Role of *Pseudomonas aeruginosa* Lipase in Inflammatory Mediator Release from Human Inflammatory Effector Cells (Platelets, Granulocytes, and Monocytes)

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Previously, we have shown that *Pseudomonas aeruginosa* lipase and phospholipase C (PLC), two extracellular lipolytic enzymes, interact with each other during 12-hydroxyeicosatetraenoic acid (HETE) generation from human platelets. In this regard, the addition of purified *P. aeruginosa* lipase to PLC-containing crude *P. aeruginosa* culture supernatants enhances the generation of the chemotactically active 12-HETE from human platelets. Therefore, we analyzed the interaction of purified *P. aeruginosa* lipase and purified hemolytic *P. aeruginosa* PLC with regard to inflammatory mediator release from human platelets, neutrophilic and basophilic granulocytes, and monocytes. Purified *P. aeruginosa* PLC, but not purified lipase by itself, induced 12-HETE generation from human platelets, the generation of leukotriene B₄ (LTB₄) and oxygen metabolites, enzyme release from human neutrophils, and histamine release from basophils but diminished interleukin-8 (IL-8) release from human monocytes in a dose-dependent manner. The addition of purified lipase enhanced PLC-induced 12-HETE and LTB₄ generation, did not influence enzyme, histamine, or IL-8 release, but diminished the PLC-induced chemiluminescent response. Similar results were obtained when the hemolytic PLC from *Clostridium perfringens* was used instead of *P. aeruginosa* PLC. For further comparison, we used the well-defined calcium ionophore A23187 and phorbol-12-myristate-13-acetate (PMA) as stimuli. Lipase enhanced calcium ionophore-induced LTB₄ generation and β -glucuronidase release but reduced calcium ionophore-induced and PMA-induced chemiluminescence. In parallel, we analyzed the role of lipase in a crude *P. aeruginosa* culture supernatant containing PLC and lipase. Lipase activity in the *P. aeruginosa* culture supernatant was inhibited by treatment with the lipase-specific inhibitor hexadecylsulfonyl fluoride, leaving the activity of PLC unaffected. The capacity of "lipase-inactivated culture supernatant" to induce 12-HETE and LTB₄ generation was diminished by 50 to 100%. Our results suggest that the simultaneous secretion of lipase and PLC by *P. aeruginosa* residing in an infected host may result in severe pathological effects which cannot be explained by the sole action of the individual virulence factor on inflammatory effector cells.

Pseudomonas aeruginosa is involved in a variety of acute and chronic infectious diseases (4, 14, 20, 38). In cystic fibrosis (CF), colonization of the respiratory tract with *P. aeruginosa* is correlated with an increase in lung destruction (2, 24). The microorganism produces a variety of extracellular products, e.g., elastase, phospholipase C (PLC), rhamnolipid, lipopolysaccharide, and lipase (5, 10, 21, 22, 34, 39, 45). While great effort has been directed toward the analysis of individual pathogenicity factors, such as the *P. aeruginosa* PLC (6, 51) the interaction of specific virulence factors has received little attention (28, 51).

Antisera obtained from CF patients with increasing duration of *P. aeruginosa* infection contained increasing amounts of anti-lipase and anti-PLC antibodies, indicating the presence of lipase and PLC in the *P. aeruginosa*-infected patient (22, 51). In vitro studies demonstrated that the major lung surfactant lipid, e.g., dipalmitoylphosphatidylcholine, was completely degraded only in the presence of both lipase and PLC (22). In vitro studies of the function of cells mediating the immune response revealed that lipase significantly inhibited monocyte chemotaxis and chemiluminescence (21). Furthermore, a combina-

tion of lipase and PLC led to a dramatic increase in the formation of 12-hydroxyeicosatetraenoic acid (12-HETE) from human platelets (28). Thus, the present state of knowledge classifies the *P. aeruginosa* lipase as an important virulence factor which induces its harmful effects in combination with other bacterial enzymes, in particular PLC. These pathogenicity factors may activate or suppress the immune response from various cells and thus contribute to the pathophysiology of *P. aeruginosa* infection in a distinct manner (4, 13, 17, 22, 26, 27, 36).

Among the inflammatory cells, neutrophils, basophils, monocytes, and platelets participate as effector cells because of the release of inflammatory mediators. These cells respond to a variety of stimuli (bacteria, toxins) with the release of mediators which proceeds via degranulation of preformed constituents (e.g., histamine and enzymes) or via generation of novel substances (e.g., oxygen metabolites, leukotrienes [LT], HETEs, and interleukin-8 [IL-8]) (4, 11, 17, 31). The newly generated arachidonic acid metabolites LTB₄ and 12-HETE, as well as the cytokine IL-8, are potent chemotactic factors for granulocytes and eosinophils (12, 15, 18, 31). All three chemotactic factors exert autocrine effects on neutrophils and induces them to degranulate, to generate superoxide, and to adhere to the vascular endothelium (31, 48, 52). Neutrophils and platelets, via metabolism of LTA₄, which is generated from stimulated monocytes, generate cysteinyl-LTs, e.g., LTC₄, LTD₄,

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and LTE₄, which have spasmogenic properties (7, 16, 47). Thus, the activation of an individual cell may trigger additional cells in the environment. Furthermore, the neutrophil produces oxygen metabolites and releases lysosomal enzymes which are directed against microorganisms and their toxic products. All these mediators are directed primarily against invading microorganisms. However, an exaggerated as well as a suppressed immune response may lead to tissue injury and promotion of inflammation (11, 14, 20, 31).

