BACTERIAL PATHOGENICITY

Effect of *Pseudomonas aeruginosa* exotoxin A on endotoxin-induced tumour necrosis factor production in murine lung

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The ability of several *Pseudomonas aeruginosa* exo-enzymes, including exotoxin A (ETA), to induce inflammation and their influence on endotoxin-induced tumour necrosis factor (TNF) production in murine lung were evaluated. Intratracheal administration of lipopolysaccharide (LPS; 0.1–10 µg/mouse), 2⁻¹ LD50 of *P. aeruginosa* alkaline protease (7.5 µg/mouse) and elastase (1.2 µg/mouse) elevated total cell number and the percentage of neutrophils in broncho-alveolar lavage fluid (BALF), whereas ETA (0.1 µg/mouse) did not. LPS induced TNF production in BALF in a dose-dependent manner, whereas the *P. aeruginosa* exo-enzymes did not. When ETA was inoculated into the respiratory tract before LPS, production of TNF in BALF was significantly suppressed in a dose-dependent manner. ETA also suppressed TNF production by alveolar macrophages (AMs) stimulated with LPS *in vitro*. Flow cytometric analysis showed that ETA markedly reduced the expression of CD14 and CD11c/CD18 on the surface of AMs. ETA also depressed partially the expression of TNF-α mRNA in AMs. These findings suggest that ETA regulates TNF production in murine lung by suppressing LPS receptor expression, mRNA expression and protein synthesis and/or secretion of TNF.

Introduction

Pseudomonas aeruginosa is the most prevalent pathogen in patients with cystic fibrosis (CF) [1–3] and diffuse panbronchiolitis (DPB) [4, 5]. Recently, high concentrations of tumour necrosis factor alpha (TNF-α) in sputum and broncho-alveolar lavage fluid (BALF) from patients with CF [6–8] and DPB [9] were reported. TNF-α, produced by alveolar macrophages (AMs) in response to *P. aeruginosa* and other organisms, may promote the destructive inflammatory process in the lung [7].

As *P. aeruginosa* is a gram-negative bacillus, endotoxin (lipopolysaccharide; LPS) of this pathogen should be an important virulence factor [10]. However, LPS from the cell wall of *P. aeruginosa* is not as toxic as that from enterobacteria [10]. *P. aeruginosa* also produces numerous extracellular products which are much more toxic than LPS and have been shown to play a role in the pathogenesis of the infections

caused by this organism [10–12]. In CF patients colonised or infected by *P. aeruginosa*, exo-enzymes such as exotoxin A (ETA), elastase (EL) and alkaline protease (AP) are detectable in the sputum and their concentrations are high during exacerbations [13, 14]. Furthermore, the high percentage of patients with serum antibodies specific for ETA [15] and proteases [16] suggests that these exo-enzymes contribute to the pathogenesis of the disease [13]. A role for ETA and EL in the virulence of *P. aeruginosa* in chronic lung infections of rats has been demonstrated [17]. A previous study [12] reported that intravenous administration of ETA, the most toxic substance produced by *P. aeruginosa*, enhanced LPS-induced TNF-α production in blood of mice, when ETA was injected before or at the same time as LPS. However, it is unclear whether ETA or other extracellular products of *P. aeruginosa* induce TNF production by themselves, or influence LPS-induced TNF production in lung. To elucidate these points, the present study evaluated the influence of intratracheal administration of *P. aeruginosa* ETA, EL and AP on inflammation and TNF production in BALF of mice. The influence

of pre-treatment with ETA and other exo-enzymes on LPS-induced TNF production was also examined. In addition, the influence of ETA on expression of LPS receptors on the surface of murine AMs and also on TNF- α mRNA expression in the cells was examined.

Materials and methods

Animals

Specific-pathogen-free male ddY mice (Japan SLC, Shizuoka, Japan) weighing 24–28 g were used.

Bacterial products

P. aeruginosa exotoxin A (ETA; List Biological Laboratories, San Mateo, Ca, USA), *P. aeruginosa* elastase (EL; Nagase Biochemicals, Kyoto, Japan), *P. aeruginosa* alkaline protease (AP; Nagase) and LPS derived from *Escherichia coli* O55:B5 (Difco Laboratories) were purchased commercially. These bacterial products were dissolved in pyrogen-free saline just before use. LD50 doses of intratracheally inoculated ETA, EL and AP in mice were determined after observation for 14 days and calculated according to the method of Karber [18]. In in-vivo studies, mice were inoculated intratracheally with 2^{-1} LD50 of exo-enzymes (ETA, 0.1 μ g/mouse; AP, 7.5 μ g/mouse; EL, 1.2 μ g/mouse) and LPS 0.1–10 μ g/mouse.

