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Generation of Leukotrienes from Human Granulocytes by Alveolysin from *Bacillus alvei*

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We investigated the effect of alveolysin on human granulocytes. Alveolysin is an exoprotein produced by *Bacillus alvei* and belongs to the group of sulfhydryl-activated cytolysins. Other members of this group are streptolysin O and theta-toxin from *Clostridium perfringens*. It is demonstrated that alveolysin leads to leukotriene generation from human granulocytes, which exert chemotactic (leukotriene B_4) and slow-reacting substance (leukotriene C_4, D_4, and E_4) activity under sublytic concentrations.

A wealth of impressive and outstanding biochemical, physiological, and genetic knowledge was obtained in the past decade which provided the basis for understanding the mechanism of pathogenesis of toxin-induced diseases such as diphtheria, cholera, and other diarrheal diseases (1, 3, 13). Previously it has been reported in a series of papers that the bacterial toxins leukocidin and streptolysin O induced as part of their overall cytotoxic effect a noncytotoxic secretion of granule enzymes (34). We recently presented evidence that the interaction of bacterial toxins such as alpha-toxin, enterotoxin B and lipase from *Staphylococcus aureus* (14), and the streptolysin O (8, 9) as well as the pseudomonas cytocidin (10, 20) lead to the transformation of arachidonic acid, with the subsequent generation of leukotrienes (6). Among the lipoxygenase products are factors with a pronounced effect on leukocyte migration and vascular permeability (leukotriene B_4 [LTB_4]) (5, 15, 17, 28); the biological activity referred to for 40 years as slow-reacting substance (SRS) of anaphylaxis consists of three related metabolites of arachidonic acid, LTC_4, LTD_4, and LTE_4 (27).

Since we had observed that various bacterial exotoxins induce the transformation of arachidonic acid via the lipoxygenase pathway (6), we analyzed the effect of alveolysin on human granulocytes with regard to leukotriene formation. Alveolysin is an exoprotein produced by *Bacillus alvei* and belongs to the group of thiolsulfhydryl-activated cytolysins (i.e., lethal, cardiotoxic, and membrane-damaging bacterial toxins) which are activated by thiol-containing reagents (1, 21, 31). Evidence has been provided that, unlike the thiol-activatable toxin streptolysin O, the alveolysin exerts a different mode of action with cell membranes. Thelestam et al. reported that, unlike streptolysin O, the toxin did not bind irreversibly to fibroblast cytoplasmic membranes; considerable membrane damage was caused at 0°C as compared with 37°C, and inhibition of amino acid uptake occurred in the absence of significant structural membrane damage (32).

MATERIALS AND METHODS

Commercial source of reagents. Phenolphthalein glucuronidate was obtained from Sigma Chemical Co., Munich, Federal Republic of Germany; Ficoll 400 was from Pharmacia, Uppsala, Sweden; Dextran-Macrodex (6%) was from Knoll, Ludwigshafen, Federal Republic of Germany. Sodium metrizoate (75%) was from Nyegaard, Oslo, Norway; [^{14}C]arachidonic acid was from Amersham Buchler, Braunschweig, Federal Republic of Germany.

Preparation of cells. Human leukocytes were obtained from heparinized blood of healthy donors and separated on a Ficoll-metrizoate gradient, followed by dextran sedimentation. This method leads to more than 97% pure polymorphonuclear leukocytes (PMNs). The cells were then washed at low speed (600 rpm) three times to remove the platelets. Less than 2% of the platelets were detected. Erythrocytes were removed by hypotonic exposure of the cell suspension. As target cells for the chemotactic assay, guinea pig peritoneal cells rich in eosinophils (30 to 80%) were obtained by injecting human serum at weekly intervals intraperitoneally. Neutrophil chemotaxis was carried out with purified human neutrophils (7, 16, 18).

Buffers. The medium used for washing the cells and for mediator release was, unless otherwise stated, a 0.025 M Tris buffer (pH 7.35) with NaCl (120 mM), KCl (4 mM), $CaCl_2$ (0.6 mM) and $MgCl_2$ (1 mM). This is referred to as TCM buffer.

