BACTERIAL PATHOGENICITY

Role of haemolytic and non-haemolytic phospholipase C from *Pseudomonas aeruginosa* in interleukin-8 release from human monocytes

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A massive accumulation of neutrophils, mainly due to enhanced interleukin-8 (IL-8) levels, is believed to contribute to the deleterious effects of *Pseudomonas aeruginosa* lung infection, e.g., in cystic fibrosis (CF). Antibodies to phospholipase C, an exoenzyme of *P. aeruginosa*, are detected early and at high levels in CF patients. However, *P. aeruginosa* produces at least two types of phospholipase C (PLC), one haemolytic (PLC-H) and the other non-haemolytic (PLC-N), both with mol. wts of c. 77 kDa. Experiments were performed to evaluate the potential contribution of *P. aeruginosa* PLC to neutrophil accumulation during infection. Therefore, *P. aeruginosa* PLC-H and PLC-N were compared with regard to IL-8 generation from human monocytes. Purified PLC-H as well as culture supernates (mol. wt > 50 kDa) of a *P. aeruginosa* strain capable of producing both PLC-H and PLC-N, and mutant strains deficient in the production of one or other phospholipase, or both, were examined. Purified PLC-H (only at low concentrations up to 1 unit/ 4×10^5 monocytes), induced a dose-dependent increase in IL-8 release and IL-8-specific mRNA expression over that of unstimulated cells (at 4-, 12- and 24-h incubation times). Higher concentrations of PLC-H led to a decrease in IL-8 release and IL-8-specific mRNA expression. These findings were confirmed by the results obtained with the supernates of cultures of mutant strains of *P. aeruginosa* PAO1 that produced either a PLC-H or PLC-N or neither. Stimulation and inhibition of IL-8 release and mRNA expression were associated with a culture supernate fraction of mol. wt > 50 kDa and containing PLC-H. These results contribute to the understanding of the role of both *P. aeruginosa* PLC in IL-8 generation during their interaction with human monocytes.

Introduction

Pseudomonas aeruginosa infections play an important role in burns and sepsis, as well as in cystic fibrosis (CF) [1–3]. In contrast to acute *P. aeruginosa* infections in burned patients, CF patients become chronically colonised by *P. aeruginosa* [3–5]. The pathogenesis of CF injury is associated with a massive accumulation of neutrophil granulocytes and neutrophil-mediated chronic pulmonary inflammation and destruction [4, 6, 7]. Although elevated levels of leukotriene B₄ – a potent chemotactic factor for neutrophils – have been found in the sputum of CF patients, it is now well established that the chemotactic cytokine

interleukin-8 (IL-8) is probably the main cause of the excessive neutrophil recruitment and activation because of its long-lasting effect [6, 8–10].

P. aeruginosa produces various virulence factors. Phospholipase C, an exoenzyme of *P. aeruginosa*, has been identified as a critical component in the pathogenesis of *P. aeruginosa* infection [11–13]. Antibodies to phospholipase C are detected early and at high levels in CF patients [14, 15]. However, *P. aeruginosa* produces at least two types of phospholipase C (PLC), one haemolytic (PLC-H) and the other non-haemolytic (PLC-N) [16–18]. The PLC-H with a mol. wt of 77 kDa haemolyses human and sheep erythrocytes and degrades not only phosphorylcholine but also sphingomyelin, which are key components of eukaryotic cell membranes. PLC-N, with a mol. wt of c. 78 kDa, is non-haemolytic for red blood cells and does not degrade sphingomyelin [16–18].

Supernates of bacterial cultures of *P. aeruginosa* containing PLC induced the generation of the chemotactic active leukotriene B₄ from human neutrophils and monocytes [2]. Similar results were obtained with partially purified PLC as described by Meyers *et al.* in in-vivo and in-vitro models [12]. The role of *P. aeruginosa* PLC in IL-8 generation has not been studied yet. Furthermore, previous studies did not distinguish between the two types of PLC with regard to their interaction with human effector cells. Current investigations regarding PLC activity by *Pseudomonas* have adopted a genetic approach. In this regard, the results of Graham *et al.* suggest that it is predominantly PLC-H that plays a role in the inflammation in the model of chronic lung infection in the rat [18, 19]. Studies performed by Wiener-Kronish *et al.* with isogenic *P. aeruginosa* PAO1 strains indicated that PLC is predominantly important in mediating injury to the alveolar epithelial barrier in acute *P. aeruginosa* pneumonia in rabbits [7]. PLC production enhanced virulence when tested in a model of burns in the mouse [7, 16].

