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Effect of Thiol-Activated Toxins (Streptolysin O, Alveolysin, and Theta Toxin) on the Generation of Leukotrienes and Leukotriene-Inducing and -Metabolizing Enzymes from Human Polymorphonuclear Granulocytes

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The generation of leukotrienes (LTC_4, LTD_4, LTE_4, and LTB_4; 12-epi-LTB_4 isomer) from human granulocytes by thiol-activated toxins (streptolysin O, alveolysin from *Bacillus alvei*, and theta toxin from *Clostridium perfringens*) is described. The release occurs under noncytolytic conditions. Although LTB_4 is the major component after calcium ionophore stimulation, more LTC_4 as compared with LTB_4 is released with the toxins. The 5-lipoxygenase pathway of toxin-mediated activation can effectively be inhibited by caffeic acid, a lipoxygenase inhibitor. The toxins also induce the release of leukotriene-metabolizing enzymes such as γ-glutamyltranspeptidase, which transfers LTC_4 into LTD_4, and dipeptidase, which metabolizes LTD_4, into LTE_4. Dipeptidase activity is more pronounced than the γ-glutamyltranspeptidase activity but still does not reach the levels obtained when cells were triggered with opsonized zymosan.

In the past, increasing knowledge has been obtained as to the physiological, genetic, and biochemical mechanisms of pathogenicity. Of great importance are the interactions of lipopolysaccharides, pili, and bacterial exoproducts with the host. It has been described that the sulfhydryl (SH)-activated cytolysins, among which the prototype is streptolysin O (SLO), are potent membrane-disrupting agents of eucaryotic cells as evidenced by the morphological damage of cytoplasmic and organelle membranes (7, 17), inhibition of lymphocyte function, alteration of transport processes, and release of intracellular constituents of target cells such as erythrocytes, platelets, leukocytes, macrophages, and fibroblasts (2, 33). An important common feature of the toxins is the implication of free thiol groups in their lytic activities, which are suppressed by oxidation of thiol group reagents and restored by reduction. Another essential property shared by the SH-activated toxins is that their cytolytic and lethal activity are specifically and irreversibly inhibited by preincubation with cholesterol and related sterols possessing a 3β-OH group and aliphatic side chain of sufficient length at carbon 17 (31). The reduced form of the toxins binds to specific receptors, which subsequently leads to morphological changes in the cell membrane. In many cases, cytolysins have been shown to be accompanied by the occurrence of circular and semicircular structures on the target membranes. It has been suggested that membrane damage ensued as the result of cholesterol rearrangement and disruption of the bilayer and that the (semi-) circular structures viewed in the electron microscope represented complexes of the toxin with tightly bound cholesterol (3, 16, 18, 30). Bhakdi et al. (8) reported that SLO polymers generated large transmembrane channels and suggested the classification of SLO as a member of the channel-forming proteins. However, the actual molecular mechanisms of toxin action remains unclear, as does the role played by the free thiol group(s) of the toxin molecule.

It is well established that granulocytes, upon stimulation, generate free arachidonic acid from phospholipids which is metabolized to three groups of derivatives, i.e., the prostaglandins, the thromboxanes, and the leukotrienes which are formed by oxygenation and further transformation (W. König, J. Scheffer, K. D. Bremm, J. Hacker, and W. Goebel, Int. Arch. Allergy Appl. Immunol., in press). These mediators which affect vascular permeability, chemotaxis of granulocytes, and smooth muscle contraction are involved in acute and chronic inflammatory disease processes. The biosynthesis of leukotrienes is initiated in a lipoxygenase-type reaction leading to 5-hydroxyperoxyeicosatetraenoic acid (5-HPETE) (29). This product is the precursor of 5-hydroxyeicosatetraenoic acid (5-HETE) and the leukotriene LTA_4. LTA_4 can be hydrolyzed to the chemotactic and chemokinetic active compound LTB_4 [5(*S*)-12(*R*)-dihydroxy-6(*cis*)-8,10-(*trans*)-14(*cis*)-eicosatetraenoic acid] by a hydrolase and nonenzymatically to other dihydroxy acids, e.g., LTB_4 isomers with less potent chemotactic activity; to these belong 5(*S*)-12(*R*)-6-*trans*-LTB_4 and 5(*S*)-12(*S*)-12-epi-6-*trans*-LTB_4 (11, 27). Alternatively, LTA_4 can conjugate with glutathione to produce LTC_4 catalyzed by glutathione *S*-transferase. LTC_4 can be transformed to LTD_4 by removal of the γ-glutamyl moiety with the γ-glutamyltranspeptidase. The last step in the metabolism is the cleavage of the carboxy-terminal glycine from LTD_4 to form LTE_4 by LTD_4 dipeptidase (22, 28).

In the past, we presented evidence that various stimuli induce lipid chemotactic factor activity from human granulocytes, e.g., the calcium ionophore, the phagocytosis of opsonized zymosan, and the interaction of the cells with phospholipase and arachidonic acid (15, 21). Data were also recently provided which show that bacterial endotoxins as well as bacterial adhesion induce arachidonic acid transformation with the subsequent formation of chemotactic

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(LTB_4) and spasmogenic (slow-reacting substance [LTC_4, LTD_4, and LTE_4]), factors. Möllby et al. (24) suggested two different modes of membrane-damaging action by bacterial thiol-activated hemolysins. In comparison to alveolysin and the theta toxin, SLO proved to be 10-fold less active when fibroblasts, as compared with erythrocytes, were used as target cells. Furthermore, streptolysin bound to the membrane at 0°C as well as 37°C but induced membrane damage only at 37°C. Alveolysin and theta toxin were shown to be active at both temperatures. It was the purpose of this investigation to analyze whether the different modes of action are reflected in leukotriene generation from human polymorphonuclear granulocytes on stimulation with alveolysin, SLO, and theta toxin from *Clostridium perfringens*. In addition, the effect of the toxins on the leukotriene-inducing and -metabolizing enzymes was analyzed.

