The effect of *Pseudomonas* exotoxin A on cytokine production in whole blood exposed to *Pseudomonas aeruginosa*

Marc J. Schultz a,b,*, Peter Speelman a, Sebastiaan A.J. Zaat c, C. Erik Hack d, Sander J.H. van Deventer b, Tom van der Poll a,b

a Department of Infectious Diseases, Tropical Medicine and AIDS, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
b Laboratory of Experimental Internal Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
c Department of Medical Microbiology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
d Department of Pathophysiology of Plasma Proteins, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands

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Abstract

To determine the effect of *Pseudomonas aeruginosa* exotoxin A (P-ExA) on cytokine production, we studied cytokine release induced by heat-killed *P. aeruginosa* (HKPA) in human whole blood in the presence or absence of P-ExA. P-ExA (0.01–1 W g ml⁻¹) caused a dose-dependent decrease in HKPA-induced production of tumor necrosis factor-α (TNF), interleukin (IL-) 10, IL-6 and IL-8 (all P < 0.05). P-ExA-induced inhibition of IL-10, IL-6 and IL-8 release was not dependent on reduced TNF concentrations, since the relative attenuation of the production of these cytokines was similar in the presence or absence of a neutralizing anti-TNF antibody. The effect of P-ExA on cytokine production may offer a disadvantage to the host with respect to clearance of the infection. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Cytokine; Host defense; *Pseudomonas aeruginosa*

1. Introduction

Pneumonia is the second most frequent cause of hospital-acquired infections in the USA. Patients receiving mechanical ventilation have the highest occurrence rates, varying between 9 and 60% depending on the diagnostic criteria used [1]. *Pseudomonas aeruginosa* is a frequently isolated pathogen in ventilator-acquired pneumonia (VAP) and mortality attributable to VAP may be higher if the etiologic agent is *P. aeruginosa* [2]. *P. aeruginosa* is a Gram-negative, extracellular bacterium, that causes serious infections especially in an immunosuppressed host. One of the virulence factors of this opportunistic pathogen is exotoxin A (P-ExA) [3].

P-ExA is produced as a single-chain 66-kDa polypeptide that binds the α2-macroglobulin receptor on the target cell [4]. P-ExA can cause cell death after translocation to the cytosol of the target cell. Multiple ex vivo and in vitro studies have demonstrated that P-ExA influences the immune system also at non-cytotoxic doses: P-ExA inhibits or enhances antibody response to antigens [5], and P-ExA stimulates proliferation of mouse thymocytes [6]. Recently, it was demonstrated that P-ExA enhances interleukin (IL-) 2 concentrations in T-cell cultures, either by inhibition of IL-2 consumption or by induction of IL-2 production [7].

Evidence exists that P-ExA is an important factor in the virulence of *P. aeruginosa* [8]. Mortality in mice challenged with a single intraperitoneal injection of P-ExA-producing *P. aeruginosa* was higher than in mice challenged with P-ExA-deficient mutant bacteria. Anti-P-ExA monoclonal antibodies (mAbs) exerted a protective effect during *P. aeruginosa* infection in mice [8,9].

Cytokines and chemokines are small proteins involved in the orchestration of inflammatory processes. During pulmonary infection, they interact in a network that consists of pro-inflammatory cytokines (e.g. tumor necrosis factor α (TNF), IL-6, IL-12, interferon-γ (IFN-γ)), anti-inflammatory cytokines (e.g. IL-10) and cytokines with...
chemoattractant capabilities (e.g. IL-8). In patients with pneumonia, cytokines and chemokines are produced within the lung at the site of the infection, where they are important for host defense. Indeed, endogenous TNF, IL-6, IL-12 and IFN-γ are essential for limitation of bacterial growth in lungs in mouse models of pneumococcal and Klebsiella pneumonia [10–14], while IL-10 hampers antimicrobial defense in such models [15,16]. Since P-ExA influences the virulence of P. aeruginosa, we considered it of interest to study the effect of P-ExA on responses known to be important in host defense mechanisms against infection with P. aeruginosa. Therefore, in the present study, we sought to determine the effect of P-ExA on cytokine production in human whole blood stimulated with heat-killed P. aeruginosa (HKPA).

