Induction of Inflammatory Mediators (Histamine and Leukotrienes) from Rat Peritoneal Mast Cells and Human Granulocytes by *Pseudomonas aeruginosa* Strains from Burn Patients

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Clinical isolates of *Pseudomonas aeruginosa* from severely burned patients were analyzed with regard to their capacity to induce inflammatory-mediator release from rat mast cells or human granulocytes. The bacterial strains were characterized according to their cell-associated hemolysin activity as well as their secreted hemolysin and phospholipase C activities. *P. aeruginosa* expressing heat-labile hemolysin and phospholipase C induced histamine release from rat mast cells and leukotriene formation from human granulocytes, while bacterial strains expressing heat-stable hemolysin were potent releasers of histamine but did not lead to leukotriene formation. The mediator-inducing capacity was dependent on the growth characteristics of the bacterial strains. The purified glycolipid (heat-stable hemolysin) of *P. aeruginosa* was a potent inducer of histamine release but did not initiate leukotriene formation. Exotoxin A did not affect inflammatory-mediator release. *P. aeruginosa* with leukotriene-inducing capacity also enhanced omega oxidation of endogenous leukotriene B_4, suggesting an additional inactivation of the chemotactic potential. Our data suggest that both hemolysins of *P. aeruginosa* contribute to the pathogenicity of *P. aeruginosa* by inducing and modulating inflammatory-mediator release from various cells.

Pseudomonas aeruginosa is frequently involved in nosocomial infections. Patients suffering from tumors, extensive burns, or mucoviscidosis and patients under immunosuppressive therapy are prone to *P. aeruginosa* infections (27, 31, 32, 34). The concomitant septicemia results in a high lethality rate. Recent research has focused on the pathogenicity of *P. aeruginosa* infections. A variety of virulence factors have been described, but the roles of the individual factors are still unclear. Among these are cell-associated factors (e.g., lipopolysaccharide, pili, outer membrane proteins, and mucus) and extracellular toxins such as exotoxin A, proteases, hemolysins, exotoxin S, lipase, leukocidin, and cytotoxin (2, 6, 13, 15, 16, 19, 20, 23, 26, 28). Exotoxin A represents an ADP-ribosyltransferase which inactivates elongation factor 2 and inhibits protein biosynthesis (19). Exotoxin A is the most toxic protein produced by *P. aeruginosa*, and its role in several disease processes has been demonstrated (30, 32, 41). It is cytotoxic for a variety of cell systems and modulates immunological cell functions (6, 18, 33, 43).

P. aeruginosa produces two different hemolysins: a heat-stable hemolysin and a heat-labile protein identified as a 78-kilodalton protein with phospholipase C (PLC) activity (5, 20). Heat-stable hemolysin is a group of glycolipids with detergent-like actions which inhibit ciliar movement and enhance the enzymatic activity of PLC (17, 24). PLC is produced in high concentrations from strains isolated from urinary tract infections and blood cultures (42). Furthermore, PLC affects pulmonary surfactant, causes immunological reactions when added locally into mice, and interacts with several cell types, e.g., platelets (3, 14, 41).

In the course of a bacterial infection, granulocytes and mast cells represent the first line of defense. The release of inflammatory mediators is one of the major functions of these cells (22). Preformed mediators are histamine, heparin, and chemotactically active peptides. Histamine induces bronchoconstriction and increases vascular permeability. Newly generated mediators consist of leukotrienes, prostaglandins, thromboxane, and the platelet activating factor. The biosynthesis of leukotrienes is initiated by the enzymatic oxidation of arachidonic acid (5-lipoxygenase), which leads to the formation of leukotriene A_4 (LTA_4). LTA_4 may be hydrolyzed to LTB_4 or conjugated with glutathione to produce LTC_4. The sequential cleavage of the glutathionyl group leads to LTD_4 and LTE_4. LTB_4 is a potent chemotactic factor for granulocytes. LTC_4, LTD_4, and LTE_4 were identified as the slow-reacting substances of anaphylaxis and lead to bronchoconstriction, vasoconstriction, and an increase in vascular permeability (35). Their role in shock and septicemia has been recently elucidated (10; M. Köller, W. König, J. Brom, M. Raulf, W. Grosse-Weege, G. Erbs, and F. E. Müller, J. Trauma, in press; and M. Köller, W. König, J. Brom, G. Erbs, and F. E. Müller, J. Trauma, in press).

Bacteria and bacterial toxins are potent activators of granulocytes and peritoneal mast cells which lead to the release of histamine and leukotrienes (8, 36, 37). It was the purpose of the present investigation to analyze the expression and secretion of the various hemolysins of *P. aeruginosa* by clinical isolates from severely burned patients and to study their capacities to induce the release of preformed and newly generated mediators of inflammation from various cells. Furthermore, the influence of exotoxin A on mediator release was investigated.

(This research was part of U. Bergmann's M.D. thesis.)

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MATERIALS AND METHODS

Materials. Synthetic leukotrienes LTB_4, LTC_4, LTD_4, and LTE_4 as well as the omega oxidation products 20-OH-LTB_4 and 20-COOH-LTB_4 were generous gifts from J. Rokach (Merck Frosst, Pointe-Claire/Dorval, Québec, Canada). Ficoll 400 was obtained from Pharmacia, Uppsala, Sweden; Macrodex (6%, wt/vol) was obtained from Schiwa, Glandorf, Federal Republic of Germany; sodium metriozate solution (75%, wt/vol) was obtained from Nycomed, Oslo, Norway. p-Nitrophenyl-phosphoryl-choline, NADH, lactate, and phenolphthalein glucuronidate were obtained from Sigma, Deisenhofen, Federal Republic of Germany. All solvents used in this study were of high-pressure liquid chromatography (HPLC) grade from local suppliers.

Bacterial strains. *P. aeruginosa* strains were obtained as clinical isolates from patients hospitalized at the intensive care unit (Abteilung für Verbrennungskrankheiten und Plastische Chirurgie BG-Krankenanstalten "Bergmannsheil," Universitätsklinik, Bochum, Federal Republic of Germany). Identification of the isolates was carried out at the Institut für Medizinische Mikrobiologie, Ruhr Universität Bochum, Federal Republic of Germany. The strains were identified by the API 20 system (Bio Merieux, Nürtingen, Federal Republic of Germany), growth at 42°C, and production of oxidase.

The strains *P. aeruginosa* PAO 1 and PAO T1 were kindly donated by Barbara H. Iglewski, University of Rochester Medical Center, Rochester, N.Y. Their genetic characteristics as well as growth requirements for exotoxin A production are described elsewhere (30). The *P. aeruginosa* glycolipid was a gift from G. Döring, Institut für Hygiene, Universität Tübingen, Tübingen, Federal Republic of Germany.

Bacterial growth. Bacterial growth was carried out in a culture medium of peptone (1%), glycerol (1%), and sodium chloride (1%) in distilled water. The broth (10 ml) was inoculated with 100 µl of an overnight culture. Bacterial growth proceeded on a shaker (150 rpm) at 37°C. Subsequently, the bacteria were centrifuged at $4,000 \times g$ for 15 min, separated from the culture supernatant, and washed in TCM buffer (see below). For the actual experiments, washed bacteria at the stated concentrations as well as the bacterial culture supernatants from various growth phases were analyzed. The strains *P. aeruginosa* PAO 1 and PAO T1 were grown in an iron-depleted Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 50 mM monosodium glutamate and 1% glycerol. Under these conditions, *P. aeruginosa* PAO 1 (exotoxin A+) produces exotoxin A (30).

