MICROBIAL PATHOGENICITY

Induction of interleukin-10 and down-regulation of cytokine production by Klebsiella pneumoniae capsule in mice with pulmonary infection

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The role of the capsule of Klebsiella pneumoniae in inducing cytokine production was investigated by comparing the responses of mice with experimentally induced pneumonia caused by capsule (strain DT-8) or non-capsule (mutant strain DT-X) K. pneumoniae. Anaesthetised ICR mice were inoculated intranasally. Whereas all DT-S-infected mice died within 3 days, no deaths were observed in DT-X-infected mice by 14 days after infection. During the early stage of infection, interferon-γ (IFN-γ) levels in broncho-alveolar lavage fluid (BALF) of DT-X-infected mice were significantly higher than those in DT-S-infected mice. In contrast, in the late stage of infection, serum levels of granulocyte macrophage-colony stimulating factor (GM-CSF) and IFN-γ in DT-S-infected mice were significantly higher than those in DT-X-infected mice. Levels of interleukin-10 (IL-10) in BALF and serum of DT-S-infected mice were significantly and persistently higher than those of DT-X-infected mice. The IL-10/TNF-α (tumour necrosis factor-α) ratios in BALF and serum indicated that higher levels of IL-10 production were induced in mice infected with strain DT-S than in those infected with strain DT-X. The results suggest that the capsule of K. pneumoniae may induce IL-10 production at the site of infection and, thereafter, these high IL-10 levels may serve to down-regulate the expression of pro-inflammatory cytokines.

Introduction

Klebsiella pneumoniae is one of the most frequently isolated gram-negative bacterial pathogens in severe nosocomial infections. The rapidly progressive clinical course of klebsiella pneumonia, which is often complicated by multilobar involvement, lung abscesses and high mortality, leaves little time to institute effective antibiotic treatment. Furthermore, an increasing proportion of nosocomial K. pneumoniae isolates are resistant to multiple antibiotics commonly used in intensive care units [1–3]. Therefore, alternative approaches to prophylaxis and supportive treatment of klebsiella respiratory infections are needed.

An effective host defence against bacterial lung infection is dependent primarily upon rapid clearance of the organism from the respiratory tract, which is mediated by the influx or activation of phagocytic cells (including neutrophils and macrophages), or both. The recruitment and activation of leucocytes in the setting of bacterial challenge is a complex and dynamic process that involves coordinated expression of both pro- and anti-inflammatory cytokines. Several cytokine mediators are thought to play important roles in host defence against bacterial invasion. For example, tumour necrosis factor-α (TNF-α) expression is elevated in the alveoli of patients with bacterial pneumonia [4].

The kinetics of TNF-α, interleukin-1β (IL-1β) and IL-6 production and synthesis have already been evaluated in a murine K. pneumoniae model of pneumonia. During the early stage of infection, TNF-α and IL-6 levels in broncho-alveolar lavage fluid (BALF) of mice infected with a non-capsulate strain were significantly higher than those in mice infected with a capsule strain [5]. However, other cytokines such as IFN-γ and granulocyte macrophage-colony stimulating factor (GM-CSF) have also been reported to play roles in the inflammatory process in pulmonary infections [6, 7]. Therefore, this study investigated the influence of capsulation of K. pneumoniae on production of IFN-γ
and GM-CSF in the same experimental murine model of *K. pneumoniae* pneumonia.

Greenberger *et al.* [8] reported that IL-10 is produced during klebsiella pneumonia and that inhibition of IL-10 bioactivity in vivo results in prolonged survival. IL-10 functions as an anti-inflammatory cytokine and suppresses production of inflammatory cytokines such as TNF-α, IL-6 and IL-1β. Although the differences in cytokine production demonstrated in the previous study [5] were probably due to shielding of lipopolysaccharide (LPS) by the capsule of this organism, we hypothesised that the capsule of *K. pneumoniae* itself may suppress host immunological responses by stimulating IL-10 production. This study examined the effect of the capsule of *K. pneumoniae* on IL-10 production and also evaluated the balance of pro/anti-inflammatory cytokines in BALF and serum during *K. pneumoniae* pneumonia.

**Materials and methods**

**Animals**

Specific pathogen-free (SPF) 4-week-old male Slc; ICR mice (Japan Shizuoka Laboratory Center, Shizuoka, Japan) each weighing 18–20 g were used in the experiments. All mice were housed in SPF conditions within the animal care facility at Toho University. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Toho University School of Medicine.

**Bacteria**

*K. pneumoniae* strain DT-S (biotype *edwardsii*, capsular type 1) was a gift from Takeda Pharmaceutical Co., Osaka, Japan. The DT-S strain was established from *K. pneumoniae* strain DT, which was isolated from the sputum of a patient with pneumonia. Cloning of strain DT-S was achieved by infecting mice with *K. pneumoniae* strain DT and then re-isolating the organism and repeatedly infecting other mice with the harvested strain until cloning was complete. *K. pneumoniae* strain DT-X was a non-mucoid mutant isolated by subculture of strain DT-S. The lack of a capsule in strain DT-X was confirmed by staining with Indian ink. Both strains were maintained at −80°C in brain heart infusion (BHI) broth containing glycerol 15%.