2. Materials and methods

2.1. Reagents

P-ExA was purchased from ICN (Cleveland, OH, USA). Anti-TNF F(ab’)2 fragment (MAK 195F) was kindly provided by Knoll, Ludwigshafen, Germany. MAK 195F is derived from a murine TNF neutralizing mAb (IgG3), and neutralizes the biological activity of recombinant and naturally occurring human TNF [17]. The concentration of MAK 195F added (10 μg ml⁻¹) represented a 1–2 log excess neutralizing capacity over TNF concentrations detected after stimulation with HKPA. Mouse IgG was purchased from Fluka Chemia (Buchs, Switzerland). HKPA was obtained from a clinical isolate (serotype PA01). The bacteria were cultured overnight (20 h) and then washed twice in pyrogen-free 0.9% NaCl and inactivated for 60 min at 80°C. A sample of 500 μl on a blood agar plate did not show growth of bacteria.

2.2. Whole blood stimulation

Whole blood stimulation was performed as described previously [18–20]. Briefly, blood was collected in a sterile fashion from six healthy subjects using a collecting system consisting of a butterfly needle connected to a syringe (Becton Dickinson and Co., Rutherford, NJ, USA). Anticoagulation was obtained using sterile heparin (Heparine Leo Pharmaceutical Products B.V., Weesp, The Netherlands) (10 U ml⁻¹ blood, final concentration). Whole blood, diluted 1:1 in sterile RPMI 1640 (Gibco BRL, Life Technologies Inc., Paisley, UK), was stimulated for 4–24 h at 37°C with HKPA (10⁶ colony-forming units (CFU) ml⁻¹, final concentration) in sterile polypolyethylene tubes (Becton Dickinson and Co.). For these experiments, polypolyethylene tubes were prefilled with 0.75 ml RPMI 1640 with or without the appropriate concentrations of HKPA, P-ExA or anti-TNF, after which 0.75 ml heparinized blood was added. Tubes were then gently mixed and placed in the incubator. After the incubation, plasma was prepared by centrifugation and stored at −20°C until assays were performed.

2.3. Assays

Cytokine concentrations were measured by specific enzyme-linked immunosorbent assays (ELISAs) according to the instructions of the manufacturer, i.e. TNF (Medgenix, Brussels, Belgium), IL-6 (Pharmingen, San Diego, CA, USA), IL-10 (Pharmingen), IL-8 and IFN-γ (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands). Concentrations of IL-12 p40 and p70 were determined using sandwich ELISAs. In short, 96-well Immuno Maxisorp plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with IL-12 p40-specific mAb C11.79 (2 μg ml⁻¹) or IL-12 p70-specific mAb 20C2 (1.25 μg ml⁻¹). The plates were washed with 0.2 M phosphate-buffered saline (PBS)/0.05% Tween 20, incubated with 2% milk in PBS for 1 h as a blocking step and washed again. Samples and standards were diluted in high performance ELISA buffer (CLB). Human recombinant IL-12 was used as the standard. Samples and standards were incubated together with biotinylated anti-human IL-12 p40 mAb C8.6 (0.5 μg ml⁻¹, final concentration) for 1.5 h at room temperature. After washing five times, bound IL-12 p40 or IL-12 p70 was detected with peroxidase-conjugated streptavidin (CLB) and ortho-phenylenediamine as the substrate. The color reaction was stopped after 10 min with 1 M H₂SO₄, and the absorbance read at 490 and 650 nm. mAb 20C2 and human rIL-12 were kindly provided by Dr. Maurice K. Gately, Hoffmann La Roche Inc., Nutley, NJ, USA. The hybridomas producing the IL-12 p40-specific mAbs C11.79 and C8.6 were kindly provided by Dr. Giorgio Trinchieri (The Wistar Institute, Philadelphia, PA, USA).

2.4. Cell viability

Cell viability was determined by incorporation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [21]. MTT-tetrazolium is a reagent that is metabolized to a dark blue end product by viable cells. For MTT incorporation, aliquots of blood from four volunteers, with and without HKPA and/or P-ExA, incubated for 16 h at 37°C as described above, were subjected to NH₄Cl lysis to clear red blood cell contamination (1.5 ml culture+1.5 ml NH₄Cl lysis buffer) and centrifuged at 200×g for 5 min. The pellet was resuspended in 3 ml cold RPMI, and after a second wash step, in 0.5 ml of RPMI 1640. Duplicate aliquots of this cell suspension (200 μl) were placed in 96-well round-bottom plates, and 20 μl MTT-tetrazolium (5 mg ml⁻¹; Sigma) was added. The plates were incubated for 4 h at 37°C in a humidified atmosphere containing 5% CO₂. After removing 150 μl of the supernatant, 100 μl of 0.04 N HCl/isopropanol was
2.5. Statistical analysis

All values are given as means ± S.E.M. Serial data were compared by analysis of variance. Two sample comparisons were performed using the Wilcoxon test for matched samples. P < 0.05 was considered to represent a statistically significant difference.