Bacterial cell counting. After incubation, the bacteria were centrifuged, washed twice, and suspended in 1 ml of buffer. The bacteria were counted microscopically and diluted to the stated concentrations.

Hemolysin assay. A washed sheep erythrocyte suspension (2%) in phosphate-buffered saline was used. A 200-µl volume of either washed bacteria or bacterial culture supernatant was added to 800 µl of erythrocyte suspension. Incubation proceeded at 37°C for 60 min. Subsequently, the samples were removed and placed on ice. Unlysed erythrocytes were then removed by centrifugation (2 min at $9,700 \times g$ in an Eppendorf model 3200 centrifuge). The optical density of the supernatant at 530 nm was determined. Cells in the presence of buffer or H_2O served as controls. The hemolytic activity was measured as a percentage of that of the positive control.

PLC assay. The PLC activity of bacterial culture superna-

tants was determined as was described by Berka et al. (4). The bacterial culture supernatants were decolorized by adding carbon (10 mg) to 1 ml of the supernatant. After centrifugation, 10 µl was incubated with 90 µl of p-nitrophenyl-phosphorocholine reagent (0.25 M Tris buffer, pH 7.2, 60% glycerol, 1.0 µM $ZnCl_2$, 10 mM p-nitrophenyl-phosphorylcholine) in a microdilution test plate for 1 h at 37°C. Bacteria (5×10^8) were added to 200 µl of p-nitrophenyl-phosphorochline. After incubation for 1 h at 37°C, the samples were centrifuged at $9,000 \times g$ for 5 min and the E_{405} for 0.1 ml of the supernatant was determined. The E_{405} was measured in a spectrophotometer.

Heat inactivation. Bacterial culture supernatants (1 ml) were boiled for 15 min, placed on ice, and analyzed with regard to hemolysis, PLC activity, induction of histamine release from rat peritoneal mast cells, and leukotriene generation from polymorphonuclear leukocytes (PMNs).

Buffers. Unless stated otherwise, the medium used for washing the cells and for mediator release was a 25 mM Tris buffer (pH 7.35) containing 120 mM NaCl, 4 mM KCl, 0.6 mM $CaCl_2$, and 1 mM $MgCl_2$. This buffer is referred to as TCM buffer (Tris-calcium-magnesium). All other experiments were carried out in phosphate-buffered saline consisting of 120 mM NaCl, 10 mM Na_2HPO_4, and 3 mM KH_2PO_4, pH 7.4.

Preparation of cells. Human PMNs were obtained from heparinized blood (15 U/ml) of healthy donors and separated on a Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.)-metrizoate gradient followed by dextran sedimentation. The cells were washed at $300 \times g$ to remove the platelets. The erythrocytes were lysed by exposing the cells to hypotonic conditions. This method results in more than 97% pure PMNs (7).

For the collection of rat peritoneal cells, Wistar rats were killed by cervical dislocation. After intraperitoneal injection of TCM buffer, the cell suspension was aspirated, centrifuged at $300 \times g$, and washed twice. The content of mast cells amounted to 5 to 10% (36). Cell viability of both human PMNs and rat peritoneal cells was assessed microscopically by trypan blue exclusion analysis. In addition, enzyme analysis for human PMNs, which required up to 10^7 cells, was carried out.

Histamine release. Rat peritoneal cells containing 10^5 mast cells per ml were used as target cells. A 300-µl volume of the cell suspension was incubated with 100 µl of the stimulus (washed bacteria or bacterial culture supernatant) at 37°C. Incubation proceeded for 60 min. Cells were centrifuged for 15 min at $300 \times g$; the supernatant was removed and deproteinized by the addition of 2 ml of $HClO_4$ (2%). The supernatant was subsequently centrifuged at $2,000 \times g$ for 20 min, and the histamine content was analyzed by the fluorophotometric analyzer technique (36, 37). Cells in the presence of buffer or $HClO_4$ (4%) served as controls. The histamine release data were presented as the percentage of the total amount of histamine present within the cells. The total amount of histamine in the various experiments ranged from 1,200 to 1,500 ng for 10^5 cells.

Leukotriene release from human PMNs. Human PMNs (2×10^7) were suspended in 1,000 µl of phosphate-buffered saline. A 500-µl portion of the cell suspension was incubated with either 50 µl of washed bacteria or the bacterial supernatant. A 50-µl volume of a Ca^{2+}-Mg^{2+} solution ($CaCl_2$ [7 mM]-$MgCl_2$ [10 mM] in phosphate-buffered saline) was added. Incubation proceeded for various times at 37°C. After centrifugation of the cells at $300 \times g$ for 15 min, the

leukotrienes were analyzed by reverse-phase HPLC (8; Köller, König, Brom, Raulf et al., in press).

Determination of marker enzymes. The release of lactate dehydrogenase as a marker enzyme for cell viability and of β-glucuronidase as a marker enzyme for degranulation of azurophile granules was determined as previously described (8). Enzyme activities were calculated as the percentage of the total enzyme activity available after sonication of un-stimulated PMNs (10^7).

Analysis of leukotriene release. The supernatants of stimulated PMNs were deproteinized by addition of 2 ml of methanol-acetonitrile (1:1), overlaid with nitrogen, and frozen at $-70°C$ for 12 h. After centrifugation at $1,000 \times g$ for 15 min, the supernatants were evaporated to dryness in a freeze-dryer, suspended in 0.5 ml of methanol-water (30:70 [vol/vol]), overlaid with nitrogen, and stored overnight at $-70°C$. The probes were centrifuged $(1,000 \times g, 15$ min), and 0.2 ml was applied to reverse-phase HPLC.

HPLC was performed on reverse-phase columns (4 by 250 mm) packed with Nucleosil (C_{18}) 5-μm particles (Macherey-Nagel, Düren, Federal Republic of Germany) with a Constametric pump III (LDC-Laboratory Data Control/Milton Roy, Hasselroth, Federal Republic of Germany) and the automatic sample injection module WISP 710B (Waters Associates, Eschborn, Federal Republic of Germany). The column temperature was constantly maintained at 40°C. The absorbance of the column effluent was monitored by using a variable UV detector adjusted to 280 nm. The peak areas were calculated by using a chromatography data system (series 3000; Nelson Analytical, Mannheim, Federal Republic of Germany). The solvent system was a mixture of phosphate buffer (17 mM K_2HPO_4 containing 0.05% EDTA), acetonitrile, and methanol (50:30:20 [vol/vol]) which was adjusted to pH 5.0 with phosphoric acid.

This solvent system separated LTB_4, its omega-oxidation products, LTC_4, and 5S,12S-DiHETE simultaneously. The flow rate was maintained at 1 ml/min. All solvents were degassed before use and were constantly stirred during HPLC analysis. Identification and quantitation of leukotrienes were performed as has been described elsewhere (Köller, König, Brom, Raulf et al., in press). Standard curves of the individual leukotrienes were obtained with five different concentrations (5 to 500 ng) and showed the following correlations: LTB_4, 0.998; 20-OH-LTB_4, 0.998; 20-COOH-LTB_4, 0.996; and LTC_4, 0.996. The minimum detectable quantities were 0.3 ng for LTB_4 and LTB_4 metabolites.