Generation of leukotrienes from human PMNs. Human PMNs in a volume of 1 ml were incubated with alveolysin (a gift from J. E. Alouf, Institut Pasteur, Paris) at 37°C. For the analysis of chemotactic factor activity, indomethacin (14 μM) (Sigma) was added to the incubation mixture to prevent prostaglandin formation. To improve SRS formation and its release from the cells, glutathione (1 mM), $CaCl_2$ (2 mM), and cysteine (1 mM) were added to the incubation mixture (12). After centrifugation of the cells, the supernatant was either assayed for chemotactic and spasmogenic activity or subjected to leukotriene determination by reverse-phase high-pressure liquid chromatography (HPLC).

Chemotaxis. The method for eosinophil chemotaxis has been described in detail (7). Briefly, $2{,}5 \times 10^6$ guinea pig peritoneal cells containing 30 to 80% eosinophils were placed above a nitrocellulose filter (Sartorius Membranfilter GmbH., Göttingen, Federal Republic of Germany; 8-μm pore size, 13-mm diameter) in a modified Boyden chamber. For the actual experiment, the eosinophils from the various animals were pooled. The chemotactic factor-containing supernatant was placed below the filter. After 3 h of incuba-

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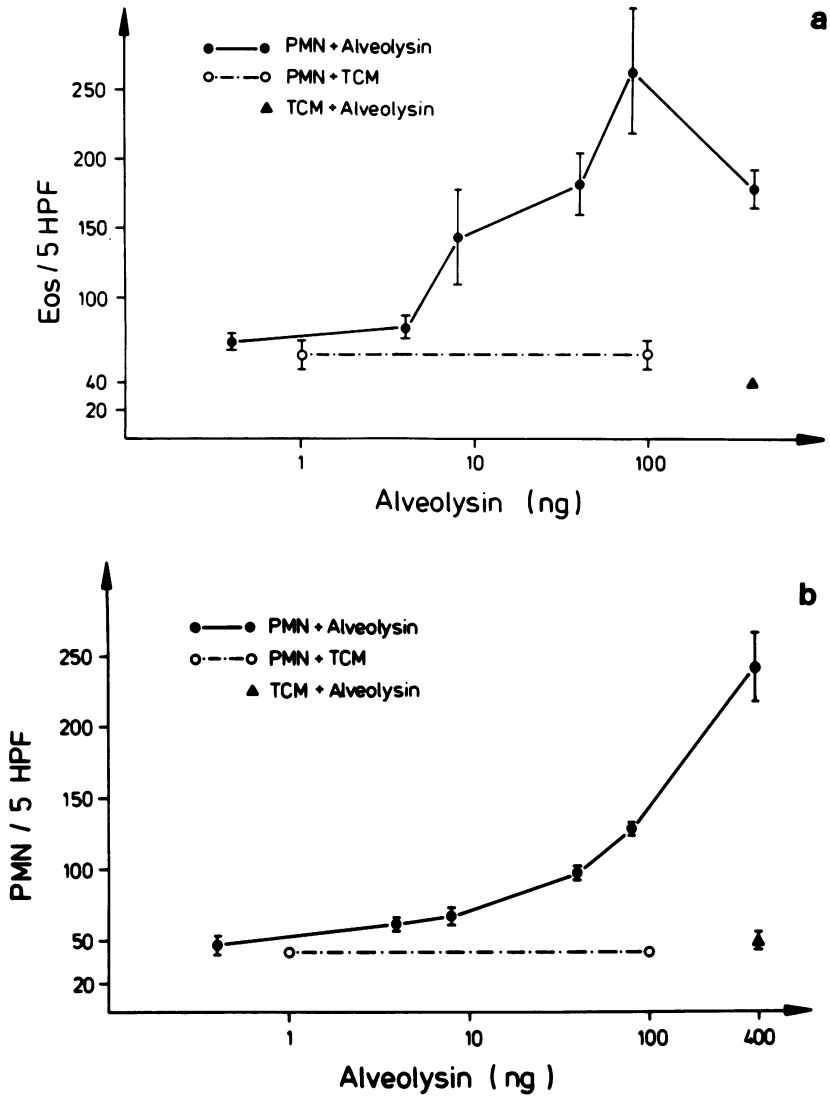

FIG. 1. Chemotactic factor generation for (a) guinea pig eosinophils and (b) human neutrophils on incubation of human granulocytes with various concentrations of alveolysin. Alveolysin alone attracted neither guinea pig eosinophils nor human neutrophils. A representative experiment out of five independent experiments performed in triplicates is depicted (see also Fig. 2).

tion, the cells that had migrated through the filter were counted at 100× magnification. Five high-power fields were evaluated. Neutrophil chemotaxis was performed with a 3-μm filter and purified human neutrophils as target cells. The assay conditions were the same as described for the eosinophil chemotaxis. The variation coefficient of the chemotactic assay ranged between 8 and 12%. All chemotactic assays were carried out in five independent determinations performed on different days; each point of the curve represents duplicate or triplicate determinations. Beta-glucuronidase determination was carried out as previously described with phenolphthalein glucuronidate as substrate (11, 16, 18). Lactate dehydrogenase was measured as previously described (16).