The present study was designed to evaluate the relative inabilities of PLC-H and PLC-N to induce IL-8 release from human monocytes – the main producers of IL-8. The study used several previously characterised isogenic *P. aeruginosa* PAO1 strains that were deficient in the production of either PLC-H or PLC-N, or both; purified haemolytic PLC from *P. aeruginosa* was also used.

Materials and methods

Materials

Peptone, sodium metrizoate solution (75% w/v) and RPMI medium 1640 (no. 430-1800) were obtained from Gibco (Eggenstein, Germany). Additional chemicals were obtained from Sigma. The IL-8 antibodies were kindly provided by M. Ceska, Sandoz, Vienna, Austria.

Buffer

The buffer used for washing the peripheral blood mononuclear cells (PBMC) and monocytes was modified Dulbecco's phosphate-buffered saline (PBS) containing 137 mM NaCl, 8 mM Na₂HPO₄, 3 mM KCl and 3 mM KH₂PO₄, pH 7.4. For stimulation assays the cells were suspended in RPMI 1640 medium supplemented with fetal calf serum (FCS) 10%.

Bacterial strains

The bacterial strains used in the study are listed in Table 1. *P. aeruginosa* PAO1, expressing both PLC-H and PLC-N, was used as the wild-type strain. The following mutants were used. *P. aeruginosa* PAOΔ SR is a deletion mutant that retains PLC-N when measured

Table 1. Bacterial strains studied

Strains	Type of phospholipase C produced	References
<i>P. aeruginosa</i> PAO1 wild-type	PLC-H; PLC-N	16–18
<i>P. aeruginosa</i> PAO1ΔSR	PLC-N	16–18
<i>P. aeruginosa</i> PAO1 PLCN ^o	PLC-H	16–18
<i>P. aeruginosa</i> PAO1ΔSRN	None	16–18

on the synthetic substrate nitrophenylphosphorylcholine. In PAO1 PLCN^o only the gene PLC-N has undergone mutation; the PLC-H was still detectable. The double deletion mutant of PAO1, PAO1ΔSRN, in which both PLC structural genes have been deleted, lacked any detectable PLC activity in the nitrophenylphosphorylcholine assay [16–18].

Bacterial growth and isolation of culture supernates

Bacteria were cultured in peptone medium (NaCl 1%, peptone 1%, glycerol 1%). Tetracycline was added at a concentration of 20 µg/ml for *P. aeruginosa* strains PAO1ΔSR and PAO1ΔSRN. Ten ml of broth were inoculated with 100 µl of an overnight culture of the appropriate *P. aeruginosa* strain and incubated with shaking (180 rpm) at 30°C for 20–24 h until the early stationary growth phase. The culture supernates were filter sterilised and used at the indicated dilutions.

Preparation of *P. aeruginosa* culture supernate fractions

P. aeruginosa PAO1 or the PAO1 mutants were cultured in phosphate-deficient peptone medium [20] to induce the synthesis of PLC. Bacterial cells were removed by centrifugation (5000 g, 10 min; Sorvall RC-5B, rotor SS34) and the supernate was filter sterilised. Twenty ml of the original culture supernate (100 mM HEPES, 500 mM NaCl, 1 mM CaCl₂, pH 7.5) were concentrated to 2 ml; the original volume was made up with buffer and again concentrated. This procedure was carried out three times. The resulting solution was considered as 'undiluted'. To achieve the various culture supernate fractions concentrators (CentriplusTM, Amicon GmbH, Witten, Germany) with mol. wt cut-offs of 50, 30 and 5 kDa were used. The culture supernate above 50 kDa contained PLC activities as indicated in the *Results* section. The concentrated culture supernate fractions were stored at 4°C.

Preparation of purified *P. aeruginosa* PLC-H

PLC-H was produced for purification by overexpression of the *plcH* gene in a T7 expression system constructed for *P. aeruginosa* [21]. Cultures of *P. aeruginosa* ADD1976 (pADD3268:plcH) were grown in brain-heart infusion media to an OD₆₀₀ of c. 0.4, 1 mM IPTG was added to induce expression and