Radioimmunoassay for LTC_4 and LTB_4. In addition to HPLC analysis, the cell supernatants were analyzed by radioimmunoassay for LTC_4 and LTB_4 (1). A 50-μl sample of the supernatants was suspended in 30% methanol, evaporated to dryness in a freeze-dryer, and suspended in 100 μl of Tris buffer (0.1 M) containing 0.1% gelatin. An appropriate antiserum dilution as well as synthetic LTB_4 and LTC_4 (at concentrations from 10 ng to 20 pg) or unknown samples were added to tubes containing [^{3}H]LTC_4 or [^{3}H]LTB_4 in a total volume of 0.6 ml (9). The minimal quantities detected were 50 pg for LTC_4 and 20 pg for LTB_4. For LTC_4 determination, the cross-reactivity for LTD_4 was 35%, and for LTB_4 and LTE_4 it was about 2%. The radioimmunoassay for LTB_4 was obtained from Wellcome, Burgwedel, Federal Republic of Germany; the cross-reactivity against peptido-leukotrienes was 2%. The correlations (r) of HPLC analysis and radioimmunoassay were 0.92 ± 0.1 for LTC_4 and 0.85 ± 0.08 for LTB_4.

Statistical analysis. The experiments described above were performed three to five times with cells of different donors.
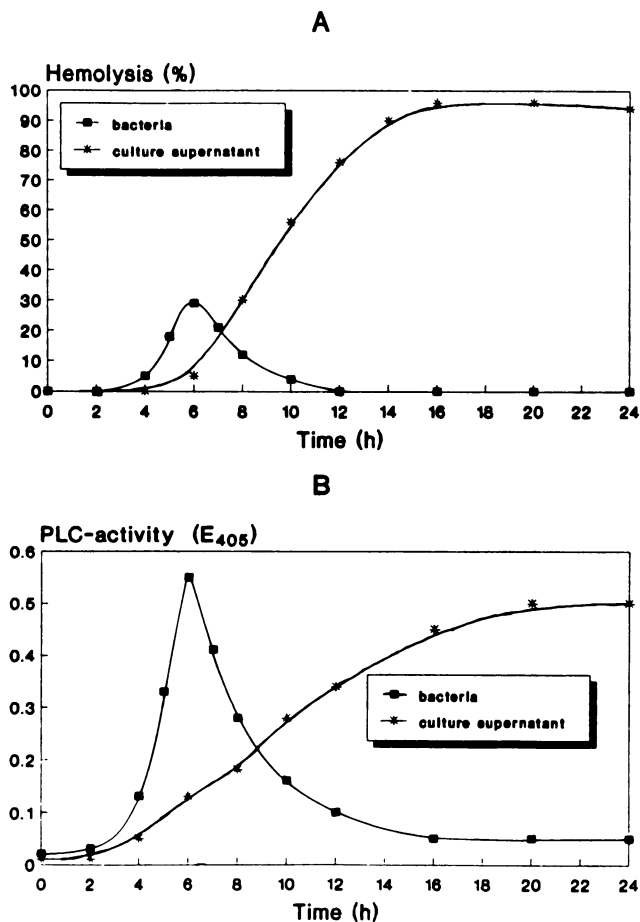

FIG. 1. Hemolysin (A) and PLC (B) activities of *P. aeruginosa* 582f. Bacteria (5 × 10^8) or bacterial culture supernatant (200 μl) were obtained at various times of growth and incubated with sheep erythrocytes (800 μl) for hemolysin activity. PLC activity was determined as described in the text. Mean values of five experiments are shown. SEM ranged from about 5 to 46%.

The individual determinations were carried out in triplicate. The amounts of leukotrienes of an individual experiment are presented as mean values ± standard error of the mean (SEM) of triplicate determinations. The residual data show mean values ± SEM of three to five different experiments. Correlations were determined by using the Spearman correlation test.

RESULTS

Characteristics of *P. aeruginosa* strains and their bacterial culture supernatants. A representative growth curve of *P. aeruginosa* 582f was obtained. Logarithmic growth was present between 4 and 12 h of growth, after which the stationary growth phase was reached (data not shown). The logarithmic growth phase was characterized by the presence of cell-associated hemolysin, which reached a maximum of 29% after 6 h of bacterial growth (Fig. 1A). At this time, hemolysin activity within the bacterial culture supernatant was 10%; after 16 h of bacterial growth, hemolysin activity reached plateau values, as indicated by the complete hemolysis of erythrocytes. PLC activity of the bacterial culture supernatants as well as of the washed bacteria revealed a time course parallel to that of the hemolysin activity (Fig. 1B).

TABLE 1. Hemolysis and PLC activity of *P. aeruginosa* bacteria and culture supernatants

P. aeruginosa strain	Bacterial activity		Bacterial culture supernatant activity		
	Hemolysis (%) (mean ± SEM)	PLC (E_{405}) (mean ± SEM)	Hemolysis (%) (mean ± SEM)	Hemolysis (%) (heat stable)[a] (mean ± SEM)	PLC (E_{405}) (mean ± SEM)
B21	23 ± 2	0.47 ± 0.04	89 ± 4	20 ± 3	1.03 ± 0.02
B10	44 ± 4	0.71 ± 0.04	47 ± 5	12 ± 1	0.41 ± 0.02
582f	29 ± 2	0.55 ± 0.05	97 ± 3	0	0.50 ± 0.07
6816A	4 ± 2	0.09 ± 0.03	95 ± 6	97 ± 2	0.14 ± 0.05
B18	18 ± 1	0.37 ± 0.06	79 ± 2	50 ± 3	0.53 ± 0.01
B1	3 ± 2	0.07 ± 0.01	97 ± 2	24 ± 3	1.21 ± 0.08
B2	0	0.06 ± 0.01	86 ± 5	19 ± 1	0.97 ± 0.04
B5	2 ± 1	0.02 ± 0.01	95 ± 3	80 ± 2	0.63 ± 0.04
B22	1 ± 1	0.04 ± 0.02	74 ± 3	37 ± 1	0.48 ± 0.02

[a] Results obtained with heat-treated culture supernatant.

We then analyzed nine clinical isolates with regard to cell-associated and cell-free hemolysin and PLC activities (Table 1). The bacteria were studied after 6 h of growth, and the bacterial culture supernatants were studied after 24 h of growth. Four strains (B21, B10, 582f, and B18) contained hemolysin activity ranging from 18 to 44%, while five strains (6816A, B1, B2, B5, and B22) expressed hemolysin activity between 0 and 4%. Within the bacterial culture supernatant, hemolysin activity of the nine strains ranged from 47 to 97%. When bacterium-associated PLC activities were evaluated, the four strains (B21, B10, 582f, and B18) expressed significant activities. PLC activity within the bacterial culture supernatant of the nine strains ranged from 0.14 to 1.21 (E_{405}); high PLC activities were secreted from strains B21, B1, and B2. No correlation was obtained when the total hemolysin and PLC activities were compared with each other in the bacterial culture supernatant ($r = 0.34$). Heating of the bacterial culture supernatants for 15 min retained the hemolysin activity to various degrees (Table 1). The culture supernatant of strain B2 showed a reduced hemolysis from 86 ± 5 to 19 ± 1%; hemolysin activity of strain 582f was completely inhibited by heat. These data demonstrate that strain 582f expressed no heat-stable hemolysin, while the other strains secreted the heat-labile hemolysin to various degrees. Strains 6816A, B18, B5, and B22 secreted mainly heat-stable hemolysin into the supernatant. These results suggest the presence of two hemolysins differing in heat sensitivity.