The purpose of our study was to explore the effect of *P. aeruginosa* lipase on the release of inflammatory mediators from human platelets and human leukocytes. The interaction between lipase and PLC in modulating the cellular responses was examined. Therefore, cells were stimulated with *P. aeruginosa* PAO1 culture supernatants expressing lipase activity or inactivated for lipase activity and with purified *P. aeruginosa* lipase alone or in combination with purified *P. aeruginosa* PLC or with PLC from *Clostridium perfringens*.

MATERIALS AND METHODS

Materials. Peptone, sodium metrizoate solution (75%, wt/vol), was obtained from Gibco, Eggenstein, Germany. Additional chemicals were obtained from Sigma, Deisenhofen, Germany. The IL-8 antibodies were kindly provided by M. Ceska, Sandoz, Vienna, Austria. Hemolytic PLC from *C. perfringens* was obtained from Sigma.

Buffer. The buffer used for washing the neutrophilic granulocytes (PMN) and the basophilic granulocytes consisted of 137 mM NaCl, 8 mM Na₂HPO₄, 3 mM KCl, and 3 mM KH₂PO₄ (pH 7.4) (modified Dulbecco's phosphate-buffered saline [PBS]). For stimulation assays, the neutrophilic and basophilic granulocytes were suspended in PBS supplemented with Mg²⁺ (1 mM) and Ca²⁺ (0.5 mM); monocytes were suspended in RPMI medium (Gibco) supplemented with 10% fetal calf serum.

Bacterial strains. *P. aeruginosa* PAO1, expressing the hemolytic (PLC-H) and the nonhemolytic (PLC-N) PLCs was used (41, 42). Purified *P. aeruginosa* PLC-H was prepared by the method of Ostroff et al. (42).

Bacterial growth and isolation of culture supernatants. Bacteria were cultured in phosphate-deficient medium (1% NaCl, 1% peptone, 1% glycerol) to induce the synthesis of PLC (19). The broth (10 ml) was inoculated with 100 µl of an overnight culture of the appropriate *P. aeruginosa* strain and incubated on a shaker (180 rpm) at 30°C for 20 to 24 h (early stationary growth phase). The culture supernatants were sterile filtered (crude culture supernatant) and used as indicated in Results.

Isolation of lipase from *P. aeruginosa* culture supernatant. *P. aeruginosa* lipase was isolated and purified as described previously (21). In brief, concentrated culture supernatant from *P. aeruginosa* PACIR was subjected to gel filtration on an FPLC-Hiload 26/60 Superdex 200 column. Fractions containing lipase activity were pooled and concentrated by ultrafiltration, and aliquots were stored at 4°C.

Isolation of PLC-H from *P. aeruginosa* culture supernatant. The hemolytic PLC (PLC-H) was produced for purification by overexpression of the *plcH* gene in a T7 expression system constructed for *P. aeruginosa* (9, 42). Purified PLC (5 µg) was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Only a single band of 78,000 Da was seen on the Coomassie blue-stained gel. PLC activity, hemolytic activity on human erythrocytes, and toxicity to NIH 3T3 contact-inhibited fibroblast (ATCC CLL 92) cells were completely abolished by heating at 100°C for 5 min. PLC-H at 100 ng was lethal for the NIH 3T3 cells. No endotoxin was associated with 1 µg of the pure PLC-H as determined by the quantitative chromogenic *Limulus* amoebocyte lysate assay (BioWhittaker, Walkersville, Md.).

Enzyme assays. (i) PLC. The PLC activity was determined photometrically (A₄₂₀) with *p*-nitrophenylphosphorylcholine as the substrate (5, 28). One unit of activity was defined as the amount of enzyme necessary to liberate 1 µmol of *p*-nitrophenol per min from *p*-nitrophenylphosphorylcholine at 37°C.

(ii) Lipase. The lipase activity was determined photometrically (A₄₂₀) with *p*-nitrophenylpalmitate as the substrate (21). One unit of activity was defined as the amount of enzyme necessary to liberate 1 µmol of *p*-nitrophenol per min from *p*-nitrophenylpalmitate.

Hemolysis. Qualitative as well as quantitative hemolysis was determined as described previously (30).

Preparation of cells. (i) Preparation of PMN and basophilic granulocytes. Human granulocytes were isolated from 200 ml of heparinized blood (15 U/ml) from healthy donors. The blood was separated on a Ficoll-metrizoate gradient and then subjected to dextran sedimentation as described previously (8). The peripheral blood mononuclear cell fraction (LMB), containing 84.6 ± 4.6% lymphocytes (L), 14.2 ± 4.1 monocytes (M), and 1.2 ± 0.5% basophilic granulocytes (B) (50), was isolated by Ficoll-metrizoate sedimentation, washed twice with PBS at 300 × g, and used as source for basophilic granulocytes. The polymorphonuclear neutrophilic granulocytes (PMN) were isolated by dextran

sedimentation and washed twice at 300 × g. This method led to more than 95% pure PMN. The cells were diluted to a final density of 2 × 10⁷ (PMN) or 1 × 10⁷ (LMB) cells per ml in PBS.

