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Effect of Staphylococcal Delta-Toxin and Bee Venom Peptide Melittin on Leukotriene Induction and Metabolism of Human Polymorphonuclear Granulocytes

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The abilities of delta-toxin from *Staphylococcus aureus* and melittin to induce and modulate the generation of leukotriene from human polymorphonuclear granulocytes (PMNs) were studied. Stimulation of PMNs with melittin (10 μg) induced leukotriene formation, whereas stimulation with delta-toxin did not. Preincubation of the PMNs with delta-toxin modulated the subsequent generation of leukotriene from PMNs induced by Ca ionophore A23187 or opsonized zymosan. The generation of leukotriene B_4 (LTB_4), induced by the Ca ionophore A23187, was increased when the PMNs were preincubated with delta-toxin for 5 min. When opsonized zymosan was used as a secondary stimulus to activate the delta-toxin-pretreated PMNs, LTB_4 generation decreased. In contrast, melittin showed no significant modulatory effect on the generation of leukotriene from PMNs. In addition, preincubation of PMNs with delta-toxin inhibited the conversion of LTB_4 to omega-oxidation products. Our data suggest that peptides with similar structures, e.g., delta-toxin and melittin, induce and modify leukotriene generation in different manners.

Staphylococcus aureus delta-toxin is a 26-amino-acid polypeptide (10) containing 14 hydrophobic residues and a high percentage of nonionizable side chain amino acids. A variety of pathological functions has been attributed to delta-toxin. It increases vascular permeability in guinea pig skin, inhibits water absorption, and activates adenylate cyclase in the ileum (1). A role of delta-toxin in the Lyell syndrome has also been suggested. In addition, it has many other effects on various cell systems, such as activation of membrane phospholipase A_2, stimulation of prostaglandin synthesis, and inhibition of the binding of epidermal growth factor to cell surface receptors (2). Delta-toxin is differentiated from the other staphylococcal hemolysins by its ability to lyse horse and human erythrocytes (14). Properties of delta-toxin, such as solubility in chloroform-methanol, inactivation by phospholipids, and strong surface activity, suggest an amphiphilic structure (3). Delta-toxin seems to possess the structural characteristics of a typical surface-active protein and can readily insert itself into hydrophobic membrane structures (8, 17). Its mode of action at the cellular level has often been compared with that of melittin, another well-known cytolytic peptide of 26 amino acids which was isolated from bee venom (9), with which it shares many physicochemical and biological properties (2). Melittin induced the release of a newly formed mediator from human polymorphonuclear granulocytes (PMNs), the eosinophil chemotactic factor for guinea pig eosinophils (15); this mediator has been shown to be identical to leukotriene B_4 (LTB_4) (13). It is well established that human PMNs, upon stimulation by extracellular signals, release free arachidonic acid from membrane phospholipids, which is metabolized to a group of potent inflammatory mediators, the leukotrienes (4). The main product of PMNs is LTB_4, which is known to stimulate chemotaxis, chemokinesis, and the release of granular enzymes. PMNs not only synthesize and respond to LTB_4 but also are able to catabolize this potent mediator rapidly by omega-oxidation to the less active products 20-hydroxy-LTB_4 and 20-carboxy-LTB_4 (6, 18, 20). Obviously, the inflammatory potential of granulocytes reflects the actual concentration of mediators resulting from both synthesis and metabolism-deactivation.

It was the purpose of this study to investigate the properties of staphylococcal delta-toxin as well as those of melittin with regard to the ability to induce and modulate leukotriene formation from human granulocytes.

Human PMNs were obtained from 200 ml of heparinized blood from healthy donors and were separated as described previously (5). The PMNs were suspended to a final concentration of 2×10^7 cells per ml in phosphate-buffered saline (PBS).