MATERIALS AND METHODS

Preparation of cells. Human polymorphonuclear leukocytes (PMNs) were obtained from heparinized blood (300 U/ml) of healthy donors and separated on a Ficoll-metrizoate cushion, followed by dextran sedimentation (10). The cells were washed at 1,400 rpm (300 × *g*) to remove the platelets. The erythrocytes were lysed by hypotonic exposure of the cell suspension. This method leads to more than 97% pure PMNs. PMNs (10^7/0.5 ml of buffer) were incubated with the various toxins in the presence of $CaCl_2$ (1 mM). Incubation proceeded at 37°C under conditions described in the experiments and was then stopped with 500 μl of ice-cold phosphate-buffered saline (PBS). Subsequently, the cells were immediately centrifuged at 300 × *g* for 15 min, and the supernatants were then placed on ice.

Reagents. ATP, NADH, Ficoll 400, lysozyme, phenolphthalein glucuronidate, and caffeic acid were obtained from Sigma, Munich, Federal Republic of Germany. Organic solvents and acetic acid were of high analytical grade (Baker Co., Deventer, The Netherlands). All other chemicals as well as the silica gel plates were of analytical grade (E. Merck AG, Darmstadt, Federal Republic of Germany).

Buffer. Cell experiments were performed in PBS which consisted of NaCl (120 mM), Na_2HPO_4 (10 mM), and KH_2PO_4 (3 mM). The pH of the buffer was 7.4.

Toxins. The toxins were previously activated with 20 mM cysteine. The preparation of alveolysin and SLO has been described by Alouf (2) and Geoffroy and Alouf (19), and that of *C. perfringens* theta toxin has been described by Thelestam and Möllby (32, 35). The samples were homogeneous as shown by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Analysis of leukotriene release. For analysis of leukotriene release, the supernatant (0.5 ml) of stimulated cells were deproteinized by the addition of 2 volumes of acidified methanol (methanol-acetic acid [1,000:1]) overlaid with argon and placed on ice for 30 min. Subsequently, centrifugation at 1,000 × *g* for 15 min at 4°C was carried out to remove the protein. The supernatant was overlaid with argon and stored overnight at −70°C. One milliliter of acidified methanol was again added, and the centrifugation step was repeated. The supernatants were evaporated to dryness under a stream of nitrogen and suspended in 500 μl of methanol-water (30:70), and 200 μl was applied for reversed-phase high-pressure liquid chromatography (HPLC). HPLC analysis was performed with a Nucleosil C18 5-μm column (diameter, 4 by 200 mm; Macherey Nagel, Düren, Federal Republic of Germany) with methanol-water-acetic acid (64:36:0.08) (pH 5.9) titered with ammonia as eluent. HPLC was performed with Consta Metric pumps I and III (Laboratory Data Control [LDC], Div. Milton Roy Co., Riviera Beach, Fla.) and the automatic sample injection module WISP 710B (Waters Associates, Inc., Milford, Mass.). The absorbance of the column effluent was monitored with a variable UV detector (LDC Spectromonitor III 1204 A) adjusted to 280 nm. The peak area or height was calculated with an LDC computing integrator 301. The flow rate was maintained at 1 ml/min. All solvents were degassed before use and constantly stirred during HPLC analysis. Identification of the leukotrienes was assessed by the determination of the retention time and the comparison with an external standard of synthetic leukotrienes (gift from J. Rokach, Merck Frosst, Pointe-Claire, Canada); 12-epi-LTB_4 (gift from P. Borgeat, Quebec, Canada) revealed under the described conditions the same retention time as did LTD_4; it was not possible to separate these peaks within one run. With the described extraction procedure, the recovery rates of leukotrienes from 250 μl of cell supernatants were 80 to 85% for LTC_4, LTD_4, and LTE_4; for LTB_4, 12-epi-LTB_4 recovery rate was 90 to 99.5% (22). Standard curves of the individual leukotrienes were obtained from five different quantities (5 to 125 ng) and showed the following correlations: LTC_4, 0.985; LTD_4, 0.995; LTE_4, 0.985; and LTB_4, 0.999. The detectable minimum quantities were as follows: LTC_4, 2 ng; LTD_4, 2 ng; LTE_4, 2 ng; and LTB_4, 1 ng.

RIA for LTC_4 and LTB_4. In addition to HPLC analysis, all supernatants were studied by radioimmunoassay (RIA) for LTC_4 and LTB_4. A 200-μl quantity of the supernatants suspended in 30% methanol was evaporated to dryness under nitrogen. The material was then suspended in 100 μl of Tris buffer (0.1 M) containing 0.1% gelatin (1). An appropriate antiplasma dilution as well as standard LTB_4, LTC_4, or unknown samples were added to tubes containing ^{3}H-LTB_4 or ^{3}H-LTC_4 in a total volume of 0.6 ml. The minimal quantities detected were ca. 50 pg for LTC_4 and ca. 20 pg for LTB_4. For the LTC_4 determination, the cross-reactivity for LTD_4 was <35%, and for LTB_4 and LTE_4 cross-reactivity was <2%. The RIA for LTB_4 was obtained from Wellcome Research Laboratories; the cross-reactivity against LTC_4 was 2%. The sensitivity for LTB_4 was ca. 10 pg. The correlation of HPLC analysis and RIA amounted to $r = 0.75 \pm 0.1$ for LTC_4 and $r = 0.74 \pm 0.08$ for LTB_4. The results presented here are derived from HPLC analysis.

Analysis of leukotriene-inducing and -metabolizing enzymes. Lipoxygenase activity in toxin-stimulated cell supernatants was analyzed as described by Koshihara et al. (23). A 500-μl quantity of the cell supernatant was incubated with 0.1 μCi of [^{14}C]arachidonic acid (58 mM/Ci) in 0.1 ml of PBS. A stabilization of enzyme activity was obtained with $CaCl_2$ (2 mM) and ATP (2 mM). For inhibition of the lipoxygenase enzyme, caffeic acid or esculetin was added (25). Incubation proceeded for 10 min and was stopped with 1 ml of acidified methanol. After centrifugation for 2 min in an Eppendorf centrifuge, the supernatants were extracted twice with ether (3 ml) and evaporated to dryness. The residue was dissolved in 200 μl of ether and spotted on a 250-μm silica gel thin-layer plate (Kieselgel 60; E. Merck AG). [^{14}C]arachidonic acid, ^{3}H-LTB_4, ^{3}H-LTC_4, ^{3}H-LTD_4, and, if no indomethacin was used, ^{3}H-labeled prostaglandins E_2 and $F_{2\alpha}$ served as reference substances. The plates were developed once in acetyl acetate-isooctane-acetic acid-water (55:25:10:50; organic phase). Radioactivity was detected with an Isomess linear analyzer (Isomess, Straubenhardt, Federal Republic of Germany). The metabolites were iden-