incubation was continued for 20 h. Bacteria were removed by centrifugation and 25 g of Whatman D52 anion exchanger washed in Tris buffer containing 50 mM NaCl, 50 mM Tris-HCl, pH 7.5 (Whatman Int., Maidstone) was added to each litre of supernate and the suspension was stirred overnight at 4°C. The DE52 with adsorbed protein was then collected by filtration, and washed twice with Tris buffer. Bound protein was eluted by two washes of buffer containing 500 mM NaCl, 50 mM Tris-HCl, pH 7.5, and then concentrated with $(\text{NH}_4)_2\text{SO}_4$ 70% w/v precipitation. The precipitate was collected by centrifugation and resuspended in Tris buffer. For final purification, portions of the concentrated protein solution were subjected to preparative gel electrophoresis with a BioRad model 491 electrophoresis apparatus (BioRad, Melville, NY, USA). Samples of the resuspended precipitate were resolved under conditions specified by the manufacturer, and collected fractions were examined for PLC-H activity. Several fractions containing the highest amount of PLC activity were pooled and analysed further. Five μg of protein from the pooled fractions as determined by absorbance at 280 nm were analysed by SDS-PAGE. Only a single band of 78 kDa was seen on the Coomassie-stained gel. The activity of PLC in the phosphatidylcholine assay, the haemolytic activity of PLC on human erythrocytes and the toxicity of PLC towards 3T3 fibroblasts cells (ATCC CLL 92) grown to confluence of this preparation were completely abolished by heating the PLC preparation at 100°C for 5 min; 100 ng of PLC-H was lethal for the 3T3 cells. No endotoxin was found to be associated with 1 μg of the pure PLC-H as determined by the Quantitative Chromogenic Limulus Amebocyte Lysate Assay (BioWhittaker, Walkersville, MD, USA).

Enzyme assays

The activity of PLC was determined spectrophotometrically ($\text{OD}_{420\text{ nm}}$) with *p*-nitrophenylphosphorylcholine as substrate [20]. One unit of activity (U) was defined as the amount of enzyme necessary to liberate 1 μmol of *p*-nitrophenol/min from *p*-nitrophenylphosphorylcholine at 37°C.

Preparation of monocytes

Human peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque sedimentation from venous blood anti-coagulated with ethylenediaminetetraacetic acid [22]. The isolated PBMC contained *c.* 15% monocytes as estimated by the non-specific esterase assay [23]. PBMC were incubated at a concentration of $1 \times 10^7/\text{ml}$ at 37°C in air with CO_2 5% in plastic flasks (Costar) for 2 h. The adherent cells were collected and 95% of the cells were esterase-positive by the esterase assay according to the method of Tucker *et al.* [23].

Cell viability

Cell viability was studied by trypan blue exclusion, by analysis of lactate dehydrogenase (LDH) release (Boehringer Mannheim, Mannheim, Germany), as well as by determination of mitochondrial activity with WST-1 (Boehringer Mannheim) in stimulated and unstimulated cells. The assays were performed as described by the manufacturer. All cellular stimulation experiments were performed under conditions of concentration and time in which the viability of the monocytes in all three assay systems was > 80%.

Cell culture conditions

Monocytes were suspended in RPMI medium plus FCS 10% at a concentration of $4 \times 10^5/\text{ml}$. The cells were cultured in the presence of the appropriate stimulus for up to 24 h. Culture supernates were collected and analysed for their IL-8 content; cell pellets were analysed for IL-8-specific mRNA expression.

IL-8 assay

IL-8 release was determined by a sandwich ELISA technique as described previously [24]. Briefly, each well of a 96-well plate (Nunc Maxisorb, Roskilde, Denmark) was pre-coated overnight at 4°C with 100 μl of Tween 20 0.1% in PBS containing a monoclonal anti-IL-8 antibody (subclone 4G9/A5/A7) at a concentration of 5 $\mu\text{g}/\text{ml}$. The plates were washed three times with PBS/Tween, the appropriate samples and the standard (a recombinant human IL-8; Calbiochem, Bad Soden, Germany) were added and incubation proceeded for 2 h at 37°C. Thereafter, polyclonal anti-IL-8 antibody labelled with alkaline phosphatase (3 $\mu\text{g}/\text{ml}$) was added. After addition of *p*-nitrophenylphosphate (15 mg/10 ml) an ELISA reader read the colour development and Mikrotek software (SLT Labinstruments, Crailsheim, Germany) was used to calculate the concentrations.

Detection of IL-8 mRNA expression by PCR

Total RNA was extracted from 4×10^5 cells with Trizol reagent (Gibco). Total RNA was analysed in 20 μl of H_2O . Expression of IL-8-specific mRNA was analysed after reverse transcription with oligo(dT) and PCR amplification of the cDNA transcripts. The reverse transcription step and the amplification steps were performed with M-MLV reverse transcriptase (Gibco) and Taq-Polymerase transcriptase (Gibco) according to the manufacturer's instructions. The primers were as follows: sense, 5'-CTTAGAT-GTCAGTGCATAAAGACATACTCC-3'; antisense, 5'-CTCAGCCCTCTTCAAAAACCTTCTCCACA-3'.