(ii) Isolation of monocytes. The LMB fraction was incubated at a density of 1 × 10⁷/ml (at 37°C under 5% CO₂) in plastic flasks (Costar) for 2 h. The adherent cells were collected and contained 95% esterase-positive cells (monocytes) as detected by the esterase assay (50).

(iii) Isolation of platelets. Platelets were prepared from freshly drawn blood. Briefly, EDTA-treated blood was centrifuged at 200 × g for 25 min to obtain platelet-rich plasma. Platelets were obtained by centrifugation at 1,050 × g at 4°C. The platelets were washed in PBS buffer and suspended at about 2 × 10⁸/ml. The purity of the platelets was 98%.

Cell viability. Cell viability was studied by trypan blue exclusion, by analysis of lactate dehydrogenase (Boehringer, Mannheim, Germany), and by determination of mitochondrial activity with WST-1 (Boehringer) in stimulated and non-stimulated cells. The assays were performed as described by the manufacturer. All experiments were performed under conditions (PLC and lipase concentration, time) where the viability of the cell types in all three assay systems was greater than 80%.

Cellular activation. For enzyme and histamine release, generation of leukotrienes, and generation of 12-HETE, human PMN (1 × 10⁷/500 µl), LMB (5 × 10⁶/500 µl), or platelets (1 × 10⁸/500 µl) in PBS in the presence of 0.5 mM Ca²⁺ and 1 mM Mg²⁺ were stimulated with 100 µl of buffer (control) or with 100 µl of the appropriate stimulus for the indicated periods at 37°C. The appropriate supernatants of stimulated cells were analyzed for LTb₄, 12-HETE, histamine, and enzymes.

Analysis of LTs and 12-HETE. PMN (1 × 10⁷/500 µl) or platelets (1 × 10⁸/500 µl) were stimulated in the presence of Ca²⁺ (0.5 mM) and Mg²⁺ (1 mM) with 100 µl of PBS (buffer control), the calcium ionophore A23187 (3 µM), sodium fluoride (20 mM), formyl-methionyl-leucyl-phenylalanine (10⁻⁴ M), lipase, the various purified PLCs, or *P. aeruginosa* PAO1 culture supernatants at the indicated concentrations for 30 min (LTb₄ analysis) or 60 min (12-HETE analysis) at 37°C. The reaction was terminated after addition of 2 ml of methanol-acetonitrile (50:50, vol/vol). Samples were processed and analyzed as described previously (27, 31). In brief, the deproteinized samples were centrifuged (1,600 × g for 15 min), the supernatant was evaporated, and the residue was suspended in 500 µl of methanol-water (30:70, vol/vol) and analyzed by high-pressure liquid chromatography. LTb₄ (total LTb₄ is made up of LTb₄ plus omega products) and 12-HETE were identified and quantified by area integration of the A₂₈₀ peaks (LTb₄) or A₂₈₀ peaks (12-HETE) and external standardization with synthetic standards.

Determination of enzyme release (β-glucuronidase, lactate dehydrogenase) from human PMN. The release of β-glucuronidase and lactate dehydrogenase was determined as previously described (27). Enzyme activities were calculated as the percentage of the total enzyme activities available after sonication of 10⁷ unstimulated PMN.

Chemiluminescence assay. Oxygen radical production was monitored by measuring the luminol-enhanced chemiluminescent response as described previously (27, 43). Briefly, 10⁶ PMN (final volume, 300 µl) were incubated for 10 min at 37°C in the presence of luminol (20 µl, 0.25 mM), Ca²⁺ (0.5 mM), and Mg²⁺ (0.5 mM). Subsequently, the various stimuli (lipase, PLC, A23187, phorbol-12-myristate-13-acetate) were added and the luminol-enhanced photoemission was monitored for an additional 30 min in a Lumacounter M2080 (Lumac, Schaerberg, The Netherlands). The luminol-enhanced photoemission is expressed as counts per minute (cpm).

Histamine release from human basophilic granulocytes. The washed cells (5 × 10⁶ LMBs per 500 µl of PBS) were stimulated in the presence of Ca²⁺ (0.5 mM) and Mg²⁺ (1 mM) with 100 µl of PBS (buffer control), A23187 (3 µM), appropriate culture supernatants, or various purified PLCs for 60 min at 37°C. Samples were centrifuged for 15 min at 300 × g, deproteinized by the addition of 2 ml of 2% HClO₄, and subsequently analyzed for their histamine content by the fluorophotometric analyzer technique (Autoanalyzer Technicon, Bad Vilbel, Germany) (44). Histamine dihydrochloride dissolved in 2% HClO₄ served as a control. Cells in the presence of buffer and the bacterial supernatants at the appropriate dilutions served as negative controls. For the determination of the total cellular histamine content (100%), the cells were disrupted by the addition of 2 ml of HClO₄.