Measurement of smooth muscle contraction. A 10-ml organ bath was used. The contraction assays were performed on the guinea pig ileum with an isometric device (Hugo Sachs) (6); the isolated guinea pig ileum was treated with mepyramine (1 μg/ml) and atropine (0.2 μg/ml); acetylcholine (10^{-7} M) served as standard. Two acetylcholine contractions were followed by one addition of leukotrienes or SRS-containing supernatant. To antagonize leukotriene contraction, FPL 55 712 (5×10^{-6} M) was added to the guinea pig ileum. Biological SRS activity was expressed as the area obtained within the first 10 min after application of the sample.

Lipoxygenase factors and leukotrienes. Either biosynthetic or synthetic compounds were used as reference substances and were obtained from P. Borgeat, L'Université Laval, Québec, Canada, B. Spur and A. Crea, Institut für Chemie, Universität Düsseldorf, Düsseldorf, Federal Republic of Germany, and J. Rokach, Merck Frosst, Canada (7, 29, 30). The following agents served as references: LTB_4, LTC_4, LTD_4, and LTE_4, synthetic (5*S*)-(12*S*)-di-HETE (HETE is hydroxyeicosatetraenoic acid), and the all-*trans* isomers of LTB_4. The identity of the compounds was checked by mass spectrometry, reverse-phase HPLC, and UV scan.

Analysis of leukotriene activity by reverse-phase HPLC. The cell supernatants were mixed with 2 ml of methanol,

