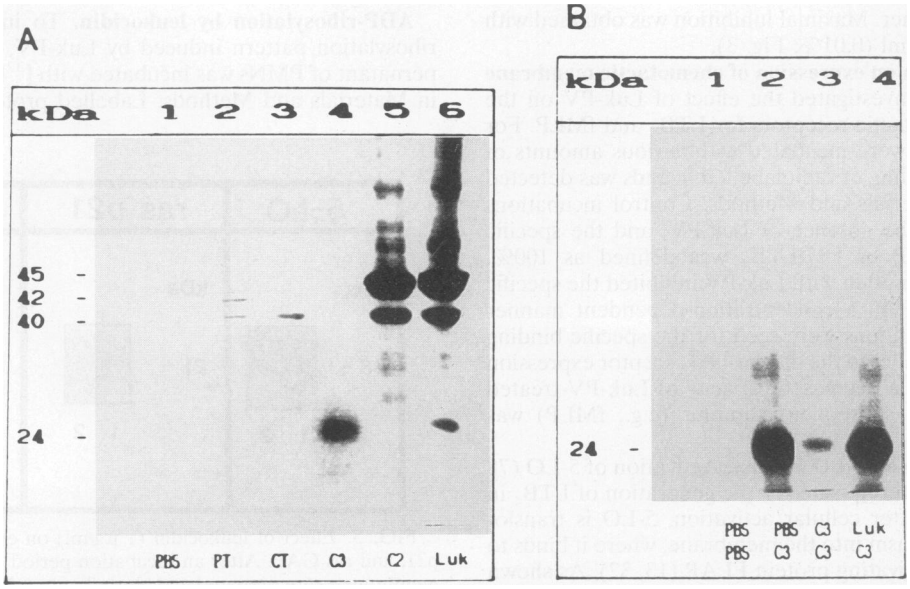

FIG. 7. ADP-ribosylation of PMNs by leukocidin. (A) The postnuclear supernatant of 2×10^7 PMNs was incubated with PBS as a control, PT (1 μg/ml), CT (10 μg/ml), *C. botulinum* exoenzyme C3 (1 μg/ml), *C. botulinum* exotoxin C2 (1 μg/ml), or leukocidin (Luk-PV; 1 μg/ml) in the presence of [^{32}P]NAD for 60 min at 30°C. (B) PMNs were pretreated with PBS as a control, C3, or Luk-PV before cellular disruption. Subsequently, the C3-stimulated binding of [^{32}P]NAD to the postnuclear supernatant was detected.

which is suspected to be important in the pathogenicity of certain staphylococcal diseases (25, 40), is commonly referred to as a membrane-damaging cytolysin with high specificity for PMNs, monocytes, and macrophages (25, 41). Moreover, clinical investigations have shown that *S. aureus* strains obtained from patients with chronic furunculosis were very often leukocidin producers (6). It is now increasingly evident that even 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 (4, 34).

To investigate the consequences of pore formation induced by leukocidin for the inflammatory response of PMNs, the generation of LTB_4, as one crucial parameter for PMN function, was analyzed. With regard to host immunity, it is evident that leukocidin activates the 5-LO activity of PMNs, which is a prerequisite for LTB_4 formation. In this context, investigations demonstrated a translocation of 5-LO from the cytoplasma to the membrane of PMNs after cellular activation, where it binds to the 18-kDa, 5-LO-activating protein (13, 32). As we observed, leukocidin directly triggered the release of LTB_4 from human PMNs in a time- and concentration-dependent manner (12). These data are in close correlation to those 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 (26). Phospholipase A_2 activation is also a prerequisite for arachidonic acid release, which is subsequently converted by 5-LO activation into LTB_4. As we observed, the separated S or F components of leukocidin did not induce LTB_4 generation by themselves. Moreover, both components acted synergistically. Similar synergistic effects of the S and F components of leukocidin on the intracellular Ca^{2+} concentration were recently described (7). The opening of cation channels in the leukocyte membrane without a subsequent effect on intracellular Ca^{2+} stores has been shown. With regard to the signal transduction mechanism involved in leukocidin-induced LTB_4 formation, we detected that the mechanism was sensitive for PT and CT. PT and CT treatment inhibited leukocidin-stimulated LTB_4 formation, whereas pretreatment of PMNs with *C. botulinum* exoenzyme C3 led to an enhanced leukocidin-induced LTB_4 formation. PT and CT ADP-ribosylate heterotrimeric G proteins in PMNs, e.g., Gi, Gs, and Gp (14, 28, 37). *C. botulinum* exoenzyme C3 ADP-ribosylates rho and rac proteins, which are members of the LMW G-protein family (24). Our results therefore indicate that both heterotrimeric and LMW G proteins are involved in the signal transduction of leukocidin-activated LTB_4 generation. One may also consider a counterregulatory mechanism by different G-protein subclasses.