The delta-toxin was prepared as previously described (1) and was dissolved in 0.01 M Tris hydrochloride, pH 7.4, at a concentration of 1 mg/ml. Melittin was obtained from Sigma Chemical Co., Munich, Federal Republic of Germany; the presence of phospholipase A_2 in the melittin preparation was determined by the method of Märki and Franson (16). The concentration of melittin which was used for cell stimulation contained no detectable phospholipase A_2 activity. Ca ionophore A23187, zymosan A, and heparin were obtained from Sigma.

The metabolism of exogenously added LTB_4 in preincubated cells was analyzed by reverse-phase high-pressure liquid chromatography (RP-HPLC), as described by Brom et al. (7). Leukotriene generation and LTB_4 metabolism were analyzed by RP-HPLC as previously described (11) with a solvent mixture of phosphate buffer (17 mM dipotassium hydrogen phosphate, containing EDTA, adjusted to pH 5.0 with phosphoric acid), acetonitrile, and methanol (50:30:20, vol/vol/vol) at 40°C.

Data from different experiments with different donor cells were combined and reported as the mean ± standard deviation. The Student *t* test for independent means was used to

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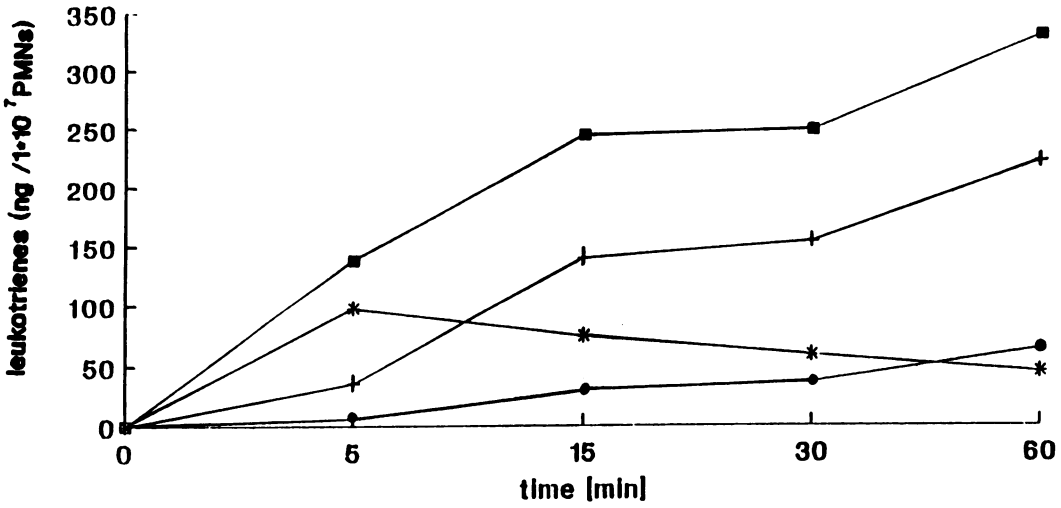

FIG. 1. Time-dependent generation of LTB_4 and its omega-oxidation products from PMNs upon stimulation with melittin (10 μg/10^7 PMNs). Leukotriene generation was analyzed by RP-HPLC. Each value represents the mean of three independent experiments performed with different donor cells. The standard deviation ranged from 5 to 10%. Symbols: ●, 20-COOH-LTB_4; *, LTB_4; +, 20-OH-LTB_4; ■, LTB_4 plus omega-oxidation products.

provide a statistical analysis ($P > 0.05$ was considered not significant).

Human PMNs (10^7) stimulated with melittin (10 μg) in the presence of calcium (1 mM) and magnesium (0.5 mM) over various time periods at 37°C generated different amounts of leukotrienes (Fig. 1). LTB_4 reached a maximum after 5 min of incubation, and with longer times of incubation, a decrease in LTB_4 was observed. Parallel to the decreased LTB_4 formation, the amounts of 20-OH-LTB_4 and 20-COOH-LTB_4 increased. The combined amounts of LTB_4 and LTB_4 omega-oxidation products, reflecting the results of de novo synthesis and metabolism, increased during the incubation time and reached a maximum after 60 min. Neither LTB_4 nor the LTB_4 omega-oxidation products were detected by RP-HPLC when the PMNs were stimulated with lower concentrations of melittin (1 μg, 100 ng, or 10 ng) over various times. In contrast to melittin, delta-toxin (10 μg, 1 μg, 100 ng, or 10 ng) was not able to induce generation of leukotriene from PMNs (data not shown). The effects demonstrated with melittin and delta-toxin were obtained under noncytolytic conditions, as determined by the release of lactate dehydrogenase (12).