Induction of inflammatory mediators from various cells. Release of histamine and generation of leukotrienes were determined as follows. Washed bacteria (10^8) of strain 582f obtained at various time points of growth were incubated with rat peritoneal cells (Fig. 2A). Histamine release was induced with bacteria from the logarithmic growth phase and reached a maximum (27% of total histamine) after 6 h of bacterial growth. The culture supernatant after 8 h of bacterial growth induced 8% histamine release. The culture supernatant obtained after 16 to 20 hours of bacterial growth induced up to 50% of total histamine release. The release occurred under noncytotoxic conditions, as was shown by trypan blue exclusion.

Preliminary experiments had demonstrated that optimal leukotriene induction was obtained with 5×10^8 *P. aeruginosa* cells (Table 2). Experiments were then carried out to study the optimal time points of bacterial growth for leukotriene release. For this purpose, *P. aeruginosa* cells (5×10^8) of strain 582f obtained from various growth phases (4, 6, 10, and 24 h) were washed and then incubated with human PMNs (10^7) for 20 min. In addition, 50-µl samples of the

bacterial culture supernatants from the various time points were analyzed (Fig. 2B). It became evident that bacteria obtained after 6 h of culture induced the release of 15.2 ± 0.3 ng of LTB_4, which correlated with the optimal time point for histamine release. The bacterial culture supernatants ob-

A

Histamine (%)
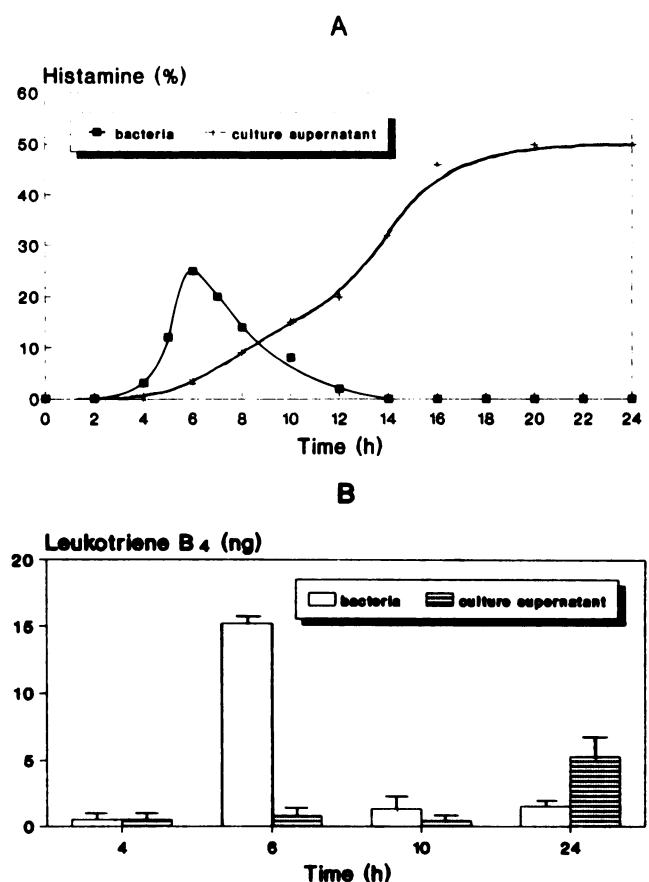

B

Leukotriene B 4 (ng)

FIG. 2. Induction of inflammatory mediators by *P. aeruginosa* 582f. (A) Histamine release from rat peritoneal mast cells. (B) LTB_4 release from human PMNs. Bacteria (1×10^8 for histamine release, 5×10^8 for leukotriene generation) or bacterial culture supernatant (50 to 100 µl) were obtained at various times of growth and incubated with rat peritoneal mast cells or human PMNs (1×10^7). Mean values of triplicate determinations (± SEM for leukotriene release) are shown. SEM for histamine release ranged from about 4 to 37%.

TABLE 2. Induction of leukotriene generation
by *P. aeruginosa* 582f

Bacteria (no. of cells)	Leukotriene generation (ng) (mean ± SEM)			
	LTB_4	$20\text{-}OH\text{-}LTB_4$	$20.COOH\text{-}LTB_4$	LTC_4
1 ± 10^7	1.5 ± 1.0	0.4 ± 0.3	0.5 ± 0.2	1.0 ± 0.7
2 ± 10^7	4.1 ± 0.6	0.5 ± 0.3	0.6 ± 0.1	1.3 ± 0.7
1 ± 10^8	5.3 ± 0.2	10.6 ± 0.7	16.3 ± 1.2	0.6 ± 0.4
2 ± 10^8	8.5 ± 1.0	18.2 ± 1.0	25.3 ± 1.5	4.3 ± 1.5
5 ± 10^8	27.7 ± 1.6	37.1 ± 1.2	12.4 ± 1.0	3.0 ± 1.2
1 ± 10^9	24.3 ± 2.0	18.6 ± 1.0	7.3 ± 0.9	1.0 ± 0.5
2 ± 10^9	23.2 ± 2.3	10.2 ± 0.8	1.5 ± 0.4	0.9 ± 0.3

tained after 10 and 24 h, respectively, induced the release of 0.4 ± 0.2 and 5.3 ± 1.5 ng of LTB_4.

Dose-response studies were performed with amounts of strain 582f ranging from 5×10^6 up to 1×10^9 bacteria obtained after 6 h of growth and with the bacterial culture supernatant obtained after 24 h of growth (Fig. 3). It was shown that bacteria at a concentration from 10^7 to 10^8 induced the release of about 22 ± 1 to 26 ± 2% of total histamine from rat mast cells. When the bacterial culture supernatant was used, about 45% of total histamine was released; a 1:20 dilution of the supernatant reduced the histamine-releasing capacity completely.
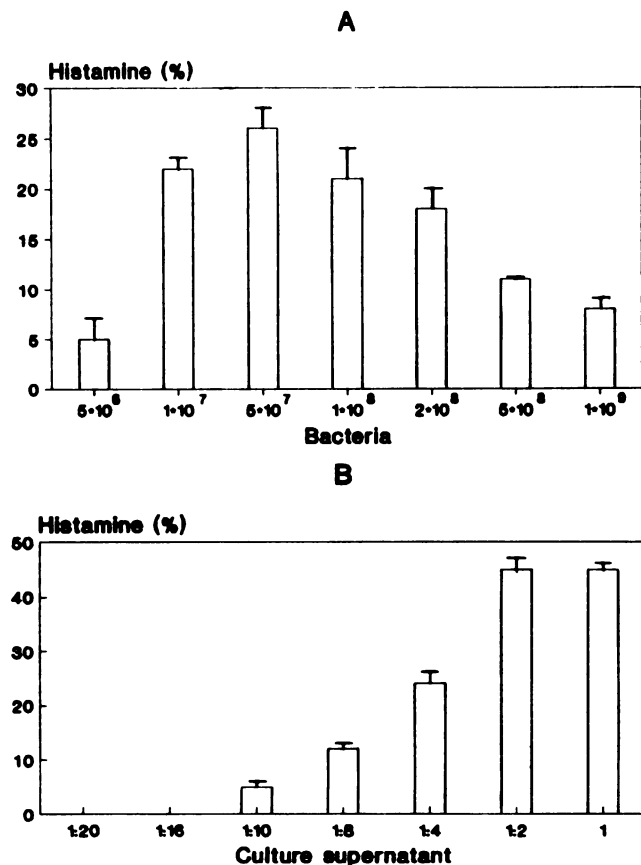
A

