Pseudomonas aeruginosa
Eicosanoid-mediated proinflammatory activity of
Pseudomonas aeruginosa ExoU

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Summary

As Pseudomonas aeruginosa ExoU possesses two functional blocks of homology to calcium-independent (iPLA2) and cytosolic phospholipase A2 (cPLA2), we addressed the question whether it would exhibit a proinflammatory activity by enhancing the synthesis of eicosanoids by host organisms. Endothelial cells from the HMEC-1 line infected with the ExoU-producing PA103 strain exhibited a potent release of arachidonic acid (AA) that could be significantly inhibited by methyl arachidonyl fluorophosphonate (MAFP), a non-selective COX inhibitor. Our results suggest that ExoU may contribute to P. aeruginosa pathogenesis by inducing an eicosanoid-mediated inflammatory response of host organisms.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that can colonize and infect virtually any human mucosal surface as well as invade tissues and blood of patients with an underlying injury or disease. Because of the seriousness of P. aeruginosa infections, and the bacterial natural resistance to antimicrobial agents, there has been a considerable interest in elucidating the pathogenesis of P. aeruginosa infections.

A number of virulence proteins produced by P. aeruginosa that contribute to its pathogenesis have been characterized (Winzer and Williams, 2001). Prominent among these virulence determinants are the proteins of the type III secretion system (TTSS). This bacterial secretory system is conserved in many Gram-negative pathogens and encodes on the order of 20 proteins assembled into a complex to secrete and translocate effectors directly into the host cells, where they subvert cell defence and signalling systems (reviewed in Hueck, 1998). To date, four TTSS effector molecules – ExoS, ExoT, ExoU and ExoY – have been identified in P. aeruginosa isolates, although few, if any, isolate, secrete all four of them. Interestingly, an inverse correlation has been noted between the presence of the exoU and exoS genes (Feltman et al., 2001). ExoS, ExoT and ExoY have clearly defined enzymatic activities, cause in vitro cell rounding and detachment and may contribute to infection by inhibiting or preventing bacterial uptake and phagocytosis (reviewed by Frank, 1997). The other protein delivered by the TTSS, ExoU, was first identified by its virulence in animal models of pneumonia (Kudoh et al., 1994; Alllewelt et al., 2000), and its rapid and potent cytotoxic activity towards different types of mammalian cells (Finck-Barbançon et al., 1997; Hauser et al., 1998; Hauser and Engel, 1999; Vallis et al., 1999). Recent stud-
ies have shown that the in vitro cytotoxic activity of ExoU is of clinical significance. Production of this toxin was shown to be a marker for highly virulent P. aeruginosa isolates obtained from patients with hospital-acquired pneumonia (Schulert et al., 2003) and bacteraemia (Berthelot et al., 2003).

In an effort to understand the mechanism by which ExoU is cytotoxic to eukaryotic cells, Sato et al. (2003) used controlled expression of the toxin in Saccharomyces cerevisiae hosts. ExoU was shown to be toxic to the yeasts and to cause a vacuolar fragmentation phenotype, leading to the hypothesis that the toxin encodes an enzymatic activity resulting in host cell membrane disruption. In tone with this hypothesis, inhibitors of human calcium-independent (iPLA_2_) and cytosolic phospholipase A_2_ (cPLA_2_) reduced strongly the ExoU cytotoxicity. Moreover, the catalytic domains of iPLA_2_ and cPLA_2_ and patatin, a plant storage glycoprotein that has lipid acyl transferase and hydrolase activities, were shown to be similar to ExoU sequences, further suggesting that ExoU cytotoxicity was related to its lipase activity. Finally, ExoU was shown to exhibit an acyl hydrolase activity that encompasses a broad range of substrates, including neutral lipids and phospholipids.

Independently and in parallel studies, Phillips et al. (2003) reported the PLA_2_2-like activity of ExoU. Consistent with this activity, the cytotoxicity induced by microinjection of purified toxin into CHO cells or by cell infection with the ExoU-producing P. aeruginosa PA103 strain could be blocked by inhibitors of the mammalian iPLA_2_ and cPLA_2_ enzymes. It was also shown that microinjecting ExoU into CHO cells resulted in the release of a large amount of free arachidonic acid.