The time-dependent generation of leukotrienes from PMNs induced by the Ca ionophore A23187 shows a characteristic pattern; 20-OH-LTB_4, the first omega-oxidation product, was the main product after 10 min of incubation. Experiments were then performed to analyze the effects of delta-toxin and melittin on leukotriene generation induced by the Ca ionophore A23187. PMNs (10^7) were incubated with delta-toxin, melittin, or an equal volume of PBS (as a control) for 5 min at 37°C. The incubation was then continued with the Ca ionophore A23187 (5 μM) for an additional 10 min at 37°C. The data obtained by HPLC analysis are shown in Fig. 2. Priming of the PMNs with delta-toxin (1 μg/10^7 PMNs) for 5 min increased the amounts of LTB_4 up to 35% after subsequent stimulation with the Ca ionophore A23187 (5 μM, $P < 0.01$). The amounts of 20-OH-LTB_4 were significantly decreased after preincubation with the delta-toxin for 5 min. The amounts of 20-COOH-LTB_4 and the combined amounts of LTB_4 and LTB_4 omega-oxidation products were not significantly affected when the cells were prestimulated for 5 min with the delta-toxin. In contrast, pretreatment of PMNs for 5 min with melittin (1 μg/10^7 PMNs) showed no significant effects on the pattern of leukotriene formation induced after stimulation with the Ca ionophore.

Experiments were then carried out to analyze the effects of toxin pretreatment of cells which subsequently were stimulated with opsonized zymosan. The amounts of leukotrienes generated after stimulation with opsonized zymosan were only 10 to 15% of those induced by the Ca ionophore. For these experiments, PMNs (10^7) were prestimulated in the presence of calcium (2 mM) and magnesium (1 mM) with the toxins or with an equal volume of PBS (as a control) for 5 min; subsequently, the cells were incubated with opsonized zymosan (2 mg) (19) for another 10 min (Fig. 3). Priming of the PMNs with the delta-toxin (1 μg/10^7 PMNs) for 5 min significantly decreased the amounts of LTB_4, 20-OH-LTB_4, and 20-COOH-LTB_4 and the combined amounts of the LTB_4 metabolites. Preincubation of PMNs for 5 min with melittin showed no significant effects on LTB_4 generation and metabolism induced after stimulation with opsonized zymosan.

In order to determine the metabolism of exogenously added LTB_4, PMNs (10^7) were pretreated with the toxins (1 μg or 100 ng) or with PBS for 5 or 30 min at 37°C. After preincubation, 100 ng of LTB_4 or the appropriate portions of buffer were added; the incubation proceeded for an additional 15 min (Table 1). It is apparent that the metabolism of LTB_4 is inhibited in a dose-dependent manner; after 5 min of preincubation, the generation of 20-COOH-LTB_4 was significantly suppressed. Extending the preincubation time from 5 to 30 min enhanced the inhibition of LTB_4 omega-oxidation, and the amounts of 20-OH-LTB_4 and 20-COOH-LTB_4 were diminished. No significant effects were observed when the PMNs were pretreated with melittin.