B

FIG. 3. Dose-dependent induction of histamine release by *P. aeruginosa* 582f bacteria and bacterial culture supernatant. Washed bacteria (A) (6-h culture) or culture supernatant (B) (24-h culture) was diluted in TCM buffer and incubated with rat peritoneal mast cells (3×10^4) for 60 min. Data represent mean values of three individual experiments. SEM was between 5 and 14%.
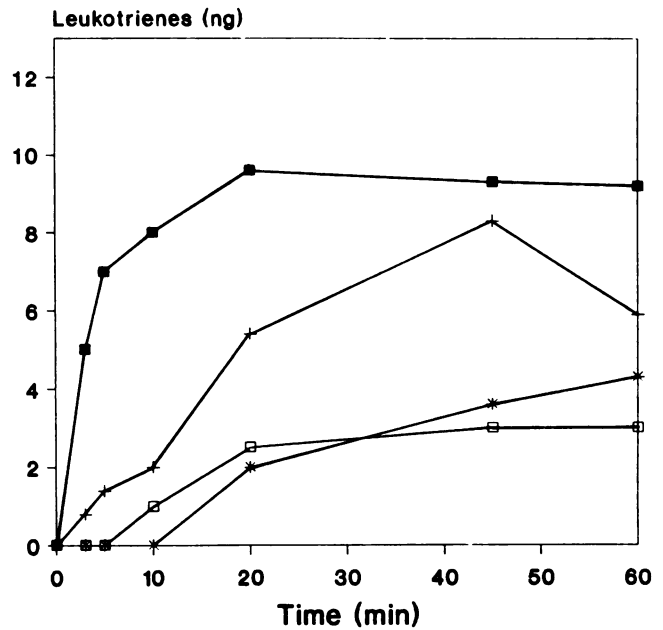

FIG. 4. Kinetics of leukotriene generation from human PMNs (1 × 10⁷) by *P. aeruginosa* 582f. Washed bacteria (5 × 10⁸) were incubated with PMNs over various times. LTB_4 (■), $20\text{-}OH\text{-}LTB_4$ (+), $20\text{-}COOH\text{-}LTB_4$ (*), and LTC_4 (□) were determined as mean values of triplicate determinations. SEM was between 3 and 12%.

Dose-response studies were carried out with amounts of washed bacteria ranging from 1×10^7 to 2×10^9 cells to study the generation of leukotrienes (Table 2). High amounts of LTB_4 (27.7 ng), $20\text{-}OH\text{-}LTB_4$ (37.1 ng), $20\text{-}COOH\text{-}LTB_4$ (12.4 ng), and LTC_4 (3.0 ng) were released from human PMNs by 5×10^8 bacteria. An increase of bacteria to 2×10^9 cells resulted in similar values for LTB_4 (23.2 ng) and pronounced reductions of $20\text{-}OH\text{-}LTB_4$ (10.2 ng), $20\text{-}COOH\text{-}LTB_4$ (1.5 ng), and LTC_4 (0.9 ng).

Kinetics of leukotriene release were then determined; washed bacteria of strain 582f were incubated with human PMNs (10^7) over various times, and the generation of LTB_4, its omega-oxidation products, and LTC_4 were monitored (Fig. 4). After 3 min of incubation, about 5.0 ± 0.4 ng of LTB_4 was detected and negligible amounts of $20\text{-}OH\text{-}LTB_4$ and $20\text{-}COOH\text{-}LTB_4$ were obtained; after 20 min of incubation, LTB_4 amounted to 9.6 ± 0.4 ng, $20\text{-}OH\text{-}LTB_4$ amounted to 5.4 ± 0.4 ng, $20\text{-}COOH\text{-}LTB_4$ amounted to 2.4 ± 0.8 ng, and LTC_4 amounted to 2.3 ± 0.6 ng. With longer incubation times (up to 60 min), no changes in LTB_4 levels were seen.

Our analysis was then extended to nine clinical isolates (Table 3). Among these isolates, four bacterial strains induced histamine release from 14 to 35%, while all bacterial culture supernatants released histamine from 46 ± 2 to 89 ± 3%. Heating of the bacterial culture supernatant suppressed the capacity of several strains to trigger histamine release, most notably strains B21, B10, 582f, B1, and B2. When the supernatants of strains 6816A, B18, and B5 were studied, heat treatment had no effect, but a slight reduction was obtained when the bacterial supernatant of strain B22 was heated. Its histamine-inducing capacity decreased from 77 ± 6 to 51 ± 3%.

Among the nine clinical isolates, only those strains expressing bacterium-bound PLC activity led to leukotriene formation from neutrophils. In these strains, LTB_4 ranged

TABLE 3. Induction of mediator release from rat peritoneal mast cells and human PMNs by *P. aeruginosa* bacteria and bacterial culture supernatants

P. aeruginosa strain	Stimulus	Histamine (%) from RPMC[a] (mean ± SEM)	Leukotrienes (ng) from PMNs (mean ± SEM)			
			LTB$_4$	20-OH-LTB$_4$	20-COOH-LTB$_4$	LTC$_4$
B21	Bacteria	14 ± 1	5.5 ± 0.8	9.7 ± 2.1	3.6 ± 1.5	1.4 ± 0.6
	Supernatant	71 ± 6 (16 ± 2)	0	0	0	0
B10	Bacteria	35 ± 1	4.6 ± 1.0	14.8 ± 1.0	5.0 ± 0.9	1.5 ± 0.6
	Supernatant	63 ± 7 (20 ± 1)	4.5 ± 1.0	0	0	2.2 ± 1.0
582f	Bacteria	29 ± 2	5.3 ± 0.5	10.7 ± 1.6	4.1 ± 0.6	3.2 ± 1.1
	Supernatant	46 ± 2 (0)	4.1 ± 0.8	0	0	1.6 ± 0.3
6816A	Bacteria	0	0	0	0	0
	Supernatant	96 ± 3 (97 ± 1)	0	0	0	0
B18	Bacteria	19 ± 2	6.7 ± 0.6	17.6 ± 9.1	6.4 ± 1.4	2.2 ± 0.8
	Supernatant	83 ± 2 (76 ± 4)	0	0	0	0
B1	Bacteria	0	0	0	0	0
	Supernatant	67 ± 6 (23 ± 1)	0	0	0	0
B2	Bacteria	0	0	0	0	0
	Supernatant	54 ± 5 (18 ± 3)	0	0	0	0
B5	Bacteria	0	0	0	0	0
	Supernatant	89 ± 3 (93 ± 5)	0	0	0	0
B22	Bacteria	0	0	0	0	0
	Supernatant	77 ± 6 (51 ± 3)	0	0	0	0

[a] Numbers in parentheses are results obtained with heat-treated culture supernatant. RPMC, Rat peritoneal mast cells.

from 4.6 ± 1.0 to 6.7 ± 0.6 ng, and LTC$_4$ ranged from 1.4 ± 0.6 to 3.2 ± 1.1 ng (Table 3). The levels of 20-OH-LTB$_4$ exceeded those of 20-COOH-LTB$_4$ by about two- to three-fold.