PLA_2_ belongs to a large family of phospholipid-hydrolising enzymes that have a biological role in the regulation of lipid metabolism, phospholipid remodelling, membrane disruption and signal transduction as well as in inflammatory response (reviewed in Six and Dennis, 2000). The enzyme hydrolyses the sn-2 ester bond of glycerophospholipids and releases lysophospholipids and unsaturated fatty acids, such as arachidonic acid, from mammalian cell membranes. Arachidonic acid can be important as storage of cell energy (Six and Dennis, 2000). More importantly, it can also function as a second messenger and act as the precursor of eicosanoids, including the cyclooxygenases (COX)-derived prostaglandins and thromboxanes and the lipooxygenases-derived leukotrienes, lipoxins, hydroxyeicosatetraenoic acids (HETES) and hydroperoxyeicosatetraenoic acids (HPETES). The eicosanoids possess a wide spectrum of potent biological activities, among which is their ability to mediate a number of phenomena associated with inflammatory reactions.

For the last several years, our laboratory has examined the interaction of P. aeruginosa with human endothelial cells in order to determine the role of endothelium as a bacterial target in the course of P. aeruginosa-disseminated infections (Plotkowski et al., 1994; Assis et al., 2000; 2004; Valente et al., 2000; Saliba et al., 2002). Bacterial toxin-mediated effects on endothelial cells have been implicated in the pathogenesis of septic shock and this may be related to the induced stimulation of the arachidonic acid pathway in the vessel wall or to frank damage of the endothelium (Suttorp et al., 1985). Scattered infectious vasculitis and microscopic abscesses in many organs are common consequences of P. aeruginosa bloodstream infections (Teplitz, 1965), but the exact role of bacterial exoproducts in the associated inflammatory reaction has not yet been determined.

In the present study we investigated whether ExoU would modulate the production of eicosanoid mediators by infected endothelial cells, thereby favouring the inflammatory response usually associated with P. aeruginosa infections.

Results

Infection by ExoU-producing P. aeruginosa induced a rapid release of arachidonic acid from human endothelial cells

An initial set of experiments was designed to compare the ExoU-producing PA103 P. aeruginosa strain with its isogenic mutant PA103DexoU, defective in ExoU synthesis, in their capability to induce the release of radiolabelled arachidonic acid previously incorporated into the membranes of endothelial cells of the HMEC-1 line (Ades et al., 1992). A high amount of radioactivity was detected in the supernatants of cells infected with the parental strain at 1 h post-infection whereas virtually no radioactivity was detected in the supernatant of cells infected with the mutant or of control non-infected cells (Fig. 1A). At 20 h post-infection, a significant increase in the release of arachidonic acid from cells infected with the ExoU-deficient bacteria and from control cells was observed but the radioactivity detected in the supernatants of PA103-infected cells was still significantly higher. To confirm that the cell supernatant radioactivity corresponded to free arachidonic acid, and not to radiolabelled phospholipid-containing membranes of cell debris, lipid composition of the supernatants of infected and non-infected cultures was analysed by thin layer chromatography (TLC). The results shown in Fig. 1B confirmed the arachidonic acid release by PA103-infected endothelial cells. Figure 1C shows that methyl arachidonyl fluorophosphonate (MAFP), an irreversible inhibitor of cPLA_2_, known to inhibit the ExoU-mediated cytotoxicity (Phillips,

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ExoU-producing *P. aeruginosa* induced a significant release of PGE$_2$ and PGI$_2$ by infected endothelial cells

As free arachidonic acid may be metabolized by one of the isoforms of cyclooxygenases to PGH2, a substrate for terminal synthases of prostanoid biosynthesis, we next investigated whether infection by the ExoU-producing *P. aeruginosa* strain would induce the overproduction of PGE$_2$ and PGI$_2$ (prostacyclin), reported to be the major arachidonic acid metabolites in the vascular endothelium (Suttorp et al., 1985). As shown in Fig. 2, endothelial cells infected with the parental bacterial strain produced significantly more prostanoids than control cells and than cells infected with the mutant strain. Interestingly, PGE$_2$ was released in higher amounts than PGI$_2$. This finding is in accordance with other studies showing that cells from the microvasculature can release PGE$_2$ in excess of PGI$_2$ (Weksler et al., 1977; Gerritsen and Cheli, 1983).

To ascertain whether the ExoU ability to induce the prostanoid production resulted from its PLA$_2$ catalytic

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**et al., 2003; Sato et al., 2003**, reduced in about 90% the amount of free arachidonic in infected culture supernatants. No difference was detected in the amount of arachidonic acid released by MAFP-treated and untreated control cells.

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Fig. 1. A. Radiolabelled arachidonic acid detected by scintillation counting in the supernatants of control non-infected HMEC-1, of cells infected with the ExoU-producing PA103 *P. aeruginosa* and of cells infected with the PA103ΔexoU deficient mutant at 1 and 20 h post-infection.