Our data demonstrate that the delta-toxin alone, in the absence of any additional stimulus, was not able to induce leukotriene generation. However, pretreatment of the cells with the toxin modulated the subsequent leukotriene gener-

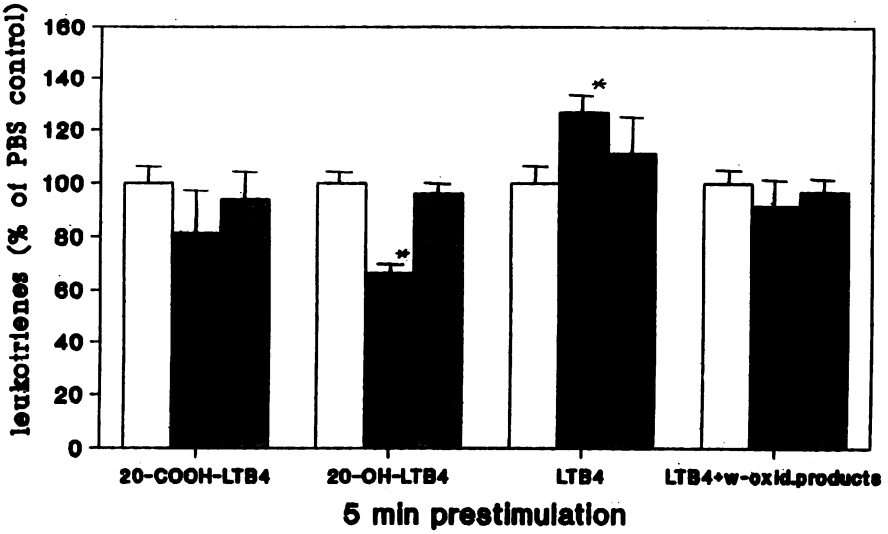

FIG. 2. Effects of delta-toxin and melittin preincubation on the generation of leukotrienes from PMNs. PMNs (10^7) were preincubated in the presence of calcium (1 mM) and magnesium (0.5 mM) with the toxins (1 μg) for 5 min at 37°C; the stimulation then proceeded for an additional 10 min in the presence of the Ca ionophore (5 μM). The values for LTB_4, 20-OH-LTB_4, and 20-COOH-LTB_4 generation and the combined amounts of LTB_4 and LTB_4-omega-oxidation products in the absence of toxins (PBS control) were expressed as 100%. The values for the amounts of LTB_4 and its metabolites in the presence of toxins were calculated as a percentage of the control value. The data represent the mean ± standard deviation of four independent experiments. 100% values (in nanograms): 20-COOH-LTB_4, 60 ± 5; 20-OH-LTB_4, 180 ± 15; and LTB_4, 80 ± 12. Symbols: □, PBS; ■, delta-toxin (1 μg); ▨, melittin (1 μg); *, $P < 0.01$.

ation induced by the various stimuli, which interact with the neutrophils via different membrane biochemical events. Significant changes in the amounts of the various leukotriene metabolites were noted. In this regard, LTB_4 formation was significantly enhanced when the cells were stimulated with the Ca ionophore A23187; the generation of 20-OH-LTB_4 was decreased after preincubation of the PMNs with the delta-toxin. When opsonized zymosan was used as a secondary stimulus, the toxin-pretreated PMNs showed a decreased generation of 20-COOH-LTB_4, 20-OH-LTB_4, and LTB_4 after 5 min of pretreatment. The data obtained with opsonized zymosan indicate that exposure of the cells to delta-toxin leads to a rapid impairment of the ability of the cells to respond to a subsequent stimulus; the pretreated cells were significantly less sensitive to stimulation with opsonized zymosan than nonpretreated cells were. In order to exclude the possibility that prestimulation of the cells exhausts the cells for further biological responses, the luminol-dependent chemiluminescence response of delta-toxin-stimulated cells was studied (data not shown). Our results show that priming of the cells with delta-toxin stimulated the granulocytes to generate reactive oxygen species; the results indicate that the cells were metabolically active. It is more likely that toxin pretreatment may result in a time-dependent deactivation of the cells, probably by post-receptor-mediated processes. Further experiments with regard to the