Furthermore, the culture supernatants of strains B10 and 582f obtained after 24 h also induced significant amounts of leukotrienes: 4.5 ± 1.0 ng of LTB$_4$ and 2.2 ± 1.0 ng of LTC$_4$ for strain B10 and 4.1 ± 0.8 ng of LTB$_4$ and 1.6 ± 0.3 ng of LTC$_4$ for strain 582f. These strains expressed no or low amounts of heat-stable hemolysin activity. The bacterial culture supernatants of the additional strains did not induce leukotriene formation.

Effect of defined pathogenicity factors from *P. aeruginosa* on inflammatory-mediator release. The question arose whether the exotoxin A also contributes to mediator release from rat mast cells and human PMNs. To answer this, *P. aeruginosa* PAO 1 (exotoxin A+) and PAO T1 (exotoxin A−) were analyzed. Washed bacteria after 6 h of growth expressed insignificant amounts of cell-bound hemolysin (up to 6%) and PLC activities (Table 4); however, the bacterial culture supernatants obtained after 24 h of growth induced high histamine release (56 and 57%) and expressed hemolysis up to 73%. Heating did not destroy the hemolysin activity within the bacterial culture supernatant. Neither the bacteria nor the bacterial culture supernatant induced leukotriene formation.

TABLE 4. Induction of hemolysin, PLC activity, and mediator release from rat peritoneal mast cells and human PMNs by *P. aeruginosa* PAO 1 (exotoxin A+) and PAO T1 (exotoxin A−)[a]

P. aeruginosa strain	Stimulus	Histamine (%) (mean ± SEM)	Hemolysis (%) (mean ± SEM)[b]	PLC activity (E_{405})
PAO 1	Bacteria	3 ± 1	6 ± 2	0.06
	Supernatant	56 ± 3	73 ± 4 (75 ± 3)	0.05
PAO T1	Bacteria	4 ± 1	5 ± 1	0.06
	Supernatant	57 ± 4	69 ± 4 (70 ± 4)	0.07

[a] No leukotriene production was observed with either bacteria or bacterial culture supernatant.

[b] Numbers in parentheses are results obtained with heat-treated culture supernatant.

Heat-stable hemolysin has been recently identified as heat-resistant glycolipid (20). The purified glycolipid in concentrations from 2 to 100 μg was incubated with either human PMNs for 20 min or rat mast cells for 60 min (Fig. 5). It was apparent that 2 μg of glycolipid only induced small amounts of histamine, whereas with 10 μg of glycolipid almost 95 ± 4% of the total histamine content was obtained. The cells assayed for viability were trypan blue positive. At the highest concentration of the glycolipid (100 μg), the release of lactate dehydrogenase from human PMNs increased from 4 to 16%, and the release of β-glucuronidase increased from 4 to 83%. At no concentration of the glycolipid was leukotriene formation obtained (data not shown).

DISCUSSION

Our data demonstrate that *P. aeruginosa* produces both heat-stable and heat-labile mediators of histamine and leukotriene release from mast cells and granulocytes. We believe that these mediators may represent hemolysins for the following reasons. (i) Hemolysins produced by *P. aeruginosa* are also heat stable and heat labile. (ii) Mediators of histamine and leukotriene release are expressed concurrently with hemolysins during bacterial growth. (iii) The purified heat-stable hemolysin (glycolipid) induces histamine release. However, actual proof that the major mediators of histamine and leukotriene release are hemolysins must await their purification and characterization.

The heat-labile hemolysin of *P. aeruginosa* obtained from the late logarithmic growth phase is cell associated and can be detected within the culture supernatant during the stationary growth phase.

In contrast to results of previously published studies (39), clinical isolates expressed cell-bound PLC activity. Four of the nine strains investigated showed a cell-bound hemolysin and PLC activity during the late logarithmic growth phase. The culture supernatants of all strains except 6816A contained PLC activity during the stationary growth phase. There may be two different modes of PLC expression: cell-bound activity with or without subsequent secretion, or non-cell-bound and only extracellular PLC activity. Vasil et al. (40) cloned the PLC gene into *Escherichia coli*, which
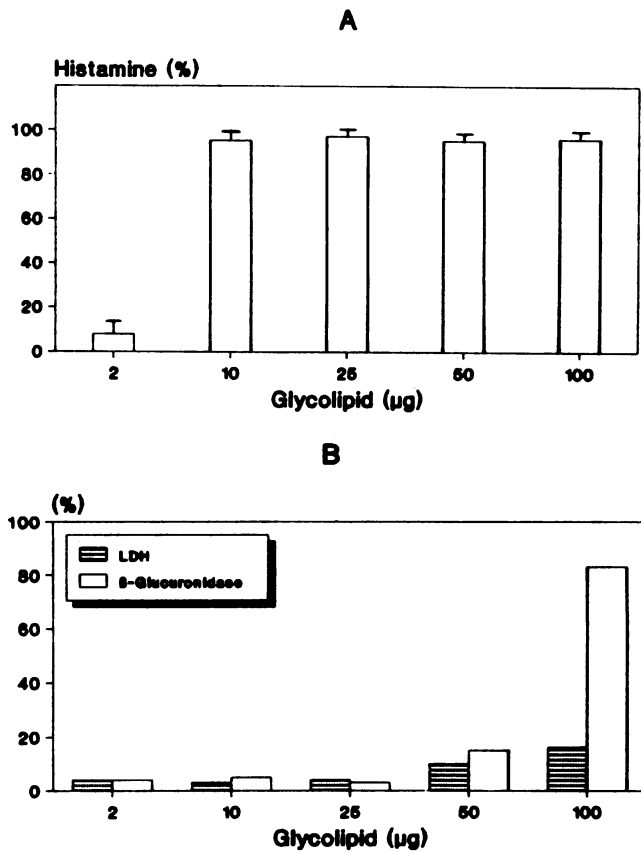
A

Histamine (%)

B

(%)

FIG. 5. Effect of purified glycolipid (heat-stable hemolysin) on various cells. (A) Induction of histamine release from rat peritoneal mast cells (3×10^4). (B) Lactate dehydrogenase and β-glucuronidase release from human PMNs (10^7). Histamine and enzyme release experiments were performed as described in the text. Data represent mean values of triplicate determinations. SEM was between 5 and 14%.

then produced a cell-bound PLC that was not secreted into the culture supernatant.

All strains expressing PLC activity induced mediator release from mast cells and PMNs. The histamine-inducing capacity depended on the bacterial concentration. Optimal release was observed with 5×10^7 to 1×10^8 bacteria. With higher bacterial counts, a remarkable decrease was observed which could be due to an inactivation or binding of histamine.

With the bacterial culture supernatant, maximal PLC activity was obtained after 20 h of incubation. In our experiments, histamine release was obtained with each bacterial culture supernatant. Heating of the bacterial supernatants induced in several cases a reduction in histamine release, indicating that in the majority of the strains at least two histamine-releasing principles are apparent, those which are heat stable and those which are heat labile.

The heat-labile component involved in histamine induction is trypsin sensitive and occurs concurrently with expression of PLC; thus, it appears likely that the PLC of *P. aeruginosa* participates in the cellular activation for histamine release from rat mast cells. Our further studies will be directed to analyze the purified PLC in the cellular models.