3. Results

3.1. Time course of cytokine induction by HKPA

Incubation of whole blood without HKPA did not result in detectable cytokine production (data not shown). Incubation of whole blood with HKPA (10⁶ CFU ml⁻¹) was associated with a time-dependent production of TNF, IL-6, IL-8, IL-10, IFN-γ, IL-12 p40 and IL-12 p70. TNF was the first cytokine detectable, peaking after 8 h (22.2 ± 4.7 ng ml⁻¹), while the other cytokines reached peak concentrations at later time points (IL-12 p40 at 12 h (9.8 ± 2.7 ng ml⁻¹), IL-10, IL-6 and IL-12 p70 at 16 h (4.7 ± 3.1 ng ml⁻¹, 166.3 ± 12.6 ng ml⁻¹, 68.4 ± 37.1 pg ml⁻¹, respectively); IL-8 and IFN-γ at 24 h (50.9 ± 6.6 ng ml⁻¹, 2.4 ± 1.0 ng ml⁻¹, respectively)). Time curves for measured cytokines are shown in Fig. 1. Based on these experiments, a 16-h incubation with HKPA was chosen for further experiments.

3.2. Effect of P-ExA on cytokine production by HKPA

P-ExA caused a dose-dependent inhibition of the production of TNF, IL-6, IL-8 and IL-10 (Table 1). TNF, IL-6 and IL-10 production appeared sensitive to P-ExA, with significant inhibition already found after incubation with P-ExA at 0.01 µg ml⁻¹. IL-8 production was inhibited by P-ExA at 0.1 and 1 µg ml⁻¹. In contrast, IFN-γ, IL-12 p40 and IL-12 p70 production was not influenced by P-ExA at the concentrations tested (Table 1). The effect of P-ExA on cytokine production was not caused by a negative influence on the viability of leukocytes, as determined by MTT incorporation (data not shown). P-ExA did not influence the different ELISAs used (data not shown).

3.3. P-ExA-induced inhibition of IL-6, IL-8 and IL-10 production is independent of the inhibitory effect on TNF production

Since it has been reported that endogenously produced TNF in part mediates the production of IL-6 induced by endotoxin [22], we were interested whether P-ExA-induced inhibition of TNF production was involved in the negative effect of P-ExA on synthesis of IL-6, IL-8 and IL-10 in HKPA-stimulated whole blood. To evaluate this possibility, we incubated whole blood with HKPA in the presence or absence of a neutralizing anti-TNF mAb (10 µg ml⁻¹).

![Fig. 1. Time-dependent release of TNF, IL-10, IL-6, IFN-γ, IL-12 p40 and IL-12 p70 in HKPA-stimulated blood. Whole blood, diluted 1:1 in sterile RPMI 1640, was stimulated for 4–24 h at 37°C with HKPA (10⁶ CFU ml⁻¹). Data are means ± S.E.M. of the means for six healthy donors.](image)

### Table 1

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>P-ExA (µg ml⁻¹)</th>
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<tbody>
<tr>
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<td>0</td>
</tr>
<tr>
<td>TNF (ng ml⁻¹)</td>
<td>9.7 ± 2.2</td>
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<tr>
<td>IL-6 (ng ml⁻¹)</td>
<td>118.5 ± 10.9</td>
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<tr>
<td>IL-8 (ng ml⁻¹)</td>
<td>52.8 ± 5.8</td>
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<tr>
<td>IL-10 (ng ml⁻¹)</td>
<td>13.5 ± 2.3</td>
</tr>
<tr>
<td>IL-12 p40 (ng ml⁻¹)</td>
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<tr>
<td>IL-12 p70 (pg ml⁻¹)</td>
<td>257 ± 78</td>
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<tr>
<td>IFN-γ (pg ml⁻¹)</td>
<td>939 ± 287</td>
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Whole blood was incubated with 10⁶ CFU ml⁻¹ HKPA, in the absence and presence of P-ExA (0.01–1.0 µg ml⁻¹) for 16 h at 37°C in 5% CO₂. Data represent means ± S.E.M. for six healthy volunteers. *P < 0.05 versus value in the absence of P-ExA.
First we assessed that mouse IgG (10 μg ml⁻¹) did not influence HKPA-induced cytokine production (data not shown). Anti-TNF inhibited HKPA-induced production of IL-6, IL-8 and IL-10 by 84.0 ± 6.9%, 84.7 ± 7.3% and 57.6 ± 11.4%, respectively, relative to concentrations of these cytokines after incubation with HKPA without anti-TNF (all P < 0.05). Hence, these data indicated that indeed TNF is partially responsible for HKPA-induced cytokine production in whole blood. In the presence of anti-TNF, P-ExA inhibited the production of IL-6, IL-8 and IL-10 to a similar extent as in the absence of anti-TNF, suggesting that the inhibiting effect of P-ExA on the production of these cytokines is not mediated via a reduction in TNF concentrations (Fig. 2).