B. Radiolabelled products recovered from the supernatants of control non-infected, of cell cultures infected with the parental PA103 *P. aeruginosa* strain and of cells infected with the deficient mutant at 1 h post-infection, analysed on silica gel TLC plates, in areas corresponding to the standard arachidonic acid (AA) or phospholipids (PL).

C. Inhibitory effect of MAFP on the release of arachidonic acid by cells infected with the ExoU-producing PA103 strain for 1 h and by control non-infected cells.

Data represent mean values of typical experiments out of two, performed in triplicate or quadruplicate. Bars represent SD. $P < 0.01$ (**) and $P < 0.001$ (***) when data obtained with PA103-infected cells were compared with data from control or from PA103ΔexoU-infected cells.

Fig. 2. PGE$_2$ (A) and PGI$_2$ (B) detected in the supernatants of control non-infected and of cell cultures infected with the ExoU-producing PA103 *P. aeruginosa* or with the PA103ΔexoU mutant at 24 h post-infection. (C) PGE$_2$ release by control non-infected cells and by cells infected with different mutants, taking the release of PA103-infected cells as 100%. Data represent mean values of typical experiments out of two performed in triplicate. Bars represent standard errors of the mean (SEM). $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***) when data from PA103-infected cells (A and B) or from PA103ΔUT/exoU-infected cells (C) were compared with those from control cells or from cells infected with the other mutants.
activity, studies were performed with HMEC cells infected with the ExoU-deficient PA103ΔexoU mutant and with bacteria complemented either with wild-type exoU (PA103 ΔUT/exoU), which secretes functionally active toxin, or with bacteria complemented with site-directed mutated exoU serine catalytic motif (PA103 ΔUT/S142A; Rabin and Hauser, 2005). As shown in Fig. 2C, complementation with the wild-type gene restored the bacterial capability to induce PGE₂ production by endothelial cells. In contrast, cells infected with bacteria complemented with the mutated gene (PA103 ΔUT/S142A) did not differ from those infected with the ExoU-deficient P. aeruginosa (PA103ΔexoU) in PGE₂ production.

**Infection by the ExoU-producing P. aeruginosa induced a marked inflammatory response in two murine experimental models**

In the first *in vivo* model used to assess the ExoU proinflammatory activity, mice footpads were infected with 10⁵ colony forming units (cfu) of PA103 or PA103ΔexoU whereas the footpads of the contralateral hind limbs were injected with a buffer. The toxin inflammatory activity was assessed by determination of the ratio of volume increase of infected to non-infected control limbs over time. Mice inoculated with the parental bacteria exhibited an early increase in the volume of the infected limbs that was significantly higher than the increase detected in mice infected with the mutant (Fig. 3A). Moreover, an important inflammatory reaction, containing mainly polymorphonuclear leucocytes, was detected in the footpad of PA103-infected mice. The infiltration of inflammatory cells was much milder in animals inoculated with the mutant (Figs 3B and 4).

The role of the PLA₂ catalytic motif in the proinflammatory activity of ExoU was assessed by injecting mice footpads with PA103 ΔUT/exoU and PA103 ΔUT/S142A followed by the determination of the ratio of volume increase of infected to non-infected control limbs. As shown in Fig. 3C, bacteria complemented with wild-type exoU induced an increase in the volume of the infected limbs similar to that induced by the parental PA103 P. aeruginosa, which was significantly higher than the increase induced by the ExoU-deficient PA103ΔexoU strain or by bacteria complemented with mutated exoU (PA103 ΔUT/S142A).

To assess the role of eicosanoids in the inflammatory response elicited by PA103, in other experiments, mice

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**Fig. 3.** Inflammatory reaction detected in mice footpads injected with PA103 or PA103ΔexoU P. aeruginosa.

A. Percentage of the increase of the volume of mice footpads detected after different periods after the bacterial injection, measured with a plethysmometer, compared with the volume of control footpads injected with diluent of the bacterial suspensions. *P* < 0.05 (*) and **P** < 0.01 (**) when mice infected with the parental bacteria were compared with those infected with the mutant.

B. Mean number of infiltrating inflammatory cells per microscopic fields (×20) of subcutaneous tissues of mice footpad. Data are means ± standard deviation obtained after quantification of 10 microscopic fields per microscopic slide (*n* = 3). ***P** < 0.001 when data obtained with PA103-infected mice were compared with those from PA103ΔexoU-infected mice.