Intratracheal cannulation and administration of bacterial products

Mice were anaesthetised with ether. The trachea was cannulated through the mouth by means of an ear pick with a light (Mimi-pick) as a laryngoscope, without any surgical operation [19]. Bacterial products in 0.2 ml of pyrogen-free saline were administered to mice intratracheally via a 20 G intravenous cannula.

Collection of BALF and serum samples

BALF was obtained by washing murine lung with 1.0 ml of saline three times. Total and differential cell counts were performed with Giemsa's stain. After centrifugation of the BALF, the supernates were frozen at -80°C until TNF assay. In some experiments, blood was obtained by cardiac puncture and serum samples were also used for TNF assay.

Influence of *P. aeruginosa* exo-enzymes on LPS-induced TNF production in murine lung

Mice were given *P. aeruginosa* exo-enzymes at a dose of 2^{-1} of LD50 in 0.2 ml of pyrogen-free saline or saline alone intratracheally via the cannula. Mice were given 0.001–1 μ g of LPS intratracheally 4 h after administration of *P. aeruginosa* exo-enzymes. BALF was obtained as described above for the TNF assay.

Alveolar macrophage culture

The cells in BALF obtained from normal mice were cultured in 96-well microtitration plates at a concentration of 1×10^5 cells/well in RPMI1640-fetal calf serum 5% overnight at 37°C . Non-adherent cells were removed by three washes and the resultant adherent cells were used as alveolar macrophages (AMs). AMs were incubated with ETA 0.008–5 μ g/ml for 4 h at 37°C . Then LPS was added to a final concentration of 1000 ng/ml. Incubation was continued for 2 h and the culture supernates were collected for TNF assay.

TNF assay

TNF activity was measured by a L929 cytotoxicity assay based on reduction of a tetrazolium dye [20]. Briefly, murine TNF-sensitive L929 cells were cultured in 96-well microtitration plates at a concentration of 5×10^4 /well in RPMI1640-fetal calf serum 5% and incubated for 20 h at 37°C . The cells were then cultured for a further 18 h in the presence of serial dilutions of the test samples with actinomycin D (Sigma) 2 μ g/ml to increase sensitivity to TNF. The viability of cells was determined by a quantitative colorimetric staining assay with MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide; Sigma) 1 mg/ml. The OD₅₉₀ of the dissolved precipitate was determined in a spectrophotometer. TNF activity was expressed in units/ml, with 1 U being the amount of TNF causing 50% lysis of L929 cells.

Detection of TNF- α mRNA expression

Mice were given ETA 0.1 μ g/mouse intratracheally followed by LPS 0.01 μ g 4 h later. BALF was obtained 90 min after LPS inoculation. Total cell RNA was isolated by lysing the cells in isogen (Nippon Gene, Toyama, Japan) [21] following the manufacturer's protocol. The influence of ETA on mRNA expression was examined by reverse transcription PCR (RT-PCR) [22]. RT-PCR was performed in 100- μ l reactions with a GeneAmpTM RNA PCR kit (Roche, Branchburg, NJ, USA) with cloned moloney murine leukaemia virus (M-MLV) reverse transcriptase (final concentration 2.5 U/ μ l) with the GeneAmp PCR System 9600-R (Roche). cDNA was analysed and amplified by PCR with primers specific to murine TNF- α and β -actin. Primers used were as follows: mouse TNF- α sense, 5'-TTC TGT CTA CTG AAC TTC GGG GTG ATC GGT CC-3'; mouse TNF- α anti-sense, 5'-GTA TGA GAT AGC AAA TCG GCT GAC GGT GTG GG-3' [23]; mouse β -actin sense, 5'-GTG GGC CGC TCT AGG CAC CA-3'; mouse β -actin anti-sense, 5'-CGG TTG GCC TTA GGG TTC AGG GGG-3' [24]. PCR was performed at 94°C for 2 min, followed by 35 cycles of 30 s at 95°C , 30 s at 55°C and 30 s at 72°C . PCR products were subjected to agarose 2% gel electrophoresis. DNA was then visualised after staining with ethidium bromide. Signals obtained for TNF- α (354 bp) and β -actin (245 bp) PCR products were

analysed by densitometry with Dendron software (Soltech, Oakdale, CA, USA) and the ratio of densitometric values for TNF- α and β -actin signals was calculated to normalise the expression of TNF- α to β -actin.