TABLE 1. Leukotriene release by alveolysin, SLO, and theta toxin[a]

PMNs incubated with:	Concn (HU/ml)	Mean leukotriene release (ng/10^7 PMNs) ± SD for:			
		LTC_4	LTD_4 + 12-epi-LTB_4	LTB_4	LTE_4
Alveolysin	100	15.6 ± 2.9	4.6 ± 0.6	4.2 ± 0.8	2.1 ± 0.4
	10	25.1 ± 15.4	1.9 ± 0.9	3.8 ± 0.6	0.7 ± 0.2
	1	11.9 ± 3.4	1.1 ± 0.2	3.4 ± 0.4	0.5 ± 0.3
	0.1	5.1 ± 3.0	0.7 ± 0.1	0.7 ± 0.3	0.2 ± 0.2
SLO	100	11.9 ± 9.4	2.5 ± 0.4	3.7 ± 0.7	4.2 ± 1.1
	10	16.8 ± 1.5	1.8 ± 1.1	2.2 ± 1.9	0.9 ± 0.4
	1	11.7 ± 1	0.8 ± 0.1	1.8 ± 1.5	0.9 ± 0.4
	0.1	3.2 ± 2.2	0.7 ± 0.1	1.8 ± 1.2	0.4 ± 0.2
Theta toxin (perfringolysin)	100	14.2 ± 3.6	1.4 ± 0.6	4.7 ± 1.1	6.1 ± 2.0
	10	22.0 ± 5.1	1.7 ± 0.7	4.2 ± 0.9	4.1 ± 0.7
	1	12.1 ± 3.1	0.5 ± 0.1	3.6 ± 1.1	3.8 ± 0.5
	0.1	10.2 ± 2.0	0.4 ± 0.1	1.8 ± 0.4	3.5 ± 0.5
PBS		1.9	0.7	0.1	0.3

[a] Human PMNs (10^7/500 μl) were incubated with the various toxins activated with 20 mM cysteine. Analysis of cell-free supernatants was performed by reversed-phase HPLC. Peaks were identified by cochromatography with standards obtained from J. Rokach. The results represent mean values calculated from five experiments. Standard deviation of an individual experiment ranged between 7 and 12%.

tified by cochromatography with reference substances. Unless otherwise stated, the amount of radioactive metabolites generated is expressed as a percentage of total radioactivity that was present in the reaction mixture (M. Stüning, M. Raulf, and W. König, Biochem. Pharmacol., in press). For analysis of the leukotriene-transforming enzymes (γ-glutamyltranspeptidase by LTC_4 conversion and LTD_4 dipeptidase by LTD_4 conversion), 250-μl samples of the stimulated cell supernatants were incubated with synthetic LTD_4 for either 30 or 15 min at 37°C, respectively. The incubation was then stopped with 2 ml of acidified methanol and extracted as has been described. Determination of metabolized leukotrienes was performed by HPLC. The γ-glutamyltranspeptidase and LTD_4-dipeptidase activities were expressed by the nanogram quantities of generated metabolites (28).

TABLE 2. Release of marker enzymes by various toxins[a]

PMNs incubated with:	Concn (HU/ml)	Distribution of enzyme release (% of total)			
		LDH[b]	β-Glucuronidase	Lysozyme	Peroxidase
Alveolysin	100	12.4	7.2	20.8	45.2
	10	6.7	4.2	8.3	4.3
	1	5.0	1.9	6.9	2.1
	0.1	4.0	2.3	5.8	1.0
SLO	100	26.3	28.4	9.8	41.3
	10	15.2	5.7	9.7	7.9
	1	5.2	4.2	6.9	2.4
	0.1	4.0	3.7	5.6	1.0
Theta toxin	100	33.0	5.2	9.7	53.2
	10	9.2	3.3	9.5	12.5
	1	7.1	3.0	5.7	2.0
	0.1	4.2	2.1	5.6	1.0
Ionophore A 23187[c]	11.4	7.8	36.0	—[d]	—
	5.7	5.2	17.0	—	—
	2.8	3.1	7.2	—	—
Buffer control		1	2.0	2.9	0
Sonicate		100	100	100	100

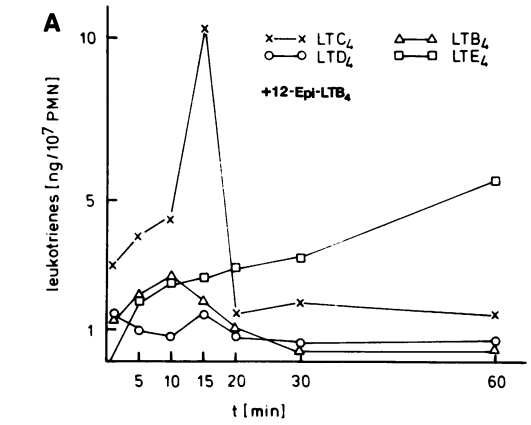
[a] PMNs (10^7/500 μl) were incubated with the activated toxins for 15 min at 37°C. Results represent mean values of three independent experiments and are expressed as percentages of the sonicate. Standard deviation ranged between 3 and 8%. Cells in the presence of buffer served as controls.
[b] LDH, Lactate dehydrogenase.
[c] Concentrations expressed are micromolar.
[d] —, Not done.