C. Increase in the volume of mice footpads infected with different mutants taking the increase detected in footpads infected with the wild type PA103 strain as 100%. * and **P** < 0.05 and 0.01 when data obtained with PA103ΔUT/exoU-infected animals were compared with those from PA103ΔUT/S142A- and PA103ΔexoU-infected mice.
were inoculated with ibuprofen, an inhibitor of both COX 1 and COX 2, or with nordihydroguaiaretic acid (NDGA), an inhibitor of 5, 12 and 15 lipoxygenases, 1 h prior to the bacterial infection. Figure 5A and B show that both drugs inhibited significantly the increase in the volume of infected mice limbs.

In the second in vivo experimental model, mice were inoculated intratracheally with $10^4$ cfu of both bacteria or with lipopolysaccharide (LPS)-free PBS and 24 h later they were submitted to bronchoalveolar lavage (BAL). In parallel, animals from another group were treated with indomethacin, a non-selective COX inhibitor, 1 h prior to the infection with the PA103 P. aeruginosa strain. The total concentration of inflammatory cells in BAL fluids recovered from mice instilled with the parental bacteria was significantly higher than the concentrations detected in control mice, in animals infected with the ExoU-deficient bacteria as well as in animals previously treated with indomethacin (Fig. 6A). Whereas in infected animals most BAL cells were polymorphonuclear leucocytes, in control mice up to 90% of the cells were mononuclear. Figure 6B shows that the concentration of PGE$_2$ in BALs from PA103-infected mice was significantly higher than the concentrations in control non-infected and in indomethacin-treated animals. Finally, the lung histopathology showed a massive infiltration of inflammatory cells in the pulmonary parenchyma and in the airway lumen of animals infected with the ExoU-producing strain, which could be reduced when the animals were pretreated with indomethacin (Fig. 7). Cell infiltration was also detected in the lungs of animals infected with the mutant bacteria, but the inflammatory reaction was much milder.

Discussion

Phospholipases are a heterologous group of enzymes indispensable to the metabolism of eukaryotic cells, as they govern the turnover of lipids and the biogenesis of membranes. Similar enzymatic activities also release precursors of lipid second messengers, accounting for the role of these enzymes in cell signal transduction pathways.

Phospholipases are also produced by a diverse group of bacterial pathogens, playing an important role as viru-
The best characterized phospholipases that play a role in bacterial virulence are phospholipases C (PLCs) (reviewed in Schmiel and Miller, 1999), but bacterial phospholipases A (PLAs) can be as cytolytic as PLCs (Winkler and Daugherty, 1989; Grant et al., 1997; Flieger et al., 2000). Phospholipid hydrolysis by PLAs results in the release of fatty acid from the position 1 or 2 of the glycerol backbone. As the arachidonic acid is the fatty acid most commonly found in position 2 of mammalian phospholipids, bacterial PLA₂ has a great potential for stimulation of the arachidonic acid cascade. Nevertheless, this potential has not been as well studied nor was its role in disease as well established as for other bacterial phospholipases (reviewed in Songer, 1997).

Parallel studies carried out by two independent groups have recently demonstrated that P. aeruginosa ExoU exhibits two small but functionally critical blocks of homology to cPLA₂ and iPLA₂ families of phospholipases (Phillips et al., 2003; Sato et al., 2003). Therefore, in the present study we addressed the question whether, besides its strong cytotoxic activity, ExoU would also exhibit a proinflammatory activity by enhancing the biosynthesis of eicosanoids by host cells. To gain insight into this possibility, we first compared the ExoU-producing PA103 strain with its isogenic mutant PA103ΔexoU, defective in the production of ExoU, in its capacity to release...
arachidonic acid from endothelial cell membranes. Cell infection by the parental strain was followed by the release of great amounts of arachidonic acid that could be blocked by MAFP, an irreversible inhibitor of PLA₂. In contrast, the release by MAFP-treated control non-infected cells remained untouched.