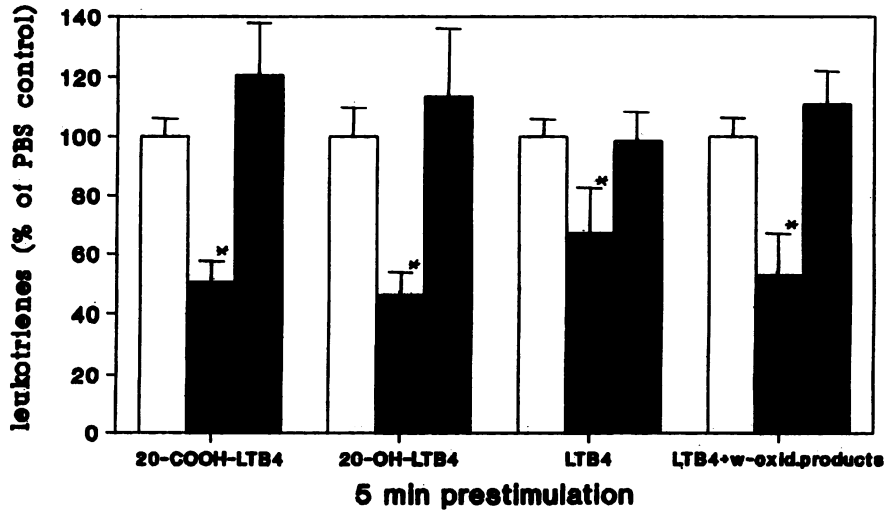

FIG. 3. Preincubation of human granulocytes (10^7) with the delta-toxin and melittin (each at 1 μg) for 5 min at 37°C, followed by stimulation with opsonized zymosan (2 mg). The data represent the mean ± standard deviation of four independent experiments. 100% values (in nanograms): 20-COOH-LTB_4, 15 ± 4; 20-OH-LTB_4, 35 ± 7; and LTB_4, 4 ± 2. Symbols: □, PBS; ■, delta-toxin (1 μg); ▨, melittin (1 μg); *, $P < 0.01$.

TABLE 1. Metabolism of exogenously added LTB_4 by toxin-stimulated PMNs[a]

Length of stimulation in PBS or peptide	% 20-COOH LTB_4	% 20-OH-LTB_4	% LTB_4	% LTB_4 metabolized
5 min				
PBS	40.1 ± 3.6	17.3 ± 5.2	42.6 ± 5.3	57.4 ± 3.9
Delta-toxin (1 μg)	16.8 ± 2.7*	20.0 ± 4.1	63.2 ± 2.1*	36.8 ± 2.5*
Delta-toxin (100 ng)	32.0 ± 1.7*	13.4 ± 3.1	54.6 ± 2.8*	45.4 ± 2.1*
Melittin (11 μg)	39.6 ± 3.9	14.6 ± 3.4	45.8 ± 7.2	54.2 ± 4.1
Melittin (100 ng)	43.0 ± 4.1	16.7 ± 3.4	40.3 ± 4.2	59.7 ± 3.7
30 min				
PBS	30.4 ± 5.2	19.6 ± 3.7	50.0 ± 7.2	50.0 ± 4.2
Delta-toxin (1 μg)	9.8 ± 4.1*	9.4 ± 2.9*	80.8 ± 3.1*	19.2 ± 2.2*
Delta-toxin (100 ng)	15.4 ± 4.0*	11.9 ± 2.0*	72.7 ± 1.7*	27.3 ± 4.9*
Melittin (1 μg)	30.9 ± 3.8	21.0 ± 2.8	48.1 ± 4.5	51.9 ± 3.8
Melittin (100 ng)	38.4 ± 3.7	15.0 ± 4.9	46.6 ± 3.0	53.4 ± 4.7

[a] PMNs (10^7) were preincubated with the toxins (1 μg and 100 ng) in the presence of calcium (1 mM) and magnesium (0.5 mM) for 5 or 30 min at 37°C. After prestimulation, LTB_4 (100 ng) was added to the cell suspension, and incubation was continued for 15 min more. The metabolites were analyzed by RP-HPLC. The sum of all identified leukotrienes is expressed as 100%. The data represent the metabolism of three independent experiments ($n = 3 \pm$ standard deviation). Asterisks indicate $P < 0.01$.

signal transduction cascade (e.g., protein phosphorylation, protein kinase C activity, and inositol turnover) are necessary to clarify the method by which delta-toxin primes the cells.