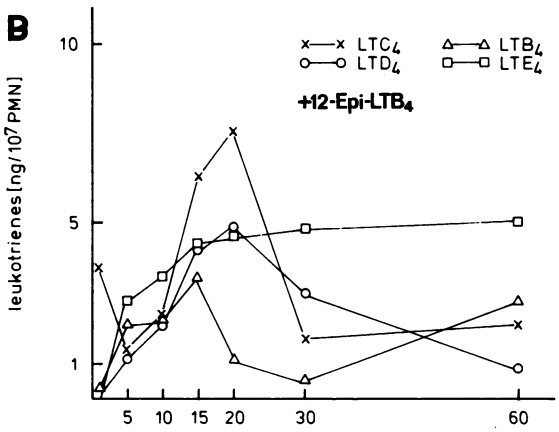
Determination of marker enzymes. Marker enzymes from granulocytes were determined as previously described (21); peroxidase was determined as described by Baggiolini et al. (6); β-glucuronidase was determined as described by Avila and Convit (5); lysozyme and lactate dehydrogenase were determined as described by Bretz and Baggiolini (13). Enzyme activities were calculated as a percentage of the total enzyme activity available after sonication of unstimulated neutrophil suspension.

Statistical analysis. The described experiments were performed at least three to seven times with the cells of various donors; the individual determinations were carried out in at least duplicate or triplicate (experiments for dipeptidase and lipoxygenase activities). All other data represent the mean values of various experiments; the absolute amount of leukotriene release as well as the enzyme activities (γ-glutamyltranspeptidase, dipeptidase) varied from donor to donor. However, a similar pattern was obtained; thus, the high standard deviation is explained by the fact that mean values are calculated from different experiments. The data derived from the RIA represent mean values of triplicates.

RESULTS

Leukotriene release by alveolysin, SLO and theta toxin. Human PMNs (10^7/500 μl) were incubated with various concentrations [0.1 to 100 hemolytic units (HU) per ml] of alveolysin, SLO, and theta toxin for 15 min at 37°C. The stimulated cell-free supernatants were then analyzed by reversed-phase HPLC for LTC_4, LTB_4, LTD_4, LTE_4, and 12-epi-LTB_4 and by RIA for LTC_4 and LTB_4. Five experiments were performed, and mean values were calculated (Table 1). At a concentration of 0.1 HU of alveolysin, 5.1 ± 3.0 ng of LTC_4 was released which increased to 25.1 ± 5.6 ng





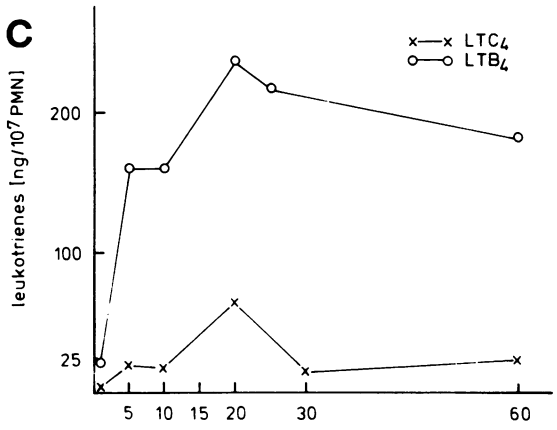


FIG. 1. Human PMNs were stimulated with alveolysin (10 HU/ml) (A), SLO (10 HU/ml) (B), or the calcium ionophore A 23187 (5.7 μM) (C) over time. The extracted supernatants were then analyzed by reversed-phase HPLC for LTC_4, LTB_4, LTE_4, and LTD_4, which revealed the same retention time as 12-epi-LTB_4, the nonenzymatically biosynthesized LTB_4 isomer. The results represent mean values obtained from experiments with the cells of four different donors.

at a concentration of 10 HU. At higher concentrations, a decrease was observed. With SLO and theta toxin, a similar pattern became apparent. The amount of LTC_4 released proved to be optimal at concentrations of 10 HU for the various toxins and decreased with higher concentrations. Maximal LTB_4 activity was obtained with 100 HU of either toxin. In general, at the optimal stimulatory concentrations LTB_4 levels were approximately three- to fivefold less as compared with LTC_4 levels. With theta toxin, significant amounts of LTE_4 were released from the cells, ranging from 6.1 ± 2.0 to 3.5 ± 0.5 ng. In contrast, alveolysin and SLO demonstrated the highest LTE_4 levels at 100 HU of the toxins.

The analysis of enzyme release (cytosol and granular) shows that only at 100 HU do toxins induce a clear elevation of lactate dehydrogenase (Table 2). Kinetic experiments were then carried out with SLO and alveolysin. The release pattern was compared with ionophore-stimulated cells (Fig. 1A to C). With alveolysin (10 HU/ml), an optimal release of LTC_4 is observed after 15 min of incubation which declined rapidly after 20 min. As has been observed in the above-described dose-response experiments, the amount of LTB_4, LTD_4, 12-epi-LTB_4, and LTE_4 was far less as compared with LTC_4. At later times of incubation, LTE_4 levels increased steadily. A similar pattern was obtained when SLO-stimulated cell supernatants were analyzed. Again, LTC_4 release exceeded that of the other leukotrienes and revealed a sharp increase followed by a steady decline after 20 min. LTE_4 levels revealed a plateau over time. These kinetics are in contrast to those observed with the calcium ionophore A 23187 (5.7 μM). A maximal response for LTB_4 with ca. 230 ng was obtained after 20 to 25 min of incubation. The amount of LTB_4 exceeded that of LTC_4 after 5 and 20 min by eight- and sixfold, respectively. In comparison to the alveolysin-induced LTC_4 release at the optimal time point, about fivefold more LTC_4 was generated from human PMNs by the calcium ionophore A 23187.

Effect of thiol-activated toxins on the release of leukotriene-inducing (5-lipoxygenase) and -metabolizing (γ-glutamyltranspeptidase, dipeptidase) enzymes from human PMNs. Experiments were performed to analyze the release of leukotriene-inducing (5-lipoxygenase) and -metabolizing (γ-glutamyltranspeptidase and dipeptidase) enzymes within the stimulated supernatant of human PMNs (Fig. 2A). Human PMNs (10^7/500 μl) were incubated with bacterial toxins at various concentrations. The supernatants were then incubated for 10 min at 37°C with 0.1 μCi of [^{14}C]arachidonic acid (1.6 nM) in the presence of $CaCl_2$ and ATP. The supernatants were deproteinized and analyzed by thin-layer chromatography. In general, the amount of metabolized arachidonic acid increased at higher concentrations of the toxins. This pattern correlated with the formation of 5-HETE and 5-HPETE. Caffeic acid, an inhibitor of the 5-lipoxygenase pathway, inhibited the formation of 5-HPETE by more than 50%. In the experiments described (Fig. 2A), granulocytes were preincubated with caffeic acid (10^{-6} M) before the cells were stimulated with the toxins. Figure 2A illustrates the results of the experiment with SLO. With higher concentrations of caffeic acid (10^{-4} M) as well as esculetin (10^{-4} M), complete inhibition was obtained (data not shown). Experiments were then carried out to analyze the metabolizing activity of supernatants collected from cells after various lengths of toxin treatment. It became apparent that all toxins metabolized arachidonic acid to a high degree after only 1 min of incubation, as is demonstrated for SLO (Fig. 2B). Optimal formation of 5-HETE and 5-HPETE was obtained with the supernatants from cells after 15 to 20 min of stimulation.