Free arachidonic acid is converted into PGH₂ via the action of COXs. PGH₂ is then converted to other prostanoids (PGD₂, PGE₂, PGF₂α, PGI₂ or thromboxane) by specific synthases (Harris et al., 2002). As the rate-limiting step in production of prostanoids is the intracellular liberation of arachidonic acid, and infection by the ExoU-producing PA103 P. aeruginosa resulted in the release of large amount of this fatty acid, we next investigated whether

Fig. 7. Light micrographs of lung sections from mice infected with the ExoU-deficient mutant (A and B), from untreated (C and D) and indomethacin-treated mice (E) infected with the parental bacteria, as well as from control non-infected mice (F). Note in A a sparse inflammatory infiltrate surrounding a blood vessel (*) and a bronchiole (**), and in B, inflammatory cells in the lumen of a bronchiole (**). In C, a dense inflammatory infiltrate surrounding blood vessels (*) and a bronchiole (**) is shown. Note in D a much denser collection of inflammatory cells in bronchiole lumen (**) and in adjacent alveolar spaces (***) of mice infected with the ExoU-producing bacteria. E shows a milder focal inflammatory infiltrate surrounding pulmonary blood vessels (*) of indomethacin-treated mice infected with the PA103 strain. No inflammatory infiltrate was detected in lung sections of control non-infected mice (F).
*P. aeruginosa* infection would overstimulate the release of PGE₂ and PGI₂, two major prostaglandins synthesized by endothelial cells (Weksler *et al.*, 1977; Gerritsen and Cheli, 1983). As hypothesized, cell cultures incubated with the parental PA103 strain produced significantly more PGE₂ and PGI₂ than did cells incubated with the defective strain and than control non-infected cells. Because endothelial prostaglandin secretion in the absence of exogenous arachidonate is dependent on PLA₂-controlled release of arachidonic acid from membrane phospholipids, our data suggest that the enhanced release of these prostanoids by infected cells has resulted from the PLA₂-like activity of ExoU, in similarity to the effect of the *Rickettsia prowazekii* PLA enzyme (Walker *et al.*, 1990).

Two isoforms of COX have been identified: COX-1, constitutively expressed in cells in which prostaglandins exert physiological functions, and COX-2, the expression of which is markedly induced in inflammation, which leads to the release of large amount of prostaglandins and thromboxane (reviewed in Smith and Langenbach, 2001). Bacterial products such as LPS (Akarsereenont *et al.*, 1995), peptidoglycan (Chen *et al.*, 2004), lipoteichoic acid (Lin *et al.*, 2001) and the *P. aeruginosa* autoducer N-acetyl-β-muramoyl-N-(3-oxododecanoyl) homoserine lactone (Smith *et al.*, 2002) are known to induce the expression of COX-2. We are therefore currently investigating whether the enhanced release of PGE₂ and PGI₂ by PA103-infected cells resulted merely from the increased disponibility of free arachidonic acid or whether ExoU could also induce the expression of COX-2 or even upregulate the expression of COX-1. This is so because studies on circulating monocytes exposed to bacterial LPS suggested that some increase in COX-1 expression can occur upon activation (McAdam *et al.*, 2000). In addition, studies using mice deficient in the expression of COX-1 or COX-2 have identified unique roles for COX-1 in the initiation of certain inflammatory responses (Tilley *et al.*, 2001). Therefore, it has been suggested that the capacity of COX-1 to modulate inflammatory responses should not be overlooked (Tilley *et al.*, 2001).

Besides COX, the enzyme that converts PGH₂ to PGE₂ also exists in two isoforms: a constitutive cytosolic PGH synthase (cPGES) and an inducible membrane-associated PGES (mPGES) (Jakobsson *et al.*, 1999). Recent evidence indicates that the mPGES is linked to the COX-2 enzyme whereas the cPGES is linked to the COX-1 enzyme (Murakami *et al.*, 2000; Tanioka *et al.*, 2000). As in our study the prostanoan release by *P. aeruginosa*-infected endothelial cells is resulted from the injection of ExoU into the host cell cytosol, it is likely that cPGES and COX-1, rather than mPGES and COX-2, have accounted for the enhanced production of PGE₂ by infected endothelial cells. However, these possibilities are also under current investigation.

The detection of the swelling of stimulated mice foot-pads is a simple experimental model that was used, in our study, to assess the in vivo significance of the inflammatory activity of *P. aeruginosa* ExoU observed in the in vitro assays. Our data clearly indicate that ExoU can activate both the COX and the lipooxygenase pathways because the footpad swelling, detected as early as 3 h after the bacterial injection, could be significantly abolished by inhibitors of those enzymes (Ibuprofen and NDGA respectively). Histopathologically, both the influx of granulocytes and oedema were strikingly more important in mice infected with the parental PA103 strain than in those infected with the defective mutant.