Flow cytometric analysis of CD14 and CD11c/18 expression on the surface of AMs

Mice were given 0.1 μ g of ETA or saline intratracheally. BALF were obtained 4 h after ETA inoculation. Total cell numbers in BALF were adjusted to 1.0×10^6 cells/ml and the cells were stained with rat fluorescein isothiocyanate (FITC)-conjugated anti-CD14 monoclonal antibody (MAb; clone rmC5-3) and hamster phycoerythrin (PE)-conjugated anti-CD11c/CD18 MAb (clone HL3) for 30 min at room temperature. After washing, labelled cells were gated in an area of macrophages on the scatter cytogram and analysed by Cytron Flow Cytometry (Ortho Diagnostic System KK, Tokyo, Japan). The percentage of positive and mean intensities for the antigens were calculated by the manufacturer's programme. Control preparations were set up by replacing the MAbs with a mouse immunoglobulin of the same isotype.

Statistical analysis

Student's *t* test was used to compare means and a level of 5% was considered significant.

Results

Augmentation of total and differential cell counts in BALF after intratracheal administration of LPS, ETA, AP or EL

Total and differential cell counts in BALF taken from the mice given LPS 0.1–10 μ g/mouse intratracheally did not change until 2 h after inoculation and increased 6 h after inoculation (Fig. 1a and b). The peak of total cell numbers was observed 12–24 h after LPS inoculation. The percentage of neutrophils was quickly elevated when LPS was inoculated. None of the mice died of intratracheal inoculation at these doses of LPS. Intratracheal inoculation of 2^{-1} LD50 of ETA (0.1 μ g/mouse), AP (7.5 μ g/mouse) or EL (1.2 μ g/mouse) did not produce any obvious change in total or differential cell counts until 6 h after inoculation (Fig. 1c and d). Thereafter, total cell numbers in BALF of mice inoculated with AP or EL, but not ETA, increased significantly (Fig. 1c). Elevation

Fig. 1. Time changes of total cell counts and percentage of neutrophils in BALF of mice after intratracheal inoculation of various doses of LPS (○, 10 μ g; ●, 1 μ g; □, 0.1 μ g) and 2^{-1} LD50 of *P. aeruginosa* AP (▲), EL (△) and ETA (■). After intratracheal inoculation with 0.1–10 μ g of *E. coli* O55:B5 LPS in 0.2 ml of pyrogen-free saline, time changes of total cell counts (a) and percentage of neutrophils (b) in BALF were examined. *P. aeruginosa* AP, EL or ETA at doses of 2^{-1} LD50 were also inoculated and time changes of total cell counts (c) and percentage of neutrophils (d) in BALF were examined. The results are expressed as the mean of at least three mice per group.

of the percentage of neutrophils was observed at 12 h and peaked at 24 h after inoculation in all groups of mice except for those given ETA (Fig. 1d).

TNF levels in BALF after intratracheal administration of LPS, ETA, AP or EL

Intratracheal administration of LPS 1 µg/mouse induced rapid augmentation of the TNF level in BALF (Fig. 2a) and the TNF levels were dose-dependent when assayed 2 h after inoculation (Fig. 2b). As LPS did not increase neutrophils within 2 h of inoculation (Figs. 1a and 2a), these findings indicate that TNF in BALF comes mainly from AMs rather than neutrophils. However, no TNF was detected in the sera (Fig. 2b). In contrast, intratracheal administration of 2^{-1} LD50 of ETA, AP and EL did not produce any detectable TNF in BALF (Fig. 2a) and sera (data not shown) until 48 h after inoculation.

Fig. 2. TNF levels in BALF and sera of mice after intratracheal inoculation of LPS (●), AP 7.5 µg (▲), EL 1.2 µg (△) or ETA 0.1 µg (■). Mice were inoculated intratracheally with 1 µg of LPS or 2^{-1} LD50 of *P. aeruginosa* AP, EL or ETA and kinetics of TNF levels in BALF were assessed by L929 cytotoxic assay (a). TNF levels in BALF (○) and in serum (●) 2 h after intratracheal inoculation of various doses of LPS were also examined (b). The results are expressed as the mean of at least three mice per group.