It has been established that PMNs stimulated with the calcium ionophore or opsonized zymosan release γ-

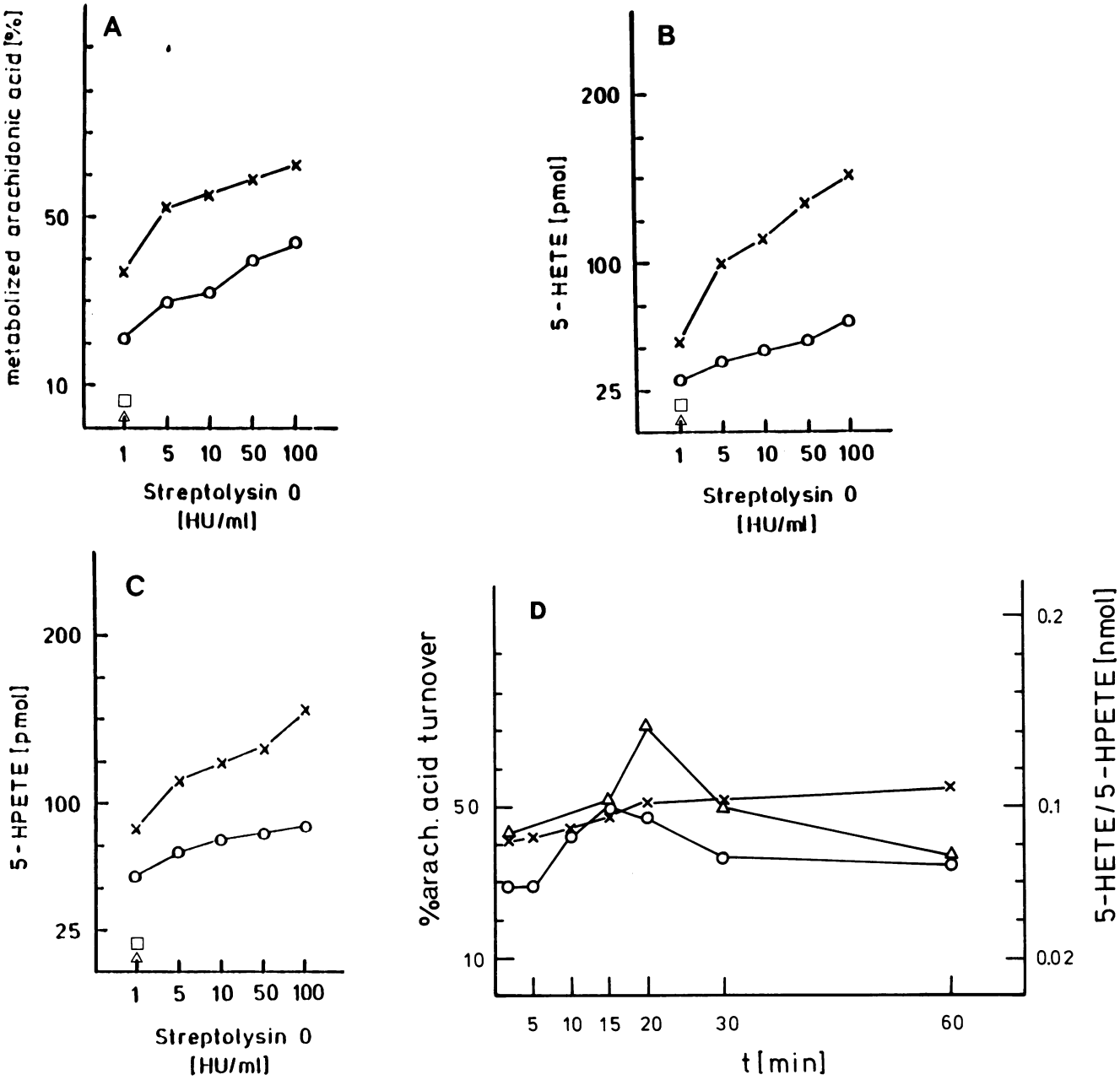


FIG. 2. (A to C) Activation of 5-lipoxygenase pathway of human granulocytes upon incubation with various concentrations of SLO (×). The inhibition of 5-lipoxygenase by caffeic acid (10^{-6} M), preincubated for 2 min and subsequently stimulated with SLO, is also shown (○). A similar pattern of lipoxygenase factor release and inhibition was observed for alveolysin and theta-toxin. Controls include the release of lipoxygenase activity from PMN incubated with buffer (□) and preincubated with caffeic acid (△). (D) Generation of lipoxygenase factors after various intervals of toxin treatment. Human granulocytes were incubated with SLO (7.5 HU). Symbols: ×, percentage of arachidonic acid; ○, 5-HETE; △, 5-HPETE.

glutamyltranspeptidase and dipeptidase activity into the supernatant which transforms LTC_4 into LTD_4 and LTD_4 into LTE_4 (28). Experiments were then performed with toxin-stimulated PMNs. Cells (10^7/500 μl) were incubated with 1 HU of SLO, alveolysin, or theta toxin (data not shown). At various times, the supernatant of stimulated cells was obtained. Synthetic LTC_4 (80 ng) was added, and incubation proceeded for 60 min at 37°C. γ-Glutamyltranspeptidase enzyme activity was quantitated by turnover studies with synthetic LTC_4. It became apparent that almost no LTD_4 was generated; the main metabolite which appeared upon incubation was LTE_4. Maximal activity was obtained with stimulated cell supernatants after 10 min of incubation.