*Pseudomonas aeruginosa* can cause life-threatening respiratory infections in cystic fibrosis patients and a marked airway inflammation is a hallmark of these infections (Berger, 2002). Excitingly, in a recent paper, *P. aeruginosa* was shown to encode a lipoxygenase that converts arachidonic acid into HETE that, together with its downstream products, is known to have regulatory actions on immune and non-immune cells (Vance *et al.*, 2004), suggesting that bacteria may regulate the host-pathogen relationship. *P. aeruginosa* is also a leading agent of nosocomial pneumonia, frequently associated with sepsis and multiple organ failure (Aksamit, 1993). Experimental lung instillation of the ExoU-producing PA103 strain has been shown to induce a potent inflammatory response in the animal lungs, alveolar epithelial injury, progressive bacteraemia and septic shock (Kurashiki *et al.*, 1999).

To further elucidate the inflammatory characteristics of ExoU we next compared the parental PA103 and the defective PA103ΔexoU strains administered in low dose (10⁴ cfu) by airway instillation in mice. In this model we examined the inflammatory cell accumulation and the local release of PGE₂. BALs from mice inoculated with the ExoU-producing strain contained significantly more cells than BALs from control or PA103ΔexoU-infected mice, as well as higher PGE₂ concentration. In contrast, BALs from animals previously treated with the COX inhibitor indomethacin exhibited both total inflammatory cell and PGE₂ concentrations that did not differ from those detected in control non-infected mice, further confirming the role of eicosanoid-mediated proinflammatory activity of *P. aeruginosa* ExoU. Mice infected with the defective strain exhibited a reduced generation of PGE₂ but there was still an influx of neutrophils. One explanation is that other components of bacterial cell may have induced the release of proinflammatory cytokines by airway cells. In fact, in recent years, much attention has been paid to the role of Toll-like receptors (TLR) as pathogen-associated molecular pattern receptors for a number of microbial-derived molecules. Signalling through TLRs leads to activation of NF-κB, and NF-κB target genes that govern the
onset of mucosal inflammatory response following microbial infection (reviewed in Akira and Takeda, 2004). Epithelial respiratory cells express TLR2, TLR4 and TLR5 (Hertz et al., 2003; Muir et al., 2004), which signal the activation of NF-κB in response to bacterial lipoprotein and peptidoglycan (Lien et al., 1999), lipopolysaccharide (Lien et al., 2000) and flagellin (Hayashi et al., 2001) respectively. Therefore, one may hypothesize that the influx of inflammatory cells detected in the airways of mice infected with the ExoU-deficient strain may have resulted from the airway cell response to P. aeruginosa components different from ExoU.

Patatin, cPLA₂ and iPLA₂ have a serine-aspartate catalytic dyad which utilizes a catalytic serine (Sato and Frank, 2004). A number of observations suggest that this dyad also accounts for ExoU activity: (i) the alignment of ExoU with patatin, cPLA₂ and iPLA₂ identified serine 142 (S142) and aspartate 344 (D344) as putative catalytic amino acids (Phillips et al., 2003; Sato et al., 2003); (ii) inhibitors containing a serine-reactive group, such as MAFP, inhibit cPLA₂ and ExoU activity; and (iii) site-specific mutagenesis of either S142 or D344 to alanine suppressed the proinflammatory activity of ExoU (Rabin and Hauser, 2005). In our study, the proinflammatory activity of ExoU was suppressed by MAFP treatment. Moreover, exoU-depleted bacteria complemented with site-directed mutate exoU serine catalytic motif (Rabin and Hauser, 2005) did not differ from exoU-depleted bacteria in their capability to induce either PGE₂ release by infected endothelial cells or the inflammatory reaction in the mouse footpad experimental model. Together, these results clearly show that the ExoU proinflammatory activity correlates with its PLA₂ activity and is dependent on the enzyme hydrolase motif containing a catalytic serine residue but the role played by the catalytic aspartate motif remains to be determined.

In conclusion, in this paper we demonstrated that ExoU is a potent inflammatory agent, as hypothesized by Sato and Frank (2004). Intratracheal injection of bacteria induced the accumulation of inflammatory cells and the release of prostanoid mediators. These effects were not seen when the defective mutant was injected. We also showed, in in vitro experiments, that endothelial cells can be stimulated to produce arachidonic acid metabolites. Therefore, besides being the TTSS effector responsible for the majority of the cytotoxic effects that have been reported for cultured mammalian cells (Finck-Barbançon et al., 1997; Fleiszig et al., 1997; Hauser et al., 1998), ExoU seems to play a role in P. aeruginosa pathogenesis by increasing the availability of arachidonic acid at local sites of bacterial infection. Moreover, as the metabolism of membrane phospholipids by PLA₂ results also in the release of lysophospholipids, which also have biological significance (Moolenaar et al., 2004), further studies are necessary to establish the full range of activities that ExoU may play in the pathogenesis of P. aeruginosa infections.