Previous investigations have clearly demonstrated that reduced capacity to generate LTB_4 via a subsequent stimulus correlates with an outcome of shock and sepsis in severely burned patients (5, 18). In this regard, we have demonstrated that leukocidin significantly inhibited the subsequent stimulation of PMNs with the calcium ionophore A23187, NaF, and fMLP 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 the influx of calcium, one may suggest that a subsequent calcium signal, e.g., by the calcium ionophore, cannot trigger the cells. In another set of experiments we demonstrated that the translocation of 5-LO into the membrane of PMNs was significantly inhibited after 30 min of incubation with leukocidin. This may be a prerequisite for the significant inhibition of the calcium ionophore A23187-induced LTB_4 formation, which may depend on the membrane-damaging action of leukocidin.

NaF activates heterotrimeric G proteins directly (15). This occurs via binding of AlF^{4-} to GDP, which then imitates the GTP-bound active state of G_{alpha}. Inhibition of NaF-stimulated LTB_4 formation by leukocidin treatment indicates disturbed heterotrimeric G-protein functions, e.g., on the GTP-binding site of G_{alpha}. Indeed, we demonstrated inhibition of crucial G-protein parameters, such as GTPase activity and Gpp(NH)p binding, after treatment with leukocidin-concentrations ranging from 100 to 1,000 ng/ml. In contrast, low leukocidin concentrations increased both G-protein parameters and may be responsible for the previous onset of LTB_4 synthesis by leukocidin. The introduction of ras p21 LMW G protein into permeabilized PMNs did not modulate the subsequent leukocidin-induced LTB_4 generation as was observed for fMLP stimulation. In this context, ras p21 is not the only member of the ras family of LMW G proteins in cell activation. There are others (e.g., rho, rac, and rab) which commonly regulate vital functions in actin polymerization, respiratory burst, phagocytosis, motility, and secretion. Thus, ras p21 was assayed as a major representative of the LMW G proteins, since its activity is linked to important steps in signal transduction (10). Translocation of ras p21 and its regulatory GTPase-activating protein ras GAP into the membrane of leukocidin-treated PMNs was impaired, as was demonstrated for the 5-LO translocation.

One possible reason for dysregulated G-protein functions might be the ADP-ribosylative property of leukocidin. As we observed, leukocidin induced the ADP-ribosylation of 40-, 45-, and 24-kDa proteins in PMNs. In the case of the 24-kDa proteins, we excluded that these proteins were substrates for the *C. botulinum* exoenzyme C3 by additional experiments. In 1989, Kato and Noda described ADP-ribosylative effects of leukocidin on rabbit PMNs (16). ADP-ribosylation of 37-kDa (by LukS-PV) and 41-kDa proteins (by LukF-PV) was detected. These obvious differences may be dependent on the use of different leukotoxins. The leukotoxin described here was Luk-PV PVL. In contrast, Noda et al. used a leukocidin with a nucleotide sequence which is 91% homologous to that of gamma-hemolysin. The gamma-hemolysin itself possesses 74% homology on the nucleotide level, as well as in its peptide sequence, to Luk-PV, which was used in our experiments (31a). Whether there is any pathophysiological relevance for the ADP-ribosylative activity of leukocidin and whether ADP-ribosylation is crucial for the disturbed inflammatory response of PMNs must be clarified by further studies.

Bacterium-derived fMLP (35) interacts with specific receptors on the surface of PMNs (38). G proteins transduce the signal into the cell interior and activate inflammatory cell functions, such as LTB_4 formation (33). Our data demonstrate that leukocidin significantly inhibits fMLP-induced LTB_4 formation. This inhibition was paralleled by a disturbed 5-LO translocation, by disturbed G-protein functions, and by a loss of cellular surface receptors for fMLP and LTB_4. Downregulation of chemotaxin receptors is not exclusive for leukocidin. In previous publications we observed downregulation of chemotaxin receptors by different bacterial exotoxins (streptolysin O, alveolysin, and theta toxin) and endotoxins (4). Similar observations have been made with cells of burned patients (5). We suggest that the reduced receptor expression impairs the functional activity of PMNs by inhibiting their recruitment to the site of microbial infection. Currently, experiments to

analyze the binding of labelled toxin components to cells are in progress. We assume that the downregulation of receptor sites is not a passive masking but a consequence of a direct activation process. Previous investigations have shown that receptor expression on PMNs is partially regulated by G proteins (36). Interestingly, LMW G proteins have been reported to copurify with the fMLP receptor (29). Therefore, the receptor downregulation may be a consequence of modulated G-protein functions. Obviously, the precise mechanism of cellular deactivation remains unknown and must be the subject of further studies.

In summary, our results indicate that staphylococcal leukocidin modulates the cellular reactivity of PMNs in two different ways. On the one hand, leukocidin activates the generation of LTB_4 from PMNs; on the other hand, it deactivates the neutrophil inflammatory response to a second stimulus. Inhibited cellular function is paralleled by a disturbed signal transduction from the membrane into the cell, which may favor microbial invasion.

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