**Pulmonary infection with *K. pneumoniae***

Bacteria grown on BHI agar for 24 h at 37°C were suspended in sterile saline and adjusted to 1 × 10⁶ cfu/ml. Each mouse was anaesthetised with an intradermal injection of 0.1 ml of xylazine/ketamine HCl/saline mixture, and then inoculated intranasally with 40 μl of *K. pneumoniae* suspension (4 × 10⁶ cfu).

Animal survival was monitored every 24 h until 14 days after inoculation with *K. pneumoniae*.

**Broncho-alveolar lavage (BAL)**

Each mouse was killed by deep ether anaesthesia followed by immediate exposure of the trachea. Mice were intubated with a polyethylene catheter (1.0 mm OD) and BAL was performed with 1 ml of sterile saline; c. 0.5–0.6 ml of lavage fluid was retrieved from each mouse. Lavage fluid was centrifuged at 3000 g for 5 min. Supernates were collected and stored at −80°C for assessment of cytokine levels.

**Cytokine measurement**

Concentrations of murine GM-CSF, IFN-γ and IL-10 in serum and BALF were determined with commercial enzyme-linked immunosorbent assay (ELISA) kits purchased from Enzyme (Cambridge, MA, USA) and Biosource International (Camarillo, CA, USA). Assays were performed according to the protocol recommended by the manufacturers.

**Statistical analysis**

Data were expressed as means and SEM. Differences in survival rates were analysed by the χ² test, and differences in the number of bacteria, BAL cells and cytokine levels were analysed by the Mann-Whitney U-test. Data were considered statistically significant when p values were < 0.05.

**Results**

**Survival of mice after pulmonary infection with *K. pneumoniae***

 Intranasal inoculation of strain DT-S induced toxemia within 24 h, and symptoms of lethargy, reduced food intake and ruffled fur. Significant and progressive respiratory distress developed at 48 h. As shown in Fig. 1, all strain DT-S-infected mice died within 72 h of inoculation. In comparison, all strain DT-X-infected mice remained symptom-free and healthy 14 days after inoculation.

**Kinetics of inflammatory cytokines in serum and BALF**

During the early stage of infection, there were no significant differences in the GM-CSF levels in serum or BALF between mice infected with strains DT-S or DT-X. However, at 24 h after inoculation, serum GM-CSF levels in DT-X-infected mice were significantly higher than those in strain DT-S-infected mice. In contrast, by the late stages of infection (48 h after inoculation), serum GM-CSF levels in strain DT-S-infected mice were significantly higher than those in strain DT-X-infected mice (Fig. 2).
BALF IFN-γ levels in strain DT-X-infected mice were significantly higher than those in strain DT-S-infected mice at all times between 6 and 48 h after inoculation. In contrast, serum IFN-γ levels in mice infected with *K. pneumoniae* strain DT-S were higher than those in mice infected with strain DT-X at all times after inoculation (statistically significant at 24 h and 48 h) (Fig. 3).

**Kinetics of anti-inflammatory cytokines in serum and BALF**

Determination of IL-10 levels in BALF and serum of mice after pulmonary infection with *K. pneumoniae* revealed that at all times BALF and serum IL-10 levels in strain DT-S-infected mice were significantly higher than those in strain DT-X-infected mice (Fig. 4).

**IL-10/TNF-α ratio**

The ratio of IL-10 to TNF-α in BALF of strain DT-S-infected mice was higher than in strain DT-X-infected mice at all times. The serum IL-10/TNF-α ratio was

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**Fig. 1.** Survival of mice after pulmonary infection with *K. pneumoniae* strain DT-S or DT-X. Mice were inoculated intranasally with 40 μl of bacterial suspension containing 4 × 10⁷ cfu of *K. pneumoniae*. ○, DT-S-infected mice; ●, DT-X-infected mice (n = 17 in each group).

**Fig. 2.** BALF (a) and serum (b) GM-CSF concentrations in mice infected with *K. pneumoniae* strain DTX or DT-S. Values are means and SEM. ○, DT-S-infected mice (n = 8); ●, DT-X-infected mice (n = 8). *p < 0.05, #p < 0.01.

**Fig. 3.** BALF (a) and serum (b) IFN-γ concentrations in mice infected with *K. pneumoniae* strain DTX or DT-S. Values are means and SEM. ○, DT-S-infected mice (n = 8); ●, DT-X-infected mice (n = 8). *p < 0.05, #p < 0.01.
also higher in strain DT-S-infected mice at 12 h after infection (Fig. 5).

**Discussion**

An earlier study evaluated the role of the bacterial capsule in the inflammatory response in a mouse pneumonia model induced by either capsulate (DT-S) or non-capsulate (DT-X) strains of *K. pneumoniae* [5]. Strain DT-S was found to be a more potent pathogen than strain DT-X in this model and the retardation of inflammatory responses, including production of inflammatory cytokines (TNF-α, IL-1β) and IL-6, due to the capsule of the organism, resulted in severe infection. The present study investigated the kinetics of production of other immunostimulatory cytokines (GM-CSF and IFN-γ) and the immunosuppressive cytokine IL-10 in the same model of infection.

It has been reported that recombinant G-CSF and GM-CSF can be used as pharmacological agents to accelerate production of neutrophils and monocytes/