4. Discussion

*P. aeruginosa* is an important etiologic pathogen in respiratory tract infection. Several host responses are considered to contribute to the defense against bacterial invasion of the pulmonary compartment. During clinical pneumonia, cytokines are produced at the site of the infection [23,24]. Mouse studies have indicated that locally produced pro-inflammatory cytokines are required for an effective host defense against bacterial pneumonia [11–13,25]. Nearly all clinically important strains of *P. aeruginosa* produce P-ExA. Since P-ExA is considered to play an important role in the virulence of *P. aeruginosa* infections [8,9], we were interested in the effects of P-ExA on cytokine production. P-ExA profoundly inhibited the production of TNF, IL-1α, IL-1β and IFN-γ by PHA, and the production of TNF induced by *S. aureus*. The effect of P-ExA on cytokine production stimulated by *P. aeruginosa* was not investigated in that study [26]. Hence these data are in line with our results except for the fact that in our hands P-ExA did not influence IFN-γ production. Possible explanations for this discrepancy include differences in stimuli used (PHA versus HKPA) and differences in culture conditions. Michalkiewitz et al. reported transient P-ExA-induced inhibition of IL-2 synthesis by T-cells [7]. However, in cultures of PBMCs lasting longer than 48 h, IL-2 concentrations were enhanced, regardless of the dose of P-ExA. The explanation for these findings is uncertain, but may be the result of superinduction of mRNA, or prolonged mRNA transcription [27,28]. In contrast, after stimulation of PBMCs with P-ExA for 48 and 96 h, IL-4 and IFN-γ production was reduced by P-ExA in a dose-dependent manner [7].

Monocytes, the main source of TNF, IL-6, IL-8 and IL-10 in blood, serve as a target for P-ExA. P-ExA inhibits the expression of the CD14 molecules on monocytes [7]. As CD14 has been shown to trigger monocyte activation and to serve as a ligand-binding receptor for complexes of endotoxin [29,30], P-ExA-induced diminished CD14 expression may inhibit many functions of monocytes induced by Gram-negative pathogens, including cytokine production. However, a possible effect of P-ExA on monocyte CD14 expression is a less likely explanation for our findings since (a) P-ExA did not influence HKPA-induced production of IL-12, a cytokine mainly derived from monocytes, and (b) cytokine production induced by HKPA is at least in part endotoxin-independent [26].

TNF is considered an endogenous mediator of IL-6...
release induced by endotoxin in human in vivo [31,32]. We hypothesized that P-ExA-induced reduction of IL-6, IL-8 and IL-10 release was in part the result of reduced TNF concentrations in the presence of P-ExA. Therefore, the effect of P-ExA on the release of these cytokines was investigated in the presence of a neutralizing anti-TNF mAb. Indeed anti-TNF inhibited HKSP-induced IL-6, IL-8 and IL-10 production in whole blood. However, in the presence of anti-TNF, the potency of P-ExA to inhibit IL-6, IL-8 and IL-10 release remained unaltered. Hence these data suggest that P-ExA-induced inhibition of IL-6, IL-8 and IL-10 release is independent of inhibition of TNF release by P-ExA.

Pneumonia is associated with local production of cytokines and chemokines. Locally produced TNF prevents outgrowth of bacteria in lungs, thereby playing a protective role in experimental pneumonia with Streptococcus pneumoniae [12,33], Klebsiella pneumoniae [11] and Legionella pneumophila [34]. Like TNF, IL-6 plays an important role in host defense in pneumococcal pneumonia [12], as do chemokines in K. pneumoniae pneumonia [25] and in P. aeruginosa infection [35]. The anti-inflammatory cytokine IL-10, although playing a detrimental role in the clearance of K. pneumoniae [15] and S. pneumoniae [16] from the lungs, may exert favorable effects in P. aeruginosa infections [36]. Hence, the overall effect of P-ExA on cytokine production induced by P. aeruginosa is likely to impair host defense during respiratory tract infections with these pathogens.

In conclusion, P-ExA inhibits TNF, IL-6, IL-8 and IL-10 production in whole blood stimulated with P. aeruginosa in vitro. Inhibition of cytokine production by P-ExA may negatively influence aspecific host defense mechanisms during pneumonia with P. aeruginosa. Together with other reported effects of P-ExA, these data suggest that the immunomodulatory actions of this toxin may offer a potential disadvantage to the host with respect to clearance of the infection.

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References