IL-8 assay. Monocytes were suspended at a concentration of 2 × 10⁵/ml in RPMI medium plus 10% fetal calf serum. The cells were cultured in the presence of the appropriate stimulus for up to 24 h. Culture supernatants were collected and analyzed for their IL-8 content. IL-8 release was determined by a sandwich enzyme-linked immunosorbent assay performed by (ELISA) the published method (3). In brief, each well of a 96-well plate (Nunc Maxisorb, Roskilde, Denmark) was coated overnight at 4°C with 100 µl of PBS-0.1% Tween 20 containing anti IL-8 antibodies at 5 µg/ml. The plates were washed three times with PBS-Tween 20, the appropriate samples or IL-8 standard (recombinant human IL-8 [Calbiochem, Bad Soden, Germany]) were added, and incubation proceeded for 2 h at 37°C. Thereafter, alkaline phosphatase-linked anti-IL-8 antibody was added. After addition of *p*-nitrophenylphosphate (15 mg/ml), an ELISA reader was used for quantification and Mikrotek software (SLT Labinstruments, Crailsheim, Germany) was used for calculation.

TABLE 1. Effects of purified PLC from *P. aeruginosa* and *C. perfringens* on inflammatory mediator release from human granulocytes

PLC (U)	Release of ^a :			Chemilumi- nescence ^b (10 ³ cpm)
	β -Glucuronidase (%)	Histamine (%)	LTB ₄ (ng/10 ⁷ cells)	
<i>P. aeruginosa</i>				
0	5.1 $\pm$ 1.0	5.1 $\pm$ 1.5	0.2 $\pm$ 0.1	3 $\pm$ 1
0.2	9.8 $\pm$ 0.9	5.4 $\pm$ 1.2	0.2 $\pm$ 0.1	3 $\pm$ 1
1	10.6 $\pm$ 1.2*	10.9 $\pm$ 2.5*	2.3 $\pm$ 1.2*	8 $\pm$ 3.1
2.5	11.8 $\pm$ 0.8*	23.9 $\pm$ 2.9*	5.4 $\pm$ 0.8*	19 $\pm$ 2.8*
5	13.7 $\pm$ 2.0*	22.3 $\pm$ 4.8*	5.9 $\pm$ 1.4*	25 $\pm$ 7.2*
10	15.8 $\pm$ 1.1*	25.8 $\pm$ 6.2*	6.1 $\pm$ 2.0*	30 $\pm$ 5.4*
<i>C. perfringens</i>				
0	5.0 $\pm$ 1.5	5.1 $\pm$ 1.0	0.2 $\pm$ 0.1	3 $\pm$ 1
0.0001	5.2 $\pm$ 0.3	5.2 $\pm$ 1.0	ND ^c	ND
0.001	4.3 $\pm$ 0.4	4.4 $\pm$ 1.0	ND	3 $\pm$ 4
0.01	19.8 $\pm$ 2.3	9.3 $\pm$ 1.8	ND	7 $\pm$ 2
0.1	42.3 $\pm$ 6.1*	15.3 $\pm$ 0.8*	2.6 $\pm$ 0.4*	13 $\pm$ 2*
5	54.0 $\pm$ 7.5*	17.8 $\pm$ 2.1*	ND	ND
10	52.1 $\pm$ 8.2*	32.2 $\pm$ 4.2*	7.65 $\pm$ 1.2*	27 $\pm$ 5*

^a Human PMN ($1 \times 10^7/500 \mu\text{l}$) or human basophilic granulocytes ($5 \times 10^6/500 \mu\text{l}$) were stimulated with the indicated purified PLCs at various concentrations for 30 min (β -glucuronidase, LTB₄) or 60 min (histamine) at 37°C. The cell supernatants from PMN or basophilic granulocytes were assayed for β -glucuronidase release and LTB₄ generation/histamine release. Data represent means $\pm$ SD of three independent experiments. *, significant difference compared with unstimulated cells (PLC, 0 U), $P < 0.05$.

^b Human PMN (2×10^6) were stimulated with the indicated purified PLCs at various concentrations for up to 30 min at 37°C. The maxima $\pm$ SD for three independent experiments are presented.

^c ND, not determined.

Statistical analysis. Unless stated otherwise, all data show mean values and the standard deviation (SD) for at least three individual experiments with cells from different donors. Significance was examined by Student's *t* test for independent means.

RESULTS

Effects of purified lipase on inflammatory mediator release from human granulocytes by purified PLC from *P. aeruginosa* and *C. perfringens*. Previously, we have shown that lipase modulates the PLC-induced (crude *P. aeruginosa* PLC, purified *C. perfringens* PLC) 12-HETE generation from human platelets. Therefore, we analyzed the interaction of purified hemolytic PLC and purified lipase from *P. aeruginosa* with regard to inflammatory mediator release from a variety of effector cells (neutrophilic granulocytes, basophilic granulocytes, monocytes, platelets). For comparison, we used the hemolytic PLC from *C. perfringens*, which possesses similar substrate specificities to those of the hemolytic *P. aeruginosa* PLC (37, 42).

(i) Neutrophilic granulocytes. Human PMN (10^7) were left untreated or were treated with purified PLC from *P. aeruginosa* (10, 5, 2.5, 1, or 0.2 U) or with PLC from *C. perfringens* (10, 5, 1, 0.1, 0.01, or 0.001 U) in the presence of purified lipase at different concentrations (from 0 to 433 U) for 30 min at 37°C. As shown in Table 1, PLC from both *P. aeruginosa* and *C. perfringens* induced significant β -glucuronidase release, chemiluminescent response, and LTB₄ generation. It became evident from Fig. 1 for a concentration of 5 U of *P. aeruginosa* PLC or 1 U of *C. perfringens* PLC that the addition of lipase caused a dose-dependent inhibition of the PLC-induced chemiluminescent response. In this regard, lipase at 433 U diminished the *P. aeruginosa* (Fig. 1A) and *C. perfringens* (Fig. 1B)-induced chemiluminescent response to baseline levels. In contrast,

PLC-induced LTB₄ generation was enhanced in the presence of lipase. Similar results were obtained for both PLCs (Fig. 2). The addition of lipase over the concentration range tested did not modulate β -glucuronidase release from PMN (data not shown).