Studies on the metabolism of exogenously added LTB_4 revealed decreased formation of omega-oxidation products, e.g., 20-COOH-LTB_4 and 20-OH-LTB_4, when the PMNs were prestimulated with the delta-toxin.

Thus, it appears likely that preincubation of PMNs with delta-toxin affects leukotriene induction by enhancing LTB_4 generation and/or inhibiting LTB_4 metabolism. The resulting effects were dependent on the subsequent stimulus used for leukotriene formation.

It appears from our data that these alterations are due to several facts: (i) Delta-toxin could affect the enzymes of the LTB_4 omega-oxidation pathway (e.g., cytochrome oxidase P450). (ii) Delta-toxin changes LTB_4 receptor expression (data not shown), which is involved in the metabolism of LTB_4. (iii) Delta-toxin may modulate components of the signal transduction cascade for ligand (opsonized zymosan)- and nonligand (Ca ionophore A23187)-induced cell activation.

In contrast to the results observed with delta-toxin, melittin induced leukotriene generation by itself at high but noncytotoxic concentrations. Preincubation of PMNs with melittin did not induce a significant modulation of leukotriene formation after stimulation of the cells with the Ca ionophore A23187 or opsonized zymosan. Melittin revealed no effects on LTB_4 metabolism. These differences between delta-toxin and melittin can be explained only if one assumes the presence of different receptor sites and/or membrane mechanisms for the generation and modulation of leukotrienes from human PMNs. Tomita et al. (21) compared oxygen production from the cells induced by delta-toxin and suggested a different transductional pathway for delta-toxin than for stimuli such as melittin. They suggested that the surface-active properties of delta-toxin affect the phagocyte membrane and induce a calcium influx from the medium.

Although bee venom melittin and delta-toxin are amphiphilic polypeptides with strong membrane surface activities, differences do exist between the two peptides. Unlike delta-toxin, melittin is predominantly hydrophobic and the charge distribution is highly asymmetric (3). The overall charge of delta-toxin is maintained by positively and negatively charged residues (22). These structural differences and the resulting properties of delta-toxin and melittin may be responsible for the different effects observed. Therefore, the data suggest that peptides, e.g., delta-toxin and melittin, behave in different ways. The effects of delta-toxin described here may contribute to a better understanding of its biological role and its implication in staphylococcal infections.

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LITERATURE CITED

1. **Alouf, J. E., J. Dufourcq, O. Siffert, and C. Geoffroy.** 1988. Comparative properties of natural and synthetic staphylococcal delta toxin and analogues, p. 39–46. *In* F. J. Fehrenbach et al. (ed.), Zentralblatt fur Bakteriologie, Supplements, vol. 17. Bacterial protein toxins. VCH Publishers, Inc., New York.
2. **Bernheimer, A. W., and B. Rudy.** 1986. Interactions between membranes and cytolytic peptides. Biochim. Biophys. Acta **864**:123–141.
3. **Bhakoo, M., T. H. Birkbeck, and J. H. Freer.** 1982. Interaction of staphylococcus aureus delta-lysin with phospholipid monolayers. Biochemistry **21**:6879–6883.
4. **Borgeat, P., and B. Samuelsson.** 1979. Metabolism of arachidonic acid in polymorphonuclear leukocytes: structural analysis of novel hydroxylated compounds. J. Biol. Chem. **254**:7865–7869.
5. **Böyum, A.** 1968. Isolation of mononuclear cells and granulocytes by one step centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1g. Scand. J. Clin. Lab. Invest. **21**(Suppl. 97):51–76.
6. **Brom, J., W. König, M. Stüning, M. Raulf, and M. Köller.** 1987. Characterization of leukotriene B4-omega hydroxylase activity within human polymorphonuclear granulocytes. Scand. J. Immunol. **25**:283–294.
7. **Brom, J., W. Schönfeld, and W. König.** 1988. Metabolism of leukotriene B4 by activated human polymorphonuclear granulocytes. Immunology **64**:509–518.
8. **Colacicco, G., M. K. Basu, A. R. Buckelew, and A. W. Bernheimer.** 1977. Surface properties of membrane systems. Transport of staphylococcal delta-toxin from aqueous to membrane phase. Biochim. Biophys. Acta **465**:378–390.
9. **Dufourcq, J., J. F. Faucon, G. Fourche, J.-L. Dasseux, M. Le Maire, and T. Gulik-Krzywicki.** 1986. Morphological changes of phosphatidylcholine bilayers induced by melittin: vesicularization, fusion, discoidal particles. Biochim. Biophys. Acta **859**: 33–48.
10. **Fitton, J. E., A. Dell, and W. V. Shaw.** 1980. The amino acid sequence of the detla haemolysin of *Staphylococcus aureus*.