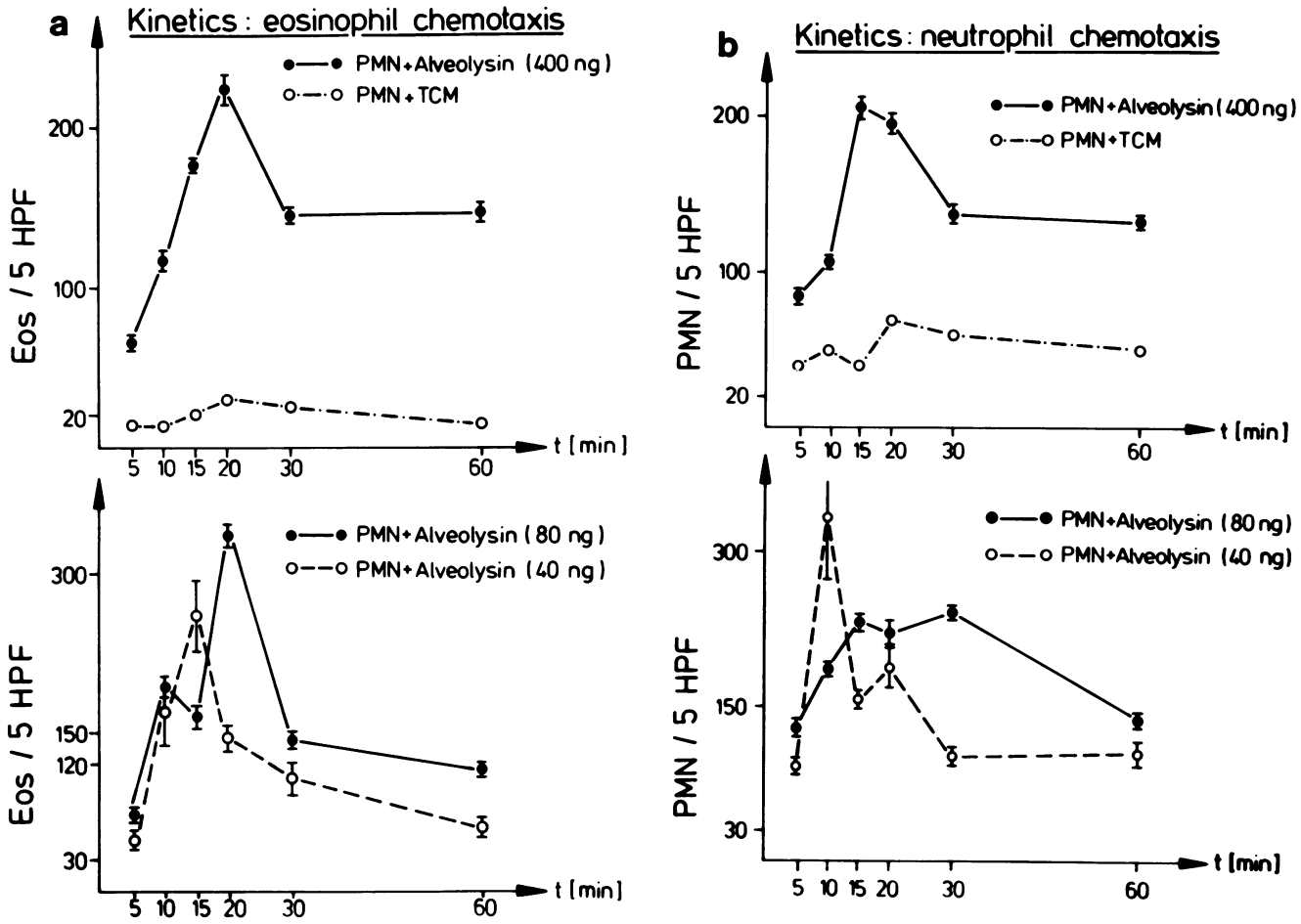

FIG. 2. Kinetics of chemotactic factor generation for (a) guinea pig eosinophils and (b) human neutrophils. Human granulocytes were incubated with different concentrations of alveolysin, and cells were centrifuged after various times. The supernatant (200 μl) was assessed for chemotactic activity.

which was adjusted to pH 3.8 by acetic acid; the samples were centrifuged at 4°C for 20 min at 1,000 × *g* to remove the residual protein; prostaglandin B_2 (100 ng) (Sigma) was added as internal standard. The supernatant was extracted three times with ether (3 ml); the ether phase was then evaporated under a stream of nitrogen. The residual water phase was lyophilized overnight. The concentrated material was suspended in methanol-water (30:70) and subjected to a silica gel column which was thoroughly washed with water, methanol, and benzene. The leukotrienes were eluted with a methanol-water solvent (80:20). The eluate was evaporated under a stream of nitrogen. Under these experimental conditions, the recovery rate for LTC_4 and LTD_4 from the cell supernatants was 50 to 60% and 80 to 90% for LTB_4.

The material was again concentrated and suspended in methanol-water (30:70). The separation of the leukotrienes was performed on a Nucleosil 5-μm C18 column (Machery/Nagel, Düren, Federal Republic of Germany; diameter, 4 by 200 mm). The solvent system was methanol-water-acetic acid (63:37:0.08, pH 5.7). The leukotrienes were determined by their absorption at 280 nm with a spectromonitor III (Milton Roy, Hasselroth). The absorption peaks of the analyzed supernatants were identical with the HPLC analysis of the relevant leukotriene standards. Further confirmation was also obtained by collecting the isolated HPLC fractions and assaying for neutrophil chemotactic and SRS activity. In addition, radioimmunoassays for LTC_4 and LTD_4 were carried out recently which confirmed the HPLC data (data not shown). The quantification of the leukotrienes was performed in relation to reference leukotrienes, with prostaglandin B_2 as the internal standard.

Statistical analysis. All experiments were performed with at least three to five individual donors. Although the absolute quantities of released leukotrienes varied from donor to donor, the release pattern was very similar at the analyzed concentrations of alveolysin. The data obtained by HPLC analysis represent duplicate extractions and determinations. The results express mean values.