In the past, Bremm et al. (8, 9) and Scheffer et al. (36, 37) showed that bacteria and various bacterial exotoxins induce activation of granulocytes with subsequent production of

leukotrienes. Receptor-mediated as well as non-receptor-linked activation leads to the influx of calcium, the activation of cellular PLC, and the generation of arachidonic acid transformation products.

As is shown here, the pattern of leukotriene release induced by washed *P. aeruginosa* bacteria or culture supernatants is similar to that observed with the *E. coli* alpha-hemolysin (36, 37). More LTB_4 than LTC_4 was generated. In comparison, stimulation of PMNs with thiol-activatable toxins induced more LTC_4 than LTB_4. This fact has been attributed to the release of leukotriene-metabolizing enzymes, which transform the cysteinyl-leukotriene LTC_4 into LTD_4 and LTE_4 (8). An increase in bacteria to 5×10^8 led to an enhanced generation of omega-oxidation products which exceeded the amount of LTB_4 generation.

The heat-stable hemolysin activity, which has been identified as a glycolipid, was detected in bacterial culture supernatants of eight strains. These supernatants and the purified glycolipid were potent activators of mast cells. At higher concentrations, the glycolipid was toxic for the cells. On the other hand, the glycolipid did not trigger cells for leukotriene generation. Only culture supernatants with low hemolysis or without heat-stable hemolysis (strains B10 and 582f) induced leukotriene generation, depending on their PLC activities. The cytotoxic effect of the glycolipid may explain the failure of induction of leukotriene generation by culture supernatants containing both hemolysins.

A great deal of effort has been directed towards analyzing the pathogenicity factors of *P. aeruginosa* in both in vivo and in vitro models (12, 13, 25, 30, 33, 43). The PLC of *P. aeruginosa* was purified by Berk et al. (3), who isolated two different hemolytic and enzymatically active proteins. These enzymes induce in 24 h a wheal and flare reaction which lasts for 7 days. Increasing amounts initiate a necrotic lesion after 2 to 4 days and lead to abscesses (3). PLC induces platelet aggregation and seems to exert cytotoxic effects on macrophages and lymphocytes (3, 14). When added locally into mice, both enzymes induce paw edema, necrotic lesions, and formation of sterile abscesses (3, 25).

These findings might be caused by the release of inflammatory mediators. LTB_4 is a chemoattractant that supports the accumulation of neutrophil granulocytes. LTC_4, one part of the slow-reacting substance of anaphylaxis, is able to enhance vascular permeability leading to hyperemia and edema.

Quite recently it has been shown that perfusion of pig lungs with *P. aeruginosa* induces the generation of cyclo-oxygenase products (12). Sordelli et al. (38) found that *P. aeruginosa* induced accumulation of granulocytes in the lung when aerosolyzed to C5-deficient mice.

Patients suffering from cystic fibrosis complicated by recurrent *P. aeruginosa* infections showed enhanced levels of leukotrienes in their sputa (29); the accumulation of granulocytes in the lungs of these patients is a typical finding during *P. aeruginosa* infection. These clinical and experimental observations could be evoked by mediators released from inflammatory cells.

The release of inflammatory mediators is a physiological function of mast cells and PMNs. Both histamine and leukotrienes enable the inflammatory response of the host against bacterial infection. Nevertheless the uncontrolled release of these potent factors provokes pathophysiological local (abscess formation and tissue destruction) and systemic (increase of vascular permeability, vasodilatation, and shock) reactions. Therefore, the amount of active inflammatory mediators must be regulated.

The human neutrophil is a cell that controls the LTB_4 concentration in its environment by generating and metabolizing LTB_4. After active LTB_4 is bound, it is internalized and inactivated by omega oxidation (11). The dysregulation of this complex enzymatic system results in increased or decreased mediator concentration.

The granulocyte dysfunction in severely burned patients is accompanied by a reduced leukotriene generation (M. Köller, W. König, J. Brom, M. Raulf et al., in press; M. Köller, W. König, J. Brom, G. Erbs et al., in press), an enhanced omega oxidation (10), and a decreased LTB_4 receptor expression (J. Brom, M. Köller, W. Schönfeld, J. Knöller, G. Erbs, F. E. Müller, and W. König, Prostaglandins Leukotrienes Essent. Fatty Acids, in press). The influence of P. aeruginosa with regard to the inactivation of LTB_4 by omega oxidation may contribute to the dysfunction of neutrophils and thus facilitate the bacterial invasion. In this regard, PLC-deficient Pseudomonas strains showed a reduced lethality in the burned-mouse model (41). Furthermore, it has been shown that toxin-pretreated granulocytes of healthy donors revealed an enhanced omega-oxidation capacity for LTB_4 compared with non-toxin-treated normal cells (9).

Exotoxin A is a potent pathogenicity factor of P. aeruginosa. Exotoxin A suppresses T and B lymphocytes (18), is cytotoxic for macrophages, and inhibits the proliferation of progenitor cells (33). In addition, exotoxin A induces the proliferation of murine splenocytes and activates cytolytic T lymphocytes (43). In our studies, exotoxin A-producing and -nonproducing strains did not differ in their capacities to induce histamine release.

Both strains revealed minor or no PLC activity, and their supernatants expressed heat-stable hemolysin, histamine release up to 57%, and no leukotriene generation. PMNs exposed to exotoxin A show structural variation (e.g., pyknosis of nuclei) but no functional impairment (e.g., phagocytosis) (6). These findings correspond to those of our investigation concerning the interference with the generation of inflammatory mediators.

Thus, out data emphasize that preformed (histamine) as well as newly generated (e.g., leukotrienes) mediators are induced by various pathogenicity factors from P. aeruginosa and that a correlation exists between PLC content and leukotriene induction; in contrast, the glycolipid proved to be a potent and probably toxic inducer for histamine release and exerted its effects by a detergent-like activity. It is obvious that under in vivo conditions a variety of factors (e.g., proteases) may in addition modulate the release of inflammatory mediators. The variety of mediators may contribute to the development of acute and chronic inflammatory responses during P. aeruginosa infection.

ACKNOWLEDGMENTS

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. Bartell, F. B. 1983. Determinants of the biologic activity of surface slime in experimental Pseudomonas aeruginosa infections. Rev. Infect. Dis. 5(S5):971–978.

3. Berk, R. S., D. Brown, I. Coutinho, and D. Meyers. 1987. In vivo studies with two phospholipase C fractions from Pseudomonas aeruginosa. Infect. Immun. 55:1728–1730.

4. Berka, R. M., G. L. Gray, and M. L. Vasil. 1981. Studies of phospholipase C (heat-labile hemolysin) in Pseudomonas aeruginosa. Infect. Immun. 34:1071–1074.

5. Berka, R. M., and M. L. Vasil. 1982. Phospholipase C (heat-labile hemolysin) of Pseudomonas aeruginosa: purification and preliminary characterization. J. Bacteriol. 152:239–245.

6. Bishop, M. B., A. L. Baltch, L. A. Hill, R. P. Smith, F. Lutz, and M. Pollack. 1987. The effect of Pseudomonas aeruginosa cytotoxin and toxin A on human polymorphonuclear leukocytes. Med. Microbiol. 24:315–324.

7. 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):56–76.

8. Bremm, K. D., W. König, P. Pfeiffer, I. Rauschen, K. Theobald, M. Telestam, and J. E. Alouf. 1985. 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. Infect. Immun. 50:844–851.

9. Bremm, K. D., W. König, M. Thelestam, and J. E. Alouf. 1987. Modulation of granulocyte functions by bacterial exotoxin and endotoxins. Immunology 62:363–371.