Experimental procedures

Bacterial strains, deletion of exoU and culture conditions

The ExoU-producing P. aeruginosa PA103 strain and its isogenic mutant PA103ΔexoU were used in this study. To delete the exoU gene, upstream and downstream DNA fragments of PA103 were amplified by polymerase chain reaction (PCR) and re-amplified by PCR overlapping extension. The primers used were 5′TATAGGATCCGAGTTTACCCGGCCAG3′ (sense) and 5′ATGGACCCGGGCTCTTTCAATAAC3′ (antisense), upstream exoU, and 5′GAGGCGCCGGGTCACATGATTGATACA3′ (sense) and 5′GGCTCTAGAAAAGCGACACTAAC3′ (antisense), downstream exoU. The PCR product was subcloned in pCR2.1 cloning vector (Invitrogen), sequenced and then cloned in the pKNG101 suicide vector (Kaniga et al., 1991). This plasmid permits the positive selection of double recombination events in Gram-negative bacteria, as it contains genes encoding streptomycin resistance and sucrose sensitivity. As this vector only replicates in bacterial hosts supplying in trans the π protein encoded by the pir gene, the recombinant pKNG101 was maintained in Escherichia coli CC118pir, a strain lysogenized with a pir transducing λ phage. The construct was transferred to PA103 by triparental conjugation and the transconjugants were selected in Pseudomonas isolation agar plates containing streptomycin at 2 mg ml⁻¹. Plasmid insertion at exoU locus was verified by PCR. Plasmid excision and loss of exoU were obtained by growing the P. aeruginosa clones in presence of 5% sucrose. The absence of exoU in the P. aeruginosa chromosome was confirmed by PCR and Southern blot. SDS-PAGE analysis revealed that, in contrast with PA103, PA103ΔexoU was unable to secrete ExoU when grown in presence of 5 mM ethylene glycol-bis-(β-aminoethyl) ether N,N′,N′,N′'-tetraacetic acid (EGTA) and 20 mM MgCl₂.

To confirm that the proinflammatory activity of ExoU was dependent on its predicted catalytically active sites, assays were also performed with bacteria in which endogenous exoU had been deleted from the chromosome complemented with either wild-type exoU gene (PA103ΔUT/exoU) or exoU with site-specific mutation from serine to alanine at aminoacid 142 of the toxin (PA103 ΔUT/S142A) (Phillips et al., 2003; Sato et al., 2003; Tamura et al., 2004; Rabin and Hauser, 2005). PA103ΔUT/exoU and PA103 ΔUT/S142A were a generous donation of Dr Alan Hauser (Northwestern University, Chicago).

Cell culture

Immortalized human dermal capillary endothelial cells from the HMEC-1 line (Ades et al., 1992) were cultured in tissue culture flasks in MCDB-131 medium (Sigma-Aldrich St. Louis, MO) containing 10% fetal calf serum, 10 ng ml⁻¹ EGF (Sigma), 1 μg ml⁻¹ hydrocortisone (Sigma) (complete culture medium) and antibiotics. Confluent cultures were trypsinized, cells were suspended in complete culture medium, seeded in 24-well (2.0 × 10⁵ cells per well) tissue culture plates and cultured for 48 h.
Release of arachidonic acid

Cells cultured in 24-well plates for 24 h were radiolabelled with 0.2 μCi 3H-arachidonic acid (New England Nuclear, Boston, MA) in 1 ml complete MCDB-131 medium and allowed to incorporate the label for additional 24 h at 37°C, before they were subjected to the experiments. About 60% of the radioactivity was taken up by the cells. For the assays, cells were washed three times with PBS supplemented with 0.2% delipitated BSA (Sigma), to bind non-incorporated 3H-arachidonic acid, and incubated with 500 μl of PA103 or PA103ΔexoU suspensions in MCDB-131 medium. Bacteria had been pretreated or not with 100 μM of the cPLA2 inhibitor MAFP (Sigma), for 30 min. Control non-infected cultures were incubated with MCDB-131 medium. As translocation of TTSS effector proteins depends on a close contact between bacteria and the host cells, bacteria were centrifuged (1000 g for 10 min) onto the endothelial cell monolayer. After incubation at 37°C for 1 h, the cell culture supernatants were removed and counted in 4 ml of scintillation counting liquid BCS (Amersham, Little Chalfont, UK) in a LKB scintillation counter. In other assays, after incubation with bacteria for 1 h, cell cultures were rinsed and incubated for 20 h with complete culture medium containing gentamicin at 300 μg ml−1, to kill infecting bacteria. The radioactivity of the different cell supernatants were then determined in the scintillation counter. To confirm whether radioactivity detected in the cell supernatants corresponded to released free arachidonic acid, and not to radiolabelled cell debris, lipid composition of the supernatants of infected and non-infected cultures was analysed by TLC. In brief, samples from these supernatants were extracted with chloroform/methanol (v/v), according to Bligh and Dyer (1959), loaded on silicagel TLC plates (Merck, Darmstadt, Germany) and resolved using H2O/acetic acid/methanol/chloroform (3/1/43/65; v/v/v/v) as solvent system. The presence of arachidonic acid and phospholipids in the supernatants was localized in the silica gels by comparison with corresponding standards. The radiolabelled spots were then cut and placed into scintillation vials containing 4 ml of the scintillation counting liquid.