RESULTS

Induction of chemotactic factor release. Human PMNs (2×10^7/ml) were incubated with various concentrations (0.4 to 400 ng) of alveolysin for 20 min at 37°C. The supernatants were then analyzed for their chemotactic properties towards guinea pig eosinophils and human neutrophils (Fig. 1a and b). It is apparent that chemotactic activity increased steadily when alveolysin was added to human PMNs at a concentration ranging from 8 to 80 ng. With higher concentrations,

chemotactic activity for guinea pig eosinophils decreased. This pattern was different when human granulocytes were used as target cells in the chemotactic assay. It is apparent that increasing concentrations of alveolysin induce higher amounts of chemotactic activity. To exclude cytolytic activity, the release of beta-glucuronidase, a granular and cytoplasmic enzyme marker, was also studied. At the described concentrations, the beta-glucuronidase release amounted to 2.6% at a concentration range of alveolysin which induced optimal eosinophil chemotactic activity (80 ng) and increased up to 11% of the total enzyme content at the highest alveolysin concentration (400 ng) used in our studies (data not shown). Under the experimental conditions, no significant lactate dehydrogenase activity was released. These results clearly suggest that alveolysin induces chemotactic factor generation under sublytic conditions.

The kinetics of chemotactic factor release indicate an optimum after 15 to 20 min of incubation at the three concentrations (40, 80, and 400 ng) of alveolysin studied (Fig. 2a and b). In general, a rapid increase in chemotactic activity occurred, which decreased at later times of incubation when guinea pig eosinophils or human neutrophils were used as target cells in the chemotactic assay.

Biological analysis of SRS activity. Experiments were then carried out to analyze whether alveolysin induces SRS activity from human granulocytes. Human PMNs (2×10^7/ml) were incubated with various concentrations of alveolysin for 20 min at 37°C in the presence of glutathione (1 mM), cysteine (1 mM), and $CaCl_2$ (2 mM). To exclude cyclooxygenase activity, all incubations were performed in the presence of indomethacin (14 μM). It is apparent that higher concentrations of alveolysin induced an increased release in SRS activity, as was determined on the guinea pig ileum (Fig. 3). As was previously demonstrated for other bacterial toxins (alpha-toxin, enterotoxin B from *S. aureus*, and the streptolysin O as well as the pseudomonas cytocidin) (6), the alveolysin-induced contractile activity was inhibited in the presence of FPL 55 712 (5×10^{-6} M) (data not shown).

Determination of leukotrienes by HPLC. The biological data were further supported by HPLC analysis for the various leukotrienes. Human granulocytes were incubated with various concentrations of alveolysin in the presence of glutathione, cysteine, and $CaCl_2$, which favor SRS formation. The activity of LTB_4 increased starting with an alveolysin concentration of 4 ng, and it reached a maximum with 80 ng. With higher concentrations of alveolysin (400 ng), a decrease in LTB_4 release was observed. The release of LTB_4 isomers (all-*trans* isomers), however, increased steadily at the concentrations studied in the cell supernatants. LTC_4 and LTD_4 activities were demonstrated beginning at alveolysin concentrations of 0.4 and 4 ng, respectively. LTC_4 activity increased steadily with higher concentrations of alveolysin, whereas LTD_4 activity showed a sharp decline at a concentration of 400 ng (Table 1). Further support for LTC_4 and LTD_4 generation was recently obtained by radioimmunoassay (data not shown; manuscript in preparation).

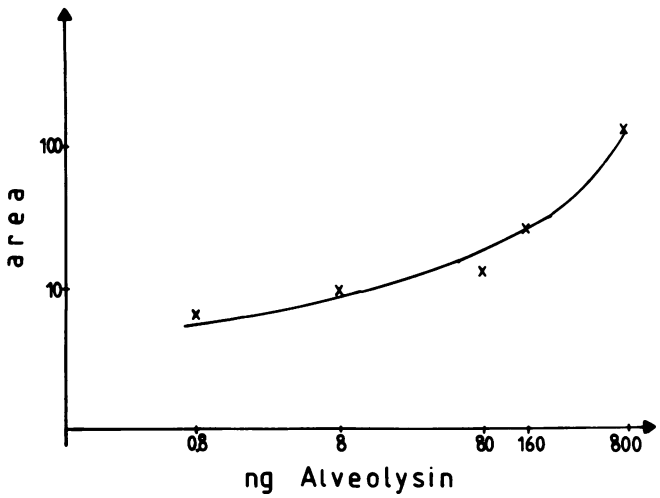

FIG. 3. Generation of SRS activity from human granulocytes by alveolysin and its evaluation on the terminal guinea pig ileum.