(ii) Basophilic granulocytes. To analyze the modulatory role of *P. aeruginosa* lipase for histamine release, a human LMB cell fraction ($5 \times 10^6/500 \mu\text{l}$) was stimulated with purified PLC from *P. aeruginosa* (10, 5, 2.5, 1, or 0.2 U) or with PLC from *C. perfringens* (10, 5, 1, 0.1, 0.01, or 0.001 U) in the presence of purified lipase at different concentrations (from 0 to 433 U) for 60 min at 37°C. Over the whole concentration range tested, *P. aeruginosa* lipase failed to influence histamine release from human basophils induced by PLC (data not shown).

(iii) Platelets. Previously, we have shown that a crude PLC preparation from *P. aeruginosa* PAO1, composed of both hemolytic and nonhemolytic PLC, induces the generation of 12-HETE. In the following experiments, we analyzed the effect of purified hemolytic *P. aeruginosa* PLC (10, 5, 1, and 0.2 U) in the absence and in the presence of purified *P. aeruginosa* lipase (0.43, 4.3, 108, 216, and 433 U) for 12-HETE generation from 10^8 human platelets (Fig. 3). For comparison, we used different concentrations of

FIG. 1. Effects of purified *P. aeruginosa* lipase on the PLC-induced chemiluminescent response from human PMN. Human PMN (2×10^6) were treated with purified *P. aeruginosa* PLC (1 U) in the presence of purified *P. aeruginosa* lipase (0, 4.3, 43, or 433 U) for up to 30 min at 37°C, and buffer values were subtracted (a), or treated with purified *C. perfringens* PLC (0, 0.01, 0.1, or 1 U) in the presence of purified *P. aeruginosa* lipase (0, 0.43, 4.3, or 433 U) for up to 30 min at 37°C (b). Data are means (maximal chemiluminescent responses are presented) from three independent experiments. *, significant difference compared with stimulation in the absence of lipase ($P < 0.05$).

FIG. 2. Effects of purified *P. aeruginosa* lipase on PLC-induced LTB₄ generation from human PMN. PMN ($10^7/500 \mu\text{l}$) were incubated with purified PLC (10 U of *P. aeruginosa* PLC; 10 U of *C. perfringens* PLC) in the presence of purified *P. aeruginosa* lipase (0, 0.43, 4.3, 108, 216, or 433 U). Incubation proceeded for 30 min at 37°C. Values for unstimulated cells were subtracted from all other values of LTB₄ formation. Data are means $\pm$ SDs (bars) for three independent experiments. *, significant difference compared with stimulation in the absence of lipase ($P < 0.05$).

PLC from *C. perfringens* as the stimulus (Fig. 3). As is apparent from Fig. 3, for a lipase concentration above 4.3 U, a significant increase in 12-HETE generation from human platelets by PLC was obtained. In this regard, lipase (108 U) enhanced *P. aeruginosa* and *C. perfringens* PLC-induced 12-HETE generation by approximately 100 and 600%, respectively.

(iv) **Monocytes.** IL-8, an additional chemotactically active compound, is produced mainly by monocytes. Human monocytes (2×10^5) were stimulated with purified PLC from *P. aeruginosa* (10, 5, 2.5, 1, or 0.2 U) (Fig. 4A) or from *C. perfringens* (10, 1, 0.1, 0.01, 0.001, or 0.0001 U) (Fig. 4B) for 4, 12, or 24 h. As shown in Fig. 4, for an incubation time of 24 h, both types of PLCs led to a decrease in IL-8 release in a dose-dependent manner (*P. aeruginosa*, >1 U; *C. perfringens*, >0.1 U). The presence of purified lipase (above 4.3 U) showed negligible effects on IL-8 release from human monocytes (Fig. 4).

FIG. 3. Effects of purified *P. aeruginosa* lipase on PLC (*P. aeruginosa*, *C. perfringens*)-induced 12-HETE generation. Platelets ($10^8/500 \mu\text{l}$) were incubated with purified PLC (10 U of *P. aeruginosa* PLC; 10 U of *C. perfringens* PLC) in the presence of purified *P. aeruginosa* lipase (0, 0.43, 4.3, 108, 216, or 433 U). Incubation proceeded for 60 min at 37°C. Values for unstimulated cells were subtracted from all other values of 12-HETE formation. *, significant difference compared with PLC-stimulated platelets in the absence of *P. aeruginosa* lipase ($P < 0.05$). Data are means $\pm$ SD (bars) for three independent experiments.