Of the 80 ng of LTC_4 added to the cell supernatant, only 4 to 6 ng of LTC_4 was metabolized at toxin concentrations of 14 HU. No further increase was observed with prolonged incubation. In dose-response studies (Table 3), major γ-glutamyltranspeptidase activity was obtained with 10 HU of alveolysin (17.5%), 10 HU of SLO (10.1%), and with 33 to 16.5 HU of theta toxin. The fact that only minute amounts of LTD_4 were generated and that LTE_4 appeared in the supernatant suggested that cell-free supernatants of stimulated

TABLE 3. Dose-response studies of γ-glutamyltranspeptidase release[a]

PMNs incubated with:	Concn (HU/ml)	Amt (ng) of:	
		Remaining LTC_4	LTE_4
Alveolysin	125	77.0	3.0
	62.5	74.8	5.2
	10	66.0	7.0
	1	72.0	6.0
	0.1	80.0	0
SLO	10	72.4	7.6
	5	74.0	6.0
	1	73.7	5.3
	0.5	77.6	2.4
	0.1	75.7	1.7
Thetatoxin	165	74.1	5.9
	33	71.9	8.1
	16.5	72.8	7.2
	10	73.0	1.0
	3.3	74.7	2.3
	1.0	75.8	4.2
	0.1	80.0	0
PBS		80	0

[a] Human PMNs were incubated with the various toxins at various concentrations for 15 min at 37°C. The cell-free supernatant was then incubated with synthetic LTC_4 (80 ng) for 60 min at 37°C. The conversion to LTD_4 and LTE_4 was analyzed by reversed-phase HPLC. Standard deviation ranged between 6 and 10%.

cells also contained a potent dipeptidase which rapidly transformed the generated LTD_4 into LTE_4. To prove this assumption, human granulocytes (10^7/500 μl) were stimulated with various concentrations of the toxins for 15 min at 37°C. Synthetic LTD_4 (75 ng) was added to the cell-free supernatants, and the conversion of LTD_4 into LTE_4 was analyzed after 60 min of incubation (Table 4). The dipeptidase activity analyzed from the supernatant was more active as compared with the γ-glutamyltranspeptidase activity. Of 75 ng of synthetic LTD_4 incubated with the cell supernatants, SLO at concentrations from 0.1 to 10 HU generated 20.1 ± 4.8 to 40.4 ± 4.1 ng of LTE_4; the alveolysin at concentrations from 0.1 to 10 HU generated ca. 28.2 ± 6.1 to 53.7 ± 3.9 ng of LTE_4; theta toxin at concentrations from 0.1 to 165 HU generated about 28.6 ± 4.3 to 60.4 ± 2.4 ng of LTE_4. A cell sonicate generated 69.6 ng of LTE_4 from 75 ng of LTD_4. Kinetic studies with SLO (1 HU), alveolysin (0.1 HU), and theta toxin (0.1 HU) emphasized that the major activity was released from granulocytes after 15 to 20 min of incubation. Previously, it has been observed that granulocytes in the presence of PBS released enzyme activity into the supernatant which steadily increased over time (28). The toxin-induced release however, far exceeded the control values.

DISCUSSION

Our data clearly demonstrate that the three SH-activated toxins lead to leukotriene generation from human PMNs. The leukotriene-inducing enzyme (5-lipoxygenase) as well as leukotriene-metabolizing enzymes which transform LTC_4 into LTD_4 as well as LTD_4 into LTE_4 were also detected. It is well established that the various stimuli differ in their ability to induce the release of leukotrienes as well as metabolize enzymes (12, 26, 28). Since leukotrienes are potent mediators of inflammation which induce vascular permeability, chemotaxis, and smooth muscle contraction, an important role for these mediators in acute and chronic inflammatory processes has been suggested. The release occurs under noncytolytic concentrations as was assessed by the determination of lactate dehydrogenase in the stimulated-cell supernatants. SLO and theta toxins at concentrations of 100 HU showed a clear elevation of lactate dehydrogenase release above the background control. With higher concentrations of the toxins (above 100 HU), even a decrease in leukotriene release was obtained, indicating a metabolization of these compounds (data not shown). Our observation raises the question whether toxins at non- or sublytic concentrations lead to cell activation before the process of irreversible cell death. Indeed Hirayama et al. (20) recently analyzed the cytotoxic action of leukocidin from *Pseudomonas aeruginosa*. The destruction of rabbit leukocytes by the toxin was reduced in the absence of Ca^{2+} and stimulated by the addition of calcium ionophore A 23187. Their studies with regard to the labeling and breakdown of phospholipids indicated that the initial action of the toxin was to stimulate phosphatidic acid production, presumably causing a rapid metabolic change of phosphatidylinositol correlating with the activities of phosphatidylinositol-specific phospholipase C and 1,2-diacylglycerol kinase. They also could detect and confirm arachidonic acid release from rabbit leukocyte membrane treated with leukocidin. From our studies, it appears that the toxins induce cells activation with leukotriene release before cell death. The leukotriene release is strictly dependent on exogenous calcium as was recently demonstrated for the calcium ionophore and opsonized zymosan as stimuli (28). Alouf et al. (4) described the interaction of bacterial SH-activated cytolytic toxins with monomolecular films of phosphatidylcholine and various sterols as a model for studying toxin-induced membrane disruption. The overall potency of

TABLE 4. Toxin-induced release of LTD_4 dipeptidase activity[a]

PMNs incubated with:	Concn (HU/ml)	Amt (ng) of:	
		Remaining LTD_4	LTE_4
SLO	10	34.6 ± 4.1	40.4 ± 4.1
	5	38.4 ± 4.4	36.6 ± 4.4
	1	59.2 ± 9.3	15.8 ± 9.3
	0.5	57.7 ± 1	17.3 ± 1
	0.1	54.9 ± 4.8	20.1 ± 4.8
Alveolysin	10	21.3 ± 3.9	53.7 ± 3.9
	1	18.6 ± 2.4	56.4 ± 2.4
	0.1	46.8 ± 6.1	28.2 ± 6.1
Theta toxin	165	14.6 ± 2.4	60.4 ± 2.4
	33	23.0 ± 4.8	52.0 ± 4.8
	16.5	34.9 ± 5.1	40.1 ± 5.1
	3.3	31.9 ± 4.0	43.1 ± 4.0
	1	31.8 ± 3.4	43.2 ± 3.4
	0.1	46.4 ± 4.4	28.6 ± 4.3
PBS		72	3
Sonicate		5.4	69.6