Statistical analysis

Unless stated otherwise, all data show mean values and SD of at least three individual experiments with cells

from different donors. Significance was examined by Student's *t* test for independent means.

Results

Modulation of IL-8 synthesis by purified PLC-H from P. aeruginosa

In a first set of experiments the capacity of purified PLC-H from *P. aeruginosa* to modulate IL-8 release from human monocytes was examined. Human monocytes (4×10^5) were stimulated with various concentrations of purified PLC-H at concentrations of 10, 5, 2.5, 1 and 0.2 U for 2, 4, 12 and 24 h at 37°C. The results are presented in Fig. 1 for IL-8 release and in Fig. 2 for IL-8-specific mRNA expression, both for an incubation time of 24 h. The data indicate that purified PLC-H exerts stimulatory as well as suppressive effects on the secretion and expression of IL-8 and IL-8 mRNA from human monocytes as measured by ELISA and RT-PCR. These effects were most pronounced after an incubation time of 24 h (kinetics not shown). In this

Fig. 1. Effects of purified *P. aeruginosa* haemolytic phospholipase C on IL-8 release (ng/ml) from human monocytes (4×10^5 /ml) stimulated with purified PLC from *P. aeruginosa* at the indicated concentrations for 24 h. Data are mean and SD from three independent experiments; *significant difference compared to unstimulated human monocytes (0 U PLC), $p < 0.05$.

Fig. 2. Effects of purified *P. aeruginosa* haemolytic phospholipase C on IL-8 mRNA expression in human monocytes (4×10^5 /ml) stimulated with purified PLC from *P. aeruginosa* at the indicated concentrations for 24 h. One typical experiment out of four independent experiments is shown. The arrow indicates the IL-8-specific product amplified by PCR.

regard PLC-H at low concentrations of 0.2 and 1 U/ 4×10^5 monocytes led to an increase in IL-8 release from human monocytes by up to 20 SD 4%; maximal induction of IL-8 was given by 1 U PLC (Fig. 1, $p < 0.05$). The results were confirmed by the mRNA level as shown in Fig. 2. Suppression of IL-8 production was detected at PLC levels above 1 U for PLC-H.

IL-8 release from monocytes after stimulation with P. aeruginosa culture supernates

Both PLC-H and PLC-N were present in the culture supernates of *P. aeruginosa* strains. To clarify their roles in IL-8 release from human monocytes, 10-fold concentrated culture supernates with a mol. wt > 50 kDa from *P. aeruginosa* PAO1 wild-type strain as well as from PAO1 mutants that express only PLC-N (PAO1ΔSR), only PLC-H (PAO1 PLCN^o) or no PLC (PAO1ΔSRN) were used. Human monocytes (4×10^5) were left untreated or were treated with dilutions of the appropriate culture supernates from undiluted (1 in 1) to dilutions of 1 in 2, 1 in 5, 1 in 10, 1 in 100 for 2, 4 and 24 h. The respective PLC activities of the undiluted and diluted culture supernate were 6, 3, 1, 0.5 and 0.05 U for PAO1 and for PAO1ΔSR, 3, 1, 0.5, 0.1 and 0.05 U for PAO1 PLCN^o, and no PLC activity for PAO1ΔSRN. Fig. 3 shows the results after an incubation time of 24 h: only culture supernates expressing PLC-H (PAO1 and PAO1 PLCN^o) led to a decrease in IL-8 release (Fig. 3a). In parallel to the decrease in IL-8 release a decrease in IL-8-specific mRNA expression (Fig. 3b) was observed in the corresponding monocyte pellets. The decrease in IL-8 production was observed in a time- and dose-dependent manner. In this regard, culture supernates containing PLC-H at concentrations > 1 U (PAO1 and PAO1 PLCN^o) diminished spontaneous IL-8 release; 3 U PLC-H of PAO1 PLCN^o reduced release to 0.6 SD 0.3 ng/ml compared with 78 SD 12 ng/ml from unstimulated cells. The inhibitory effects of PLC-H were observed after an incubation time of 4 h and were most pronounced after an incubation time of 24 h (Fig. 4). The data are presented for *P. aeruginosa* PAO1 in Fig. 4a and for *P. aeruginosa* PAO1 PLCN^o in Fig. 4b. In contrast to those containing PLC-H, culture supernates containing only PLC-N (PAO1ΔSR), even at the high concentration of 7 U, neither downregulated nor upregulated IL-8 release from human monocytes (data not shown). Culture supernates devoid of PLC activity (PAO1ΔSRN) also failed to modulate IL-8 release (data not shown).