FIG. 4. Effects of purified *P. aeruginosa* lipase on IL-8 release from human monocytes challenged with PLC from *P. aeruginosa* and *C. perfringens*. Human monocytes ($2 \times 10^5/\text{ml}$) were incubated with various concentrations of purified PLC from *P. aeruginosa* (10, 5, 2.5, 1, or 0.2 U) (A) or *C. perfringens* (10, 1, 0.1, 0.01, 0.001, or 0.0001 U) (B) in the presence of purified *P. aeruginosa* lipase (0 or 4.3 U [A] or 0, 0.43, 4.3, or 43 U [B]) for 24 h. The cell supernatants were analyzed for IL-8 by ELISA. The data are means $\pm$ SD for three independent experiments. *, significant difference from the buffer control ($P < 0.05$).

Inflammatory mediator release from human granulocytes and platelets by lipase-containing and lipase-inactivated culture supernatants from *P. aeruginosa* PAO1. To analyze whether lipase exerts similar effects on PLC action in crude culture supernatants, further experiments were performed to verify the above results. For this purpose, prior to stimulation, a crude culture supernatant from *P. aeruginosa* PAO1, containing PLC and lipase, was treated with hexadecylsulfonyl fluoride (AMSF), a specific inhibitor of *P. aeruginosa* lipase (21). AMSF by itself showed no effects on inflammatory mediator release from the various cell types. The respective PLC and lipase activities were 20 ± 4 and 43 ± 23 U, respectively, for untreated culture supernatants and 19 ± 2 and 0.5 ± 0.2 U, respectively, for AMSF-treated culture supernatants. The capacity of lipase-inactivated culture supernatant to induce 12-HETE and LTB₄ generation was diminished by 70 and 50%, respectively; the chemiluminescent response was enhanced by about 100%. The results are shown in Table 2. Untreated and AMSF-treated crude PAO1 culture supernatants did not differ in their capacity to induce histamine release from basophilic granulocytes or β -glucuronidase release from PMN (data not shown). Heated culture supernatants failed to induce inflammatory mediator release from the various cell types (data not shown). Thus, data obtained with purified compounds can also be obtained with crude *P. aeruginosa* culture supernatants.

TABLE 2. Generation of LTB₄ and 12-HETE from human effector cells by lipase-containing and lipase-inactivated-culture supernatants of *P. aeruginosa* PAO1

PAO1 culture supernatant	Lipase activity (U/100 μ l)	PLC activity (U/100 μ l)	LTB ₄ ^a (ng/10 ⁷ cells)	12-HETE ^a (ng/10 ⁷ cells)	Chemiluminescence ^b (10 ⁴ cpm)
Without AMSF	43 $\pm$ 23	20 $\pm$ 4	15.6 $\pm$ 2.4	169 $\pm$ 24	8 $\pm$ 3
With AMSF	0.5 $\pm$ 0.2	19 $\pm$ 2	8.82 $\pm$ 1.15	45 $\pm$ 12	20 $\pm$ 2

^a Human PMN (10⁷/ml) or human platelets (10⁸/ml) were stimulated with the indicated crude culture supernatants (100 μ l) from *P. aeruginosa* PAO1 grown up to the early stationary growth phase (20 to 24 h). Incubation proceeded for 30 min (LTB₄) or 60 min (12-HETE) at 37°C. The cell supernatants were analyzed either for LTB₄ (neutrophils) or for 12-HETE (platelets). Data are means $\pm$ SD for three independent experiments.

^b Human PMN (2 $\times$ 10⁶) were stimulated with the indicated crude culture supernatant (50 ml) from *P. aeruginosa* PAO1 for up to 30 min at 37°C. The maxima $\pm$ SD of three independent experiments are presented.

Role of purified *P. aeruginosa* lipase in inflammatory mediator release from human effector cells by defined stimuli. Further experiments were performed to analyze the modulatory effects of purified lipase on inflammatory mediator release from human granulocytes that was induced by defined stimuli. For this purpose, human PMN (1 $\times$ 10⁷/500 μ l) or LMB (5 $\times$ 10⁶/500 μ l) were left untreated or were treated with the Ca ionophore A23187 (3 μ M) sodium fluoride (20 mM), fMLP (10⁻⁴ M), or anti-immunoglobulin E (only histamine) in the presence of various concentrations of purified lipase (0, 0.43, 4.3, 216, or 433 U) for 30 min (β -glucuronidase, LTB₄, chemiluminescence) or 60 min (histamine) at 37°C. As shown in Table 3, A23187-induced LTB₄ generation by and β -glucuronidase release from human granulocytes was significantly enhanced by lipase at concentrations above 4.3 U. In this regard, 216 and 433 U of lipase enhanced the Ca ionophore-induced β -glucuronidase release by up to 200 and 500%, respectively; the Ca ionophore-induced LTB₄ generation was enhanced by up to 50%. The fMLP- and NaF-induced inflammatory mediator release were not affected by lipase. *P. aeruginosa* lipase failed to modulate histamine release from human basophils regardless of the stimulus used. In contrast to β -glucuronidase and histamine release and LTB₄ generation, lipase caused a dose-dependent decrease in the A23187-induced chemiluminescent response from human PMN (Fig. 5). Similar results were obtained when human PMN were stimulated with PMA, a potent stimulus of the chemiluminescent response (Fig. 5). In this regard, the addition of 4.3 U of lipase diminished the PMA-induced chemiluminescent response by approximately 50%.