10. Brom, J., W. König, M. Küller, W. Gross-Weege, G. Erbs, and F. E. Müller. 1987. Metabolism of leukotriene B_4 by polymorphonuclear granulocytes of severely burned patients. Protaglandins Leukotrienes Med. 27:209–225.

11. Brom, J., W. König, M. Stüning, M. Raulf, and M. Köller. 1987. Characterization of leukotriene B_4-omega-hydroxylase activity within human polymorphonuclear granulocytes. Scand. J. Immunol. 25:283–294.

12. Chin Lee, C., H. J. Sugerman, J. L. Tatum, T. P. Wriggt, P. D. Hirsch, and J. I. Hirsch. 1986. Effect of Ibuprofen on a pig Pseudomonas ARDS model. J. Surg. Res. 40:438–444.

13. Cicmanec, J. F., and I. A. Holder. 1979. Growth of Pseudomonas aeruginosa in normal and burned skin extract: role of extracellular proteases. Infect. Immun. 25:477–483.

14. Coutinho, I. R., R. S. Berk, and E. Mammen. 1988. Platelet aggregation by a phospholipase C from Pseudomonas aeruginosa. Thromb. Res. 51:495–505.

15. Engels, W., L. Endert, M. A. F. Kamps, and C. P. A. van Boven. 1985. Role of lipopolysaccharide in opsonization and phagocytosis of Pseudomonas aeruginosa. Infect. Immun. 49:182–189.

16. Franklin, A. L., T. Todd, G. Gurman, D. Black, P. M. Mankinen-Irvin, and R. T. Irvin. 1987. Adherence of Pseudomonas aeruginosa to cilia of human tracheal cells. Infect. Immun. 55:1523–1525.

17. Hingley, S. T., A. T. Hastie, F. Kuepper, M. L. Higgens, G. Weinbaum, and T. Shryok. 1986. Effect of ciliostatic factors from Pseudomonas aeruginosa on rabbit respiratory cilia. Infect. Immun. 51:254–262.

18. Holt, P. S., and M. L. Misfeldt. 1984. Alteration of murine immune response by Pseudomonas aeruginosa exotoxin A. Infect. Immun. 45:227–233.

19. Iglewski, B. H., and D. Kabat. 1975. NAD-dependent inhibition of protein synthesis by Pseudomonas aeruginosa toxin. Proc. Natl. Acad. Sci. USA 72:2284–2288.

20. Johnson, M. K., and D. Boese-Marrazzo. 1980. Production and properties of heat-stable extracellular hemolysin from Pseudomonas aeruginosa. Infect. Immun. 29:1028–1033.

21. Kharazmi, A., N. Hoiby, G. Döring, and N. H. Valerius. 1984. Pseudomonas aeruginosa exoprotease inhibit human neutrophil chemiluminescence. Infect. Immun. 44:587–591.

22. 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.

23. Kropinski, A. M., B. Jewell, J. Kuzio, F. Milazzo, and D. Berry. 1985. Structure and function of Pseudomonas aeruginosa lipopolysaccharide. Antibiot. Chemother. 36:55–73.

24. Kurioka, S., and P. V. Liu. 1967. Effect of the hemolysin of *Pseudomonas aeruginosa* on phosphatides and on phospholipase C activity. J. Bacteriol. 93:670–674.

25. Liu, P. H. 1965. The roles of various fractions of Pseudomonas aeruginosa in its pathogenesis. II. Effects of lecithinase and protease. J. Infect. Dis. 116:113–116.

26. Lutz, F. 1979. Purification of a cytotoxic protein from Pseudomonas aeruginosa. Toxicon 17:467–475.

27. Morrison, A. J., and R. P. Wenzel. 1984. Epidemiology of infections due to Pseudomonas aeruginosa. Rev. Infect. Dis. 6(S3):627–642.

28. Nicas, T. I., J. Bradley, J. E. Lochner, and B. H. Iglewski. 1987. The role of exoenzyme S in infections with Pseudomonas aeruginosa. J. Infect. Dis. 152:716–721.

29. O'Driscoll, B. R. C., O. Cromwell, and B. Kay. 1984. Sputum leukotrienes in obstructive airway diseases. Clin. Exp. Immunol. 55:397–404.

30. Ohman, D. E., R. P. Burns, and B. H. Iglewski. 1980. Corneal infections in mice with toxin A and elastase mutants of Pseudomonas aeruginosa. J. Infect. Dis. 142:547–555.

31. Pier, G. B. 1985. Pulmonary disease associated with Pseudomonas aeruginosa in cystic fibrosis: current status of the host-bacterium interaction. J. Infect. Dis. 151:575–580.

32. Pollack, M. 1984. The virulence of Pseudomonas aeruginosa. Rev. Infect. Dis. 6(S3):S617–S626.

33. Pollack, M., and S. E. Anderson, Jr. 1978. Toxicity of *Pseudomonas aeruginosa* exotoxin A for human macrophages. Infect. Immun. 19:1092–1096.

34. Pruitt, B. A., Jr., R. B Lindberg, W. F. McManus, and A. D. Mason. 1983. Current approach to prevention and treatment of Pseudomonas aeruginosa infections in burned patients. Rev. Infect. Dis. 5(S5):889–897.

35. Samuelsson, B. 1980. The leukotrienes: a group of biological active compounds including SRS-A. Trends Pharmacol. Sci. 19:227.

36. Scheffer, J., W. König, J. Hacker, and W. Goebel. 1985. Bacterial adherence and hemolysin production from *Escherichia coli* induces histamine and leukotriene release from various cells. Infect. Immun. 50:271–278.

37. Scheffer, J., K. Vosbeck, and W. König. 1986. Induction of inflammatory mediators from human polymorphonuclear granulocytes and rat mast cells by hemolysin positive and negative E. coli strains with different adhesins. Immunology 59:541–548.

38. Sordelli, D. O., M. C. Cerquetti, A. M. Hooke, and J. A. Bellanti. 1985. Effect of chemotactins released by *Staphylococcus aureus* and *Pseudomonas aeruginosa* on the murine respiratory tract. Infect. Immun. 49:265–269.

39. Stinson, M. W., and C. Hayden. 1979. Secretion of phospholipase C by *Pseudomonas aeruginosa*. Infect. Immun. 25:558–564.

40. Vasil, M. L., R. M. Berka, G. L. Gray, and H. Nakai. 1982. Cloning of a phosphate-regulated hemolysin gene (phospholipase C) from *Pseudomonas aeruginosa*. J. Bacteriol. 152:431–440.

41. Vasil, M. L., R. M. Berka, G. L. Gray, and O. R. Pavlovskis. 1985. Biochemical and genetic studies of iron-regulated (exotoxin A) and phosphate-regulated (hemolysin phospholipase C) virulence factors of Pseudomonas aeruginosa. Antibiot. Chemother. 36:23–39.

42. Woods, D. E., M. S. Schaffer, H. R. Rabin, G. D. Campbell, and P. A. Sokol. 1986. Phenotypic comparison of *Pseudomonas aeruginosa* strains isolated from a variety of clinical sites. J. Clin. Microbiol. 24:260–264.

43. Zehavi-Willner, T. 1988. Induction of cytolytic T lymphocytes by *Pseudomonas aeruginosa* exotoxin A. Infect. Immun. 56:213–218.