TABLE 1. Alveolysin-induced release of LTB_4, the LTB_4 *trans* isomer, LTC_4, and LTD_4 from human granulocytes

Alveolysin concn or TCM buffer	LTC_4 (ng/10^7 PMN)	LTD_4 (ng/10^7 PMN)	LTB_4 (ng/10^7 PMN)	LTB_4 isomer (ng/10^7 PMN)
Alveolysin				
400 ng	64.6	10.9	8.3	10.2
80 ng	29.5	31.1	20	9.8
40 ng	34.2	6.9	12.5	7.3
8 ng	24.6	1.2	2.1	
4 ng	23.9	1.6	2.3	
0.4 ng	26.5			
TCM buffer	6.9	0.5	1.0	0.5

DISCUSSION

Our data indicate that alveolysin interacts with human granulocytes and leads to leukotriene generation, as was assessed by chemotaxis and smooth muscle contraction on the terminal guinea pig ileum. The chemotactic pattern demonstrated by eosinophil chemotaxis contrasts to that shown with human neutrophils. Concentrations above 100 ng led to a striking decrease of chemotactic factor activity for guinea pig eosinophils, whereas with human neutrophils as target cells, concentrations higher than 100 ng increased the chemotactic response (Fig. 1a and b). When this pattern was compared with the biochemical analysis of LTB_4, concentrations higher than 100 ng also led to a significant decrease in LTB_4 activity, whereas LTB_4 isomers, which are 10- to 100-fold less active than LTB_4, revealed a plateau (Table 1). These results could be due to the fact that eosinophils and neutrophils differ in their sensitivity towards the leukotrienes and isomers; also, monohydroxylated eicosatetraenoic acids (e.g., 5- or 15-HETE) which were not determined could contribute to the chemotactic responsiveness of the neutrophils to a greater extent than the guinea pig eosinophils. Thus, we cannot exclude at present the possibility that the chemotactic responses reflect the fact that the dose dependence of formation of the various arachidonic acid metabolites is different, producing varying results of multiple variables; i.e., the amount of LTB_4 generated may not correlate exactly with the biological data obtained by chemotaxis (Fig. 2a and b). When SRS activity was analyzed by biological methods, it became apparent that an increase in contractile activity was observed up to 800 ng of alveolysin (Fig. 3). It should be pointed out that by biological analysis the combined activities of LTC_4, LTD_4, and LTE_4 (SRS) are determined. The biological data correlated quite well with

the biochemical analysis of alveolysin-induced LTC_4 release from human granulocytes. We cannot exclude the possibility that isomers might interfere with the biological analysis of SRS. A fivefold higher concentration of alveolysin (80 to 400 ng) led to about a 2.2-fold increase in LTC_4 release, as was assessed by reverse-phase HPLC. In contrast, the LTD_4 release decreased from 31.1 to 10.9 ng per 10^7 PMNs when concentrations beyond 80 ng were applied (Table 1). One might speculate that a gamma-glutamyltranspeptidase induces the transformation from LTC_4 to LTD_4 at concentrations between 8 and 80 ng of alveolysin per ml. The subsequent decrease in LTD_4 activity might be due to the interaction of a cellular dipeptidase which transforms LTD_4 to LTE_4.

It is by now clearly established that among the lipoxygenase factors the leukotrienes are the most effective mediators with regard to their ability to increase vascular permeability, to induce chemotaxis and chemoaggregation (LTB_4) (2, 5, 22, 28), and to initiate spasmogenic activity (SRS of anaphylaxis, LTC_4, LTD_4, and LTE_4). In the past, numerous reports have dealt with the various mechanisms of cell activation leading to the release of these newly generated mediators of inflammation (19, 24, 25). Among the stimuli which have been described are the calcium ionophore A 23 187 (7), phagocytosis (16), the anaphylatoxins (23), the platelet-activating factor (25), immunoglobulin E immune complexes (26), and the bee venom peptide melittin (17); cells which have been shown to release leukotrienes are mast cells, basophils, polymorphonuclear granulocytes, mononuclear cells, macrophages (peritoneal, alveolar), and rat basophilic leukemia cells. Quite recently it has been shown that leukotriene antagonists prevent endotoxin lethality in sensitized mice (11). The fact that defined bacterial exotoxins induce leukotriene formation could be responsible for many symptoms which evolve during bacterial infection. Furthermore, this model provides new perspectives to analyze membrane-activating steps in the course of adhesiveness of bacteria (33) and after toxin-receptor interactions with various cells.

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