Modulation of IL-8 release from human monocytes after stimulation with different fractions of P. aeruginosa culture supernates

The ability of additional exoproducts of *P. aeruginosa* to modulate IL-8 release from human monocytes was tested. For this purpose total culture supernates from

Fig. 3. Dose-dependent effects of haemolytic and non-haemolytic phospholipase C on IL-8 release from human monocytes (4×10^5) left untreated (unstimulated cells, $\square$), or cultured with undiluted (1:1; $\blacksquare$) or with diluted (1:2, 1:5, 1:10, 1:100; $\blacksquare$, $\square$, $\square$, $\square$) culture supernate (mol. wt cut-off > 50 kDa) of the appropriate *P. aeruginosa* strains (PAO1, PAO1ΔSR, PAO1 PLCN^o, PAO1ΔSRN). The respective PLC activities were 6, 3, 1, 0.5 and 0.05 U for PAO1 and for PAO1ΔSR; 3, 1.5, 0.5, 0.1 and 0.01 U for PAO1 PLCN^o; and no PLC activities for PAO1ΔSRN. The culture supernates and the cell pellets (monocytes) were isolated after 24 h. Supernates were assayed for IL-8 by ELISA (a) and the cell pellets for IL-8-specific mRNA expression (b). Data are mean and SD from three independent experiments; *significant difference compared to unstimulated cells, $p < 0.05$.

PAO1 as well as from the PAO1 mutants were fractionated and concentrated by ultrafiltration with defined filter pore sizes. Human monocytes (4×10^5) were stimulated with different dilutions ranging from

Fig. 4. Time-dependent effect of haemolytic and non-haemolytic phospholipase C on IL-8 release from human monocytes (4×10^5) left untreated (unstimulated cells, $\square$) or cultured with undiluted (1:1; $\blacksquare$) or with diluted (1:2, 1:5, 1:10, 1:100; $\blacksquare$, $\square$, $\square$, $\square$) culture supernate (mol. wt cut-off > 50 kDa) of (a) *P. aeruginosa* PAO1 and (b) PAO1 PLCN^o. The respective PLC activities were 6, 3, 1, 0.5 and 0.05 U for PAO1 and 3, 1, 0.5, 0.1 and 0.01 U for PAO1 PLCN^o. The culture supernates were isolated after 2, 4 and 24 h and assayed for IL-8 by ELISA (triplicate determination). Data are mean and SD from five independent experiments; *significant difference compared to unstimulated cells, $p < 0.05$.

undiluted (1 in 1) to 1 in 100 of the various fractions that contained components > 50 kDa; 30–50 kDa; 5–30 kDa and < 5 kDa for 2, 4, 12 and 24 h. The respective PLC values for the fractions above mol. wt 50 kDa were 6, 3, 1, 0.5 and 0.05 U for PAO1 and for PAO1ΔSR; 3, 1.5, 0.5, 0.01 and 0.01 U for PAO1 PLCN^o; PAO1ΔSRN showed no PLC activity. The fractions with components below 50 kDa showed no PLC activities. For an incubation time of 24 h, only the fractions with components > 50 kDa from PAO1 (Fig. 5a) and PAO1 PLCN^o (Fig. 5b), but not from PAO1ΔSR (Fig. 5c) or PAO1ΔSRN (Fig. 5d), modulated IL-8 release from human monocytes. Similar results were obtained for incubation times < 24 h (data not shown). Thus, LPS (a stimulus for IL-8 release) was absent or a minor component in the culture supernates from the early stationary phase. The inhibitory effects on IL-8 release were not due to an increase in trypan blue staining, LDH release or a decrease in mitochondrial activity. The results of determination of IL-8-specific mRNA expression also showed modulation by components > 50 kDa from PAO1 (Fig. 6).