[a] PMNs (10^7/500 μl) were incubated with various concentrations of the toxins. Synthetic LTD_4 (75 ng) was added to the cell-free supernatants, and the conversion to LTE_4 was analyzed. Results represent mean values of triplicates obtained from the cells of one donor. A similar pattern was observed with the cells of four different donors.

the four toxins tested was SLO > alveolysin = perfringolysin O > pneumolysin. When alveolysin, SLO, and theta toxin were analyzed at various concentrations, it appeared that theta toxin at 0.1 HU is more active as compared with SLO and alveolysin. This also holds true when LTE_4 levels were analyzed.

It is well established that among human PMNs, the major source for LTB_4 is the neutrophil after stimulation with the calcium ionophore. Lower amounts of LTB_4 were obtained after incubation with serum-treated zymosan, and undetectable amounts were obtained when cells were treated with *N*-formyl-methionyl-leucyl-phenylalanine (26). Borgeat et al. (9) recently described that eosinophil-rich human PMNs from patients with hypereosinophilia characteristically release LTC_4 upon ionophore challenge. Our cell preparations contained about 95 ± 3% neutrophils and 1 to 3% eosinophils. At present, no precise data are available as to the contribution of the low amounts of eosinophils to LTC_4 release. The interaction of SH-activated toxins with cells leads to a rapid release of 5-lipoxygenase activity as was demonstrated by the generation of 5-HETE and 5-HPETE upon the addition of exogeneous arachidonic acid to cell-free stimulated supernatants. The release of arachidonic acid was rapidly converted to subsequent metabolites as is indicated by the formation of 5-HETE and 5-HPETE. The addition of caffeic acid or esculetin (data not shown), both of which are inhibitors of the lipoxygenase pathway, significantly reduced the generation of the lipoxygenase products. In previous studies, we demonstrated that, in the course of phagocytosis, stimulated PMNs as well as the release of leukotrienes release γ-glutamyltranspeptidase and dipeptidase activities. By subcellular fractionation it became evident that γ-glutamyltranspeptidase is associated with the granular and microsomal fractions and that the dipeptidase was correlated with the specific granules (14). It is apparent from our results that the supernatant of toxin-stimulated cells is able to metabolize exogeneously added LTC_4 as well as LTD_4 into LTE_4, thus indicating the release of those enzymes.

Only minute amounts of LTD_4 were detected in these experiments. Conversely, the addition of exogeneous synthetic LTD_4 to the stimulated cell supernatants induced a transformation into LTE_4 by 21, 74.2, and 57.6% when either 1 HU of SLO, alveolysin, or theta toxin were used as stimuli. From these data, it appears that alveolysin is significantly more active in inducing the release of dipeptidase activity from human PMNs.

We recently demonstrated that cells stimulated with opsonized zymosan induced the release of γ-glutamyltranspeptidase and dipeptidase activity. It became evident that under experimental conditions similar to those described above, exogeneously added LTC_4 (80 ng) was metabolized by ca. 100%, and LTD_4 was metabolized by about 88% (28). These data support the notion that, unlike the toxin stimulated neutrophil, the phagocytic stimulus in addition to the release of leukotrienes has the capacity to release large amounts of leukotriene-metabolizing enzymes. It thus appears that phagocytosis is more likely to protect the microenvironment from the effect of inflammatory mediators. In contrast, toxin stimulation of neutrophils is more likely to damage the microenvironment by inducing leukotriene release which is badly metabolized.

Recently, evidence has been presented as to two different modes of membrane-damaging action by bacterial thiol-activated toxins. Among the properties in common, similar morphological effects and leakage patterns were noted. Distinct similarities include the effect of thiol-activated toxins on fibroblast membranes. Compared with the other two hemolysins, SLO was about 10 times less active on fibroblasts in relation to erythrocytes. SLO inhibited the uptake of AIB to a low extent, while both theta-toxin and alveolysin drastically inhibited α-aminoisobutyric acid uptake at concentrations causing almost no release of the nucleotide marker (24). Our experiments do stress the similarities of the toxins as to their capacity to release leukotrienes. In contrast to the above-mentioned studies, the biochemical processes described here occur at early times of toxin cell interaction and mostly show a maximum of leukotriene release after 5 to 15 min of incubation. As to the release of dipeptidase activity, it appears that alveolysin is more potent than the two other toxins.