FEBS Lett. **115**:209–212.

11. **Knöller, J., W. Schönfeld, M. Köller, T. Hensler, and W. König.** 1988. Arachidonic acid metabolites from polymorphonuclear leukocytes of healthy donors, severely burned patients and children with cystic fibrosis—routine monitoring by high-performance liquid chromatography. J. Chromatogr. **427**:199–208.
12. **König, W., N. Frickhofen, and H. Tesch.** 1978. Generation and secretion of eosinophilotactic activity from human polymorphonuclear neutrophils by various mechanisms of cell activation. Immunology **36**:733–742.
13. **König, W., H. W. Kunau, and P. Borgeat.** 1982. Comparison of the eosinophil chemotactic factor with endogenous hydroxyeicosatetraenoic acids. Adv. Prostaglandin Thromboxane Leukotriene Res. **9**:301–305.
14. **Kreger, A. S., K.-S. Kim, F. Zaboretzky, and A. W. Bernheimer.** 1971. Purification and properties of staphylococcal delta hemolysin. Infect. Immun. **3**:449–465.
15. **Kroegel, C., W. König, C. Mollay, and G. Kreil.** 1981. Generation of eosinophil-chemotactic factor ECF from various cell types of melittin. Mol. Immunol. **18**:227–236.
16. **Märki, F., and R. Franson.** 1986. Endogenous suppression of neutral-active and calcium-dependent phospholipase A2 in human polymorphonuclear granulocytes. Biochim. Biophys. Acta **879**:149–156.
17. **Mellor, I. R., D. H. Thomas, and M. S. P. Sansom.** 1988. Properties of ion channels formed by Staphylococcus aureus delta toxin. Biochim. Biophys. Acta **942**:280–294.
18. **Powell, S. W.** 1984. Properties of leukotriene B4 20-hydroxylase from polymorphonuclear leukocytes. J. Biol. Chem. **259**:3082–3089.
19. **Raulf, M., and W. König.** 1988. Modulation of leukotriene release from human polymorphonuclear leucocytes by PMA and arachidonic acid. Immunology **64**:51–59.
20. **Shak, S., and I. M. Goldstein.** 1984. w-Oxidation is the major pathway for the catabolism of leukotriene B ın human polymorphonuclear leukocytes. J. Biol. Chem. **259**:10181–10186.
21. **Tomita, T., K. Momoi, and S. Kanegasaki.** 1984. Staphylococcal delta toxin-induced generation of chemiluminescence by human polymorphonuclear leukocytes. Toxicon **22**:957–965.
22. **Yianni, Y. P., J. E. Fitton, and C. G. Morgan.** 1986. Lytic effects of melittin and delta-haemolysin from Staphylococcus aureus on the vesicles of dipalmitoyl phosphatidylcholine. Biochim. Biophys. Acta **856**:91–100.