Influence of pre-inoculation with ETA, AP or EL on LPS-induced TNF production in BALF

Although ETA, AP and EL themselves lack the ability to induce intratracheal TNF production, these substances may influence LPS-induced TNF production in BALF. Pre-inoculation with AP and EL did not show any obvious effect on LPS-induced TNF production in BALF (data not shown). In contrast, previous inoculation with ETA (0.05 or 0.1 µg/mouse) suppressed LPS-induced TNF production in a dose-dependent manner, and complete suppression was obtained by pre-treatment with 0.1 µg of ETA following stimulation with 0.01 µg of LPS ($p < 0.01$, Fig. 3).

Suppressive effect of ETA on TNF-α mRNA expression in BALF

As shown in Fig. 4, the expression of TNF-α mRNA (354 bp) in BALF obtained after pretreatment with

Fig. 3. Suppressive effect of previous inoculation of ETA on LPS-induced TNF production in BALF. Various doses of ETA were inoculated intratracheally 4 h in advance and then LPS (0.01 or 1.0 µg) was inoculated via the same route. BALF was obtained 2 h after LPS challenge and TNF levels in BALF were assessed. The results are expressed as the mean and SD in triplicate wells.

Fig. 4. Effect of previous treatment of ETA on LPS-induced TNF-α mRNA expression in BALF cells. Mice that had been inoculated intratracheally with ETA (0.1 µg/mouse) 4 h earlier were inoculated with LPS (0.01 µg) via the same route and 90 min later BALF was collected. TNF-α mRNA expression in the BALF cells was assessed by RT-PCR.

ETA and LPS stimulation was less than that obtained after inoculation of LPS only. No difference was seen in the expression of β -actin mRNA (245 bp) in either group. This was also confirmed by a spectrophotometric estimation (data not shown). The ratio of TNF- α mRNA/ β -actin mRNA was 0.81 in the group inoculated with LPS and 0.42 in the group pre-treated with ETA and inoculated with LPS. Cell viability was not influenced by ETA, which was estimated by MTT reduction test as described above (data not shown).

Suppressive effects of ETA on in-vitro LPS-induced TNF production by macrophages

To examine whether ETA directly affected TNF production by TNF producer cells, AMs were cultured in the presence or absence of various concentrations of ETA, and then stimulated with LPS 1 μ g/ml. As shown in Fig. 5, the greater the dose of ETA added to macrophage cultures, the lower the production of TNF.

Fig. 5. Effect of previous treatment with ETA on LPS-induced TNF production by AMs *in vitro*. AMs obtained from BALF were cultured in the presence or absence of ETA (0.008–5 μ g/ml) for 4 h and then stimulated with LPS (final concentration 1000 ng/ml) for 2 h. At the end of cultivation, culture supernates were collected and TNF levels in the supernates were assessed.

ETA itself at the concentration used did not affect cell viability as determined by the MTT reduction test over 12 h (data not shown).

Down-regulation of CD14- and CD11c/CD18-positive cells by ETA

The expression of CD14 and CD11c/CD18 on the cells in BALF, which are presumed to be receptors for LPS and participate in LPS-induced cytokine production [25, 26], was analysed by two-colour flow cytometry. As shown in Fig. 6, the cells in BALF obtained from normal mice were 98.1% CD14 positive and 94.7% CD11c/CD18 positive, including 93.3% double positive cells. On the other hand, macrophages in BALF obtained from the mice pre-treated with ETA were 41.0% CD14 positive and 49.3% CD11c/CD18 positive, including 36.3% double positive cells. These findings indicate that pre-treatment with ETA reduces the cells bearing CD14 and CD11c/CD18 molecules in BALF. ETA did not influence cell viability as determined by the MTT reduction test (data not shown).