TABLE 3. Effects of purified *P. aeruginosa* lipase on A23187-induced β -glucuronidase release and LTB₄ generation from human PMN^a

Lipase concn (U)	% release of β -glucuronidase	LTB ₄ generation (ng/10 ⁷ cells)
0	8.1 $\pm$ 1.0	187 $\pm$ 30
0.43	8.1 $\pm$ 1.0	203 $\pm$ 30
4.3	11.29 $\pm$ 2	227 $\pm$ 27
108	16.7 $\pm$ 1.8*	324 $\pm$ 75*
216	29.6 $\pm$ 4.0*	329 $\pm$ 55*
433	47.6 $\pm$ 11.1*	320 $\pm$ 70*

^a Human neutrophils (10⁷/500 μ l) were stimulated with A23187 (3 μ M) in the presence of the indicated lipase (purified) concentrations for 30 min at 37°C. The cell supernatants from PMN were assayed for β -glucuronidase release and LTB₄ generation. Data represent means $\pm$ SD of three independent experiments. *, significant difference compared with experiments in the absence of lipase (P < 0.05).

FIG. 5. Dose-dependent effects of purified *P. aeruginosa* lipase on the Ca ionophore and PMA-induced chemiluminescent response by human PMN. Human PMN (2 $\times$ 10⁶) were left untreated (buffer control) or were treated with Ca ionophore (3 μ M) or PMA (10⁻⁸ M) in the presence of various concentrations of purified *P. aeruginosa* lipase (0, 0.43, 4.3, or 433 U) for up to 30 min at 37°C. The maximum values (cpm) are presented; buffer values were subtracted. Data represent means $\pm$ SD of three independent experiments performed in triplicate. *, significant difference compared with stimulation in the absence of lipase (P < 0.05).

DISCUSSION

P. aeruginosa produces a variety of extracellular products, e.g., PLC, alkaline phosphatase, alginate, lipase, and proteases (5, 10, 21, 34, 39, 46). Up to now, the interaction between specific virulence factors, e.g., PLC and lipase, has received little attention. Our data show that lipase, although by itself inactive, exhibits a variety of effects on PLC-induced inflammatory mediator release from human platelets and leukocytes.

Phagocytes (neutrophils, monocytes) belong to the first line of defense against invading microorganisms. However, the pathogenesis of CF lung injury is associated with neutrophil-mediated chronic pulmonary inflammation (15, 20, 23). Neutrophil migration into the lung is dependent on the generation of chemotactic factors specific for the elicitation of neutrophils. Arachidonic acid metabolites such as LTB₄ and 12-HETE play an important role in the inflammatory response pattern (16, 26, 31, 35, 47, 48). Studies with PLC-containing culture supernatants as well as partially purified PLC of *P. aeruginosa* indicated a role for PLC in LTB₄ and 12-HETE generation from human neutrophils and platelets (4, 28, 36). Furthermore, elevated concentrations of LTB₄ are present in the sputum of CF patients compared with normal volunteers (32). Our studies support and extend the previous findings and show that purified *P. aeruginosa* PLC induces LTB₄ and 12-HETE generation. Our data clearly indicate that purified *P. aeruginosa* PLC is a potent stimulus for 12-HETE generation from human platelets as compared with fMLP, fluoride, and the Ca ionophore A23187. In this regard, 10 U of PLC, which is present in most clinical *P. aeruginosa* culture supernatants, was as stimulatory as sodium fluoride for 12-HETE generation. However, in comparison with the well-defined stimuli such as fMLP, fluoride, and A23187, purified PLC represents a weak stimulus for LTB₄ generation.

Lipase is frequently produced by clinical *P. aeruginosa* strains isolated from CF and burn patients; lipase activities up to 22 U/ml of culture supernatant were measured (22). Recent observations indicated that the bronchoalveolar lavage fluid from CF patients contains elevated lipase levels compared with that from normal volunteers (20a). *P. aeruginosa* lipase by itself has no PLC activity (22). Previously, we have shown that the addition of lipase to

PLC-containing culture supernatants significantly enhances 12-HETE generation from human platelets compared with generation induced by PLC-containing culture supernatants alone (28). Lipase by itself did not induce 12-HETE generation (28). In this study, we extend our previous finding and show that lipase enhances 12-HETE generation by purified *P. aeruginosa* PLC in a similar fashion to that described for the PLC in native culture supernatants. Furthermore, we extend our previous findings (4) that lipase, by itself inactive, enhanced the PLC-induced LTB₄ generation from human neutrophils. To further prove a role for lipase in 12-HETE and LTB₄ generation, PLC-containing culture supernatants from *P. aeruginosa* PAO1 were treated with AMSF, a lipase-specific inhibitor (28). In human platelets or neutrophils challenged with the AMSF-treated culture supernatant, 12-HETE and LTB₄ generation was reduced by 50 to 100%. These results clearly emphasize that PLC and lipase act together with regard to lipid mediator release.