LITERATURE CITED

1. **Aehringhaus, U., R. H. Wölbling, W. König, C. Patrono, B. M. Peskar, and B. A. Peskar.** 1982. Release of leukotriene C_4 from human polymorphonuclear leukocytes as determined by radioimmunoassay. FEBS Lett. **146**:111–114.
2. **Alouf, J. E.** 1980. Streptococcal toxins (streptolysin O, streptolysin S, erythrogenic toxin). Pharm. Ther. **11**:661–717.
3. **Alouf, J. E., and C. Geoffroy.** 1984. Structure activity relationships in sulfhydryl-activated toxins, p. 165–171. *In* J. E. Alouf, F. J. Fehrenbach, J. H. Freer, and J. Jeljaszewicz (ed.), Bacterial protein toxins. Academic Press, London.
4. **Alouf, J. E., C. Geoffroy, F. Pattus, and R. Verger.** 1984. Surface properties of bacterial sulfhydryl-activated cytolytic toxins. Interaction with monomolecular films of phosphatidylcholine and various sterols. Eur. J. Biochem. **141**:205–210.
5. **Avila, J. L., and J. Convit.** 1973. Studies on human polymorphonuclear leukocyte enzymes. I. Assay of acid hydrolases and other enzymes. Biochim. Biophys. Acta **293**:397–406.
6. **Baggiolini, M., J. G. Hirsch, and C. de Duve.** 1969. Resolution of granules from rabbit heterophil leukocytes into distinct populations by zonal sedimentation. J. Cell Biol. **40**:529–533.
7. **Bernheimer, A. W.** 1976. Sulfhydryl activated toxins, p. 85–97. *In* A. W. Bernheimer (ed.), Mechanisms in bacterial toxinology. John Wiley and Sons, Inc., New York.
8. **Bhakdi, S., M. Roth, A. Sziegoleit, and J. Tranum-Jensen.** 1984. Isolation and identification of two hemolytix forms of streptolysin-O. Infect. Immun. **46**:394–400.
9. **Borgeat, P., V. Fruteau de Laclos, H. Rabinovitch, S. Picard, P. Braquet, J. Hebert, and M. Laviolette.** 1984. Generation and structures of the lipoxygenase products. Eosinophil-rich human polymorphonuclear leukocyte preparations characteristically release leukotriene C_4 on ionophore A 23187 challenge. J. Allergy Clin. Immunol. **74**:310–315.
10. **Böyum, A.** 1968. A one stage procedure for isolation of granulocytes and lymphocytes from human blood. General sedimentation properties of white blood cells in a 1g gravity field. Scand. J. Clin. Lab. Invest. **21**(Suppl. 97):51–76.
11. **Bray, M. A., F. M. Cunningham, A. W. Ford-Hutchinson, and M. J. H. Smith.** 1981. Leukotriene B_4: a mediator of vascular permeability. Br. J. Pharmacol. **72**:483–486.
12. **Bremm, K. D., H. J. Brom, J. E. Alouf, W. König, B. Spur, A. Crea, and W. Peters.** 1984. Generation of leukotrienes from human granulocytes by alveolysin from *Bacillus alvei*. Infect. Immun. **44**:188–193.
13. **Bretz, U., and M. Baggiolini.** 1974. Biochemical and morphological characterization of azurophilic and specific granules of human neutrophilic polymorphonuclear leukocytes. J. Cell Biol. **63**:251–256.
14. **Brom, J., M. Raulf, M. Stüning, B. Spur, A. Crea, K. D. Bremm, and W. König.** 1984. Subcellular localization of enzymes involved in leukotriene formation within human polymorphonuclear granulocytes. Immunology **51**:571–583.
15. **Czarnetzki, B. M., W. König, and L. M. Lichtenstein.** 1976. Antigen induced eosinophil chemotactic factor (ECF) release by human leukocytes. Inflammation **1**:201–215.

16. **Fehrenbach, F. J., C. M. Schmidt, and H. Huser.** 1982. Early and late events in streptolysin O induced hemolysin. Toxicon **20**:223–228.
17. **Freer, J. H.** 1982. Cytolytic toxins and surface activity. Toxicon **20**:217–221.
18. **Geoffroy, C., and J. E. Alouf.** 1982. Interaction of alveolysin a sulfhydryl-activated bacterial cytolytic toxin with thiol group reagents and cholesterol. Toxicon **20**:239–241.
19. **Geoffroy, C., and J. E. Alouf.** 1983. Selective purification by thiol-disulfide interchange chromatography of alveolysin, a sulfhydryl-activated toxin of Bacillus alvei. Toxic properties and interaction with cholesterol and liposomes. J. Biol. Chem. **258**:9968–9972.
20. **Hirayama, T., and I. Kato.** 1984. Mode of cytotoxic action of pseudomonal leukocidin on phosphatidylinositol metabolism and activation of lysosomal enzymes in rabbit leukocytes. Infect. Immun. **43**:21–27.
21. **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.
22. **Köller, M., W. Schönfeld, J. Knöller, K. D. Bremm, W. König, B. Spur, A. Crea, and W. Peters.** 1985. The metabolism of leukotrienes in blood plasma studied by high performance liquid chromatography. Biochim. Biophys. Acta **833**:128–134.
23. **Koshihara, Y., T. Neichi, S. I. Murota, A. N. Lao, Y. Fujimoto, and T. Tatsuno.** 1984. Caffeic acid is a selective inhibitor for leukotriene biosynthesis. Biochim. Biophys. Acta **792**:92–97.
24. **Möllby, R., M. Thelestam, C. Geoffroy, and J. E. Alouf.** 1982. Two different modes of membrane damaging action by bacterial thiol-activated haemolysins. Toxicon **20**:229–232.
25. **Neichi, T., Y. Koshihara, and S. I. Murota.** 1983. Inhibitory effect of esculetin on 5-lipoxygenase and leukotriene biosynthesis. Biochim. Biophys. Acta **753**:130–132.
26. **Palmer, R. M. J., and J. A. Salmon.** 1983. Release of leukotriene B_4 from human neutrophil and its relationship to degranulation induced by N-formyl-methionyl-leucyl-phenylalanine, serum-treated zymosan and the ionophore A 23187. Immunology **50**:65–73.
27. **Powell, W. S.** 1984. Properties of leukotriene B_4 20-hydroxylase from polymorphonuclear leukocytes. J. Biol. Chem. **259**: 3082–3089.
28. **Raulf, M., M. Stüning, and W. König.** 1985. Metabolism of leukotrienes by L-γ-glutamyltranspeptidase and dipeptidase from human polymorphonuclear granulocytes. Immunology **55**:135–147.
29. **Samuelsson, B.** 1980. The leukotrienes: a group of biologically active compounds including SRS-A. Trends Pharmacol. Sci. **19**:227–230.
30. **Smyth, C. J., and J. L. Duncan.** 1978. Thiol-activated (oxygen-labile) cytolysins, p. 129–183. *In* J. Jeljaszewicz and T. Wadström (ed.), Bacterial toxins and cell membranes. Academic Press, London.
31. **Stephen, J., and R. A. Pietrowski.** 1980. Bacterial toxins, p. 46–49. *In* J. A. Cole and C. J. Knowles (ed.), Aspects of microbiology. Thomas Nelson Publishing, Nashville, Tenn.
32. **Thelestam, M., and R. Möllby.** 1975. Determination of toxin-induced leakage of different-size nucleotides through the plasma membrane of human diploid fibroblasts. Infect. Immun. **11**:640–648.
33. **Thelestam, M., and R. Möllby.** 1980. Interaction of streptolysin O from *Streptococcus pyogenes* and theta-toxin from *Clostridium perfringens* with human fibroblasts. Infect. Immun. **29**:863–872.