Discussion

High concentrations of TNF and other cytokines such as interleukin-1 β (IL-1 β), IL-6, IL-8 and other molecules including intracellular adhesion molecule-1 (ICAM-1) and IL-1 receptor antagonist (IL-1Ra) have been detected in the sputum or BALF from CF patients [6–8]. High levels of IL-1 β , IL-1Ra, IL-8 and TNF are also found in BALF from patients with DPB [9, 27]. This suggests that cytokines and other molecules play important roles in the pathogenesis of respiratory infections caused by *P. aeruginosa* in CF and DPB. The significance of endotoxin in the pathogenesis of *P. aeruginosa* infections has been reported extensively, while the role of exo-enzymes and the interactions of LPS and exo-enzymes are less well understood despite the strong expression of these molecules in sputum and

Fig. 6. Influence of ETA on CD14 and CD11c/CD18 expression on the surface of murine AMs. Total cells in BALF obtained from control mice, ETA (–), and mice inoculated with ETA 4 h before, ETA (+), were adjusted to 1.0×10^6 cells/ml and stained with FITC-conjugated anti-CD14 MAb and PE-conjugated anti-CD11c/CD18 MAb. Labelled cells were gated in an area of macrophages on the scatter cytogram and analysed by Cytron flow cytometry.

BALF of CF patients [13, 14]. Recently, it has been reported that low-dose and long-term treatment with erythromycin or other macrolides improves the clinical symptoms and prognosis of patients with DPB [9, 27–29]. An earlier study reported that macrolides suppress the production of *P. aeruginosa* exo-enzymes *in vitro* [28, 30]. This is one possible role for macrolides in *P. aeruginosa* infections.

Earlier work also reported that ETA was detected in the serum of mice with endogenous *P. aeruginosa* septicaemia, and that systemic administration of ETA before or at the same time as LPS injection enhanced LPS-induced TNF production in murine serum [12]. The findings suggest that ETA is responsible for enhancing the production to a lethal dose of TNF in the presence of LPS in systemic *P. aeruginosa* infection.

It is of interest that in the present study, ETA suppressed LPS-induced TNF production in murine lung; the opposite was the case in the systemic model. The strongest priming effect of ETA in LPS-induced TNF production in the systemic model was observed when ETA was administered 4 h before LPS. In the lung model reported here, ETA was also injected 4 h before LPS inoculation. These findings also showed that administration of ETA did not change total cell number or the percentage of neutrophils, suggesting that participation of neutrophils in the suppressive effect of ETA can be neglected in this experimental schedule. A difference of local concentration of ETA between systemic and lung models may be one reason for the discrepancy. However, a wide range of ETA concentrations suppressed TNF production by AMs *in vitro*, indicating that other mechanisms are involved. TNF is capable of increasing host resistance to bacterial infection [31]. The suppressive effect of ETA on LPS-induced TNF production may weaken the role of TNF in the augmentation of host resistance and favour the infecting *P. aeruginosa* organisms. Jaffar-Bandjee *et al.* [13] reported increased levels of exo-enzymes in sputum of CF patients (levels of ETA, EL and AP were 0.3–126, 0.3–79 and 0.3–32 ng/ml, respectively). The dose and concentration of ETA in the present study is similar to those in their report.

ETA is a protein toxin that inhibits polypeptide synthesis through ADP ribosylation of elongation factor 2, as does diphtheria toxin [32]. Recent studies have reported the immunological activities of ETA in addition to its direct cytotoxicity against mammalian cells. Holt and Misfeldt [33] demonstrated that ETA modulates the in-vitro and in-vivo antibody response in mice. ETA has also been reported to stimulate in-vitro production of IL-1 in murine peritoneal macrophages [34] and to induce murine cytotoxic T lymphocytes [35]. Staugas *et al.* [36] reported that ETA depresses lymphoproliferation, TNF, lymphotoxin, γ -interferon and IL-1 production in human

leucocytes *in vitro*. Although the influence of ETA on cytokine production varied in these studies, it is possible that ETA acts as an immunomodulator through regulatory cytokines or other factors.

The findings of the present study suggest that ETA suppressed the expression of CD14 and CD11c/CD18 molecules (presumed LPS receptor molecules) on the membrane surfaces of AMs, the expression of TNF mRNA in AMs and protein synthesis and/or secretion of TNF. The suppression of the expression of TNF mRNA may be secondary to the depressed expression of the LPS receptors CD14 [25] and CD11c/CD18 [26] on the surface of AMs. As production of TNF was completely inhibited in spite of only a partial depression of mRNA, protein synthesis or secretion of TNF, or both, is probably involved. The clinical significance of the TNF-suppressing effect of ETA in the lung is still unclear. It is speculated that ETA regulates the severe acute inflammation caused by LPS and induces chronic inflammation.

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