Enzyme (lysozyme, β -glucuronidase, elastase) release and oxygen metabolite generation, measured as the chemiluminescent response, are directed primarily against invading microorganisms. However, an inappropriate immune response may lead to tissue injury and promotion of inflammation or to immunosuppression. Our data show that PLC is a potent stimulus for the generation of oxygen metabolites (chemiluminescence). Whether the suppressive effects of lipase on chemiluminescence response are a tool to escape host defense mechanisms is not yet clear. We also observed the release of the lysosomal enzyme β -glucuronidase from neutrophilic granulocytes after stimulation of PMN with purified *P. aeruginosa* PLC (15.8% $\pm$ 1.1% release at 10 U of PLC). Elastase has been shown to impair specific host defense and to be responsible for the destruction of lung tissue in CF (14). Excessive amounts of lysosomal enzymes may additionally favor tissue destruction. Continuous release of degradative enzymes is also sustained by the generation of LTB₄, which may induce neutrophils to degranulate (31). Thus, lipase may, because of its enhancing effects on LTB₄ generation, indirectly favor lysosomal enzyme release. In recent years, it has become clear that basophils and mast cells not only are involved in allergic reactions but also play a crucial role in inflammatory processes. Basophils are known to be attracted into the inflamed tissue and to generate potent mediators such as arachidonic acid metabolites, performed mediators such as histamine, and even cytokines (e.g., IL-3, IL-4, and granulocyte-macrophage colony-stimulating factor). These mediators contribute to allergic and pseudoallergic reactions and also amplify or trigger immunoglobulin E-mediated immune reactions. Histamine causes edema formation and contraction of smooth muscle cells and regulates local immunity by its ability to influence lymphocyte function. Previously, we have observed that basophils are involved in the chronic process of bacterial infections of the lungs of CF patients. In this regard, an altered profile of histamine content and release from CF patients was measured (44). Our data clearly show that purified *P. aeruginosa* PLC induces histamine release from human basophils.

In addition to both arachidonic acid metabolites, LTB₄ and 12-HETE, the cytokine IL-8 is a potent inducer of the chemotactic response of PMN which proceeds via interaction with two receptor sites (1). Recently, it became apparent from a variety of studies that the potent neutrophil chemotactic peptide IL-8 participates in the chronic inflammatory events in airways of CF patients (12, 52). For our studies, we used monocytes, the main producer of IL-8. Surprisingly, purified *P. aeruginosa* PLC in concentrations up to 1 U led to only a slight dose-dependent increase in IL-8 release from human monocytes. Unlike the generation of 12-HETE and LTB₄, purified

P. aeruginosa PLC at concentrations above 1 U suppressed IL-8 release from human monocytes in a time- and dose-dependent manner. Nonetheless, our data support clinical findings, especially in CF. Indeed, *P. aeruginosa* strains isolated from CF patients during the chronic phase express only low levels of PLC and induce no LTB₄ but induce significant amounts of IL-8 from human monocytes (17, 25). Thus, our data suggest a dual role of PLC in cellular interaction. The suppression of IL-8 release, as well as release of additional cytokines, was observed for other toxins, e.g., the *Escherichia coli* alpha-hemolysin, as well (29). Although active lipase is associated with LPS, lipase failed to induce IL-8 generation from human monocytes (21). One may speculate that suppression of cytokine generation and release may lead to immunosuppression and a failure of specific host defense mechanisms.

Up to now, the mechanism of lipase action has been speculative. However, our data clearly show that lipase acts together with *Clostridium perfringens* PLC in a similar way to that shown for *P. aeruginosa* PLC. Similar to the hemolytic *P. aeruginosa* PLC, the PLC from *C. perfringens* (alpha-toxin) degrades not only phosphatidylcholine but also sphingomyelin, as shown by thin-layer chromatography (22, 33, 37, 49, 51; data not shown). It is obvious from our results that PLC from *C. perfringens* is more potent than PLC from *P. aeruginosa*. One explanation may be the different degradative activities of the two PLCs for sphingomyelin, leading to different effects on inflammatory mediator release from human cells. One end product of phosphorylcholine hydrolysis by PLC is diacylglycerol, a compound that plays an important role as a second messenger in eukaryotic cell regulation through protein kinase C-dependent and -independent pathways (30, 40). Moreover, diacylglycerol is cleaved by endogenous and bacterial lipases e.g., *P. aeruginosa* lipase, resulting in the release of arachidonic acid (16). Thus, the enhanced 12-HETE and LTB₄ generation from human platelets and neutrophils by the combined action of PLC and lipase may be due to a two-step reaction, with the first step being the hydrolysis of phosphatidylcholine by PLC from *P. aeruginosa* and from *C. perfringens*, leading to diacylglycerol, and the second step being an enhanced inflammatory response, which is induced by the metabolism of diacylglycerol to arachidonic acid by *P. aeruginosa* lipase (16, 47). Thus, large amounts of the chemotactic 12-HETE and LTB₄ can be generated without the participation of intracellular PLA₂. Whether lipase influences additional signalling pathways or whether it interacts with additional components of the signal transduction pathway is not clear. Additional experiments with activators of distinct elements of the intracellular signalling pathway are necessary to determine this. Preliminary results show that lipase modulated only the A23187-induced but not the fluoride- or fMLP-induced inflammatory mediator release (31, 37, 49). G-proteins are the target for fluoride as well as for fMLP.

Our results emphasize that PLC and lipase are critical components in the pathogenesis of *P. aeruginosa* infections. Both factors in combination modulate host responses in a distinct manner which may be beneficial for the microorganism. Thus, our in vitro findings contribute to a better understanding of the action of pathogenicity factors and the pathological mechanisms of *P. aeruginosa* infection.

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