PGE2 and PGI2 assays

HMEC-1 cells in 24-well culture plates were incubated with 500 μl of the bacterial suspensions in MCDB-131 medium and centrifuged, as described above. After incubation for 1 h at 37°C, cell cultures were rinsed and incubated with complete culture medium containing gentamicin at 300 μg ml−1, to kill infecting bacteria. Control non-infected cultures were treated with the gentamicin-containing medium as well. After incubation for 20 h, PGE2 and PGI2 in culture supernatants were assayed in duplicate by enzyme immunoassay (EIA), according to the manufacturer’s instruction (Cayman Chemical, Ann Arbor, MI). As cytotoxicity of PA103 strain led to the rapid killing of a high percentage of infected endothelial cells, after the 1 h infection period the cell concentrations in PA103- and PA103ΔexoU-infected culture wells, as well as in control non-infected culture wells, were determined. The PGE2 and PGI2 concentrations were reported as picogram of eicosanoid per 105 cells.

In vivo murine experimental models

Two murine models of inflammatory response were used to assess the effect of ExoU. In the first, bacterial suspensions (105 cfu in 50 μl of LPS-free PBS) were injected into the footpads of the right hind limbs of 8- to 10-week-old female BALB/c mice, whereas the footpads of the left limbs (control limbs) were injected with the same volume of the diluent. After different periods, the volumes of the bacteria-injected and control footpads were measured with a plethysmometer. The animals were then sacrificed by CO2 inhalation and their footpads were fixed with 10% formalin in PBS, decalcified, and embedded in paraffin. The paraffin sections were stained with haematoxylin and eosin for light microscopic histologic analysis. To assess the role of eicosanoids in the swelling of mice footpads, 1 h before the injection of the bacterial suspensions, groups of five animals were inoculated either intraperitoneally with 500 μl of ibuprofen, an inhibitor of COX 1 and COX 2, at a final dose of 25 mg kg−1, or subcutaneously with 100 μl of NDGA, an inhibitor of 5, 12 and 15 lipoxigenases, at a final dose of 50 mg kg−1.

In the second experimental model, mice were anesthetized with isoflurane and 105 μCi of PA103 or PA103ΔexoU bacteria in 50 μl of LPS-free NaCl solution were instilled into their tracheas. Control animals were inoculated with the same volume of the diluent. In parallel, other group of mice were inoculated intraperitoneally with the non-selective COX inhibitor indomethacin, 1 h before the bacterial instillation. At 24 h post infection, mice were sacrificed by CO2 inhalation and BAL was performed. Recovered BAL fluid was assessed for: (i) total cell counting using a standard haemocytometer; (ii) differential cell counting using Diff-Quick-stained (Dade Diagnostic) cytoplasmic preparations; and (iii) PGE2 determination. In parallel, the animal lungs were fixed in 10% formalin and processed for histopathological analysis, as described above.

The protocol for all animal experiments was approved by the Animal Research Committee of the State University of Rio de Janeiro and FIOCRUZ.

Statistical analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) with the Dunnett’s or Bonferroni’s test to determine significant statistical differences between groups, unless otherwise stated. P < 0.05 was taken as statistically significant.

Acknowledgements

We thank Maria Angelica Pereira da Silva for her technical assistance. A.M.S and M.C.A.S. were supported by doctoral fellowships from CAPES (Brazil) whereas L.T and B.R. were supported by the French Association ‘Vaincre-La-Mucoviscidose’. This work was supported by grants from CNPq and CAPES (Brazil), from COFECUB (France) and from the Howard Hughes Medical Institute (EUA).

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