Fig. 5. Effects of *P. aeruginosa* culture supernate fractions on IL-8 release from human monocytes (4×10^5) left untreated (unstimulated cells; ▨) or stimulated with undiluted (1:1; ■) or with dilutions (1:5; 1:100; ■, □) of the various culture supernate fractions whose whole mol. wt cut-off points ranged from > 50, 30–50, 5–30, to < 5 kDa of *P. aeruginosa* strains (a) PAO1, (b) PAO1ΔSR, (c) PAO PLCN° and (d) PAO1ΔSRN. The culture supernates were isolated after 24 h and assayed for IL-8 by ELISA (triplicate determination). Data are mean and SD from three independent experiments; *significant difference compared to unstimulated cells, $p < 0.05$.

Fig. 6. Effects of *P. aeruginosa* PAO1 culture supernate fractions on IL-8 mRNA expression in human monocytes (4×10^5) stimulated with undiluted (1:1) or with dilutions (1:5; 1:100) of the various culture supernate fractions whose mol. wt cut-off points ranged from > 50, 30–50, 5–30, to < 5 kDa of *P. aeruginosa* strain PAO1. The cell pellets were assayed for IL-8 mRNA expression by PCR. One representative experiment out of three is shown.

Discussion

P. aeruginosa infections play an important role in burns and sepsis as well as in CF [1–3]. The pathogenesis of lung injury in CF patients colonised by *P. aeruginosa* is especially associated with a massive accumulation of neutrophil granulocytes and neutrophil-mediated chronic pulmonary inflammation and destruction [4, 25]. Arachidonic acid metabolites such as leukotriene B₄ (LTB₄) and 12-hydroxyeicosatetraenoic acid (12-HETE) as well as the cytokine IL-8 are potent chemotactic factors for neutrophils [20, 26, 27]. Although elevated LTB₄ levels have been found in the sputum of CF patients, it is now established that IL-8 is probably the main cause of the excessive neutrophil recruitment and activation because of its long lasting effect [8–10]. It has been shown that PLC induces the chemotactically active LTB₄ and 12-HETE [2, 20]. Therefore, the hypothesis that PLC could contribute to inflammation by eliciting IL-8 seemed to be reasonable. Moreover, the description of two types of *P. aeruginosa* PLC, one haemolytic and the other non-haemolytic, opened up speculation as to the role of both types of PLC during infection. The present study used human monocytes, the main producer of IL-8.

The data clearly show a stimulating effect of PLC-H on

IL-8. The effects on IL-8 release induced by PLC-H were observed at concentrations that induce only negligible amounts of LTB₄. However, PLC concentrations that induced the generation of high amounts of LTB₄ led to a decrease in IL-8 and IL-8 mRNA production [28]. These data were supported by the use of mutant *P. aeruginosa* PAO1 strains capable of producing only one kind of PLC or none. In this regard, upregulation and inhibition of IL-8 release were associated with a supernate fraction of > 50 kDa. Only strains that secrete PLC-H produced the high mol. wt inhibitor; the remaining supernate fractions with components < 50 kDa did not trigger IL-8 release from the cells. In a recent study, a *P. aeruginosa* culture supernate component of < 1 kDa appeared to be a potent stimulator of IL-8 for epithelial cells [29]. This factor was not apparent under the conditions used in the present study; culture supernate components of mol. wt < 5 kDa did not induce IL-8 release from human monocytes. Until now the interaction of PLC-N with inflammatory cells has not been elucidated. PAO1 mutant strains with a deleted haemolytic PLC gene exhibited a marked reduction in virulence when tested in the burn mouse model [13, 18]. The present data support these findings and show that PLC-N failed to modulate IL-8 release.

These observations are not in contrast to the pathological picture observed in CF. While there is no doubt that PLC is important in initial *P. aeruginosa* infection because of inflammatory mediator release (enzymes, histamine, LTB₄, oxygen metabolites) the role of *P. aeruginosa* PLC-H in chronic infection must await further studies. *P. aeruginosa* isolates from CF patients at the chronic infection phase showed a decreased production of specific virulence determinants such as exotoxin A, exoenzyme S, phospholipase C and lipase [4, 7]. However, at present there is no information on the time course of LTB₄ or IL-8 production in acute and chronic infection. From these results a pathogenic role may be ascribed to PLC-H at least for the acute phase of *P. aeruginosa* infection [7, 25]. Previous studies have shown that antibodies to PLC are detected early and at high levels in CF patients [14, 15]. It seems unlikely that biologically active *P. aeruginosa* PLC plays any role in chronic *P. aeruginosa* infection; however, there is no information about the neutralising capacity of these antibodies. Furthermore, PLC-antibody complexes may damage the host by Fc and complement-mediated pathophysiological reactions. The role of PLC-N for such a versatile pathogen as *P. aeruginosa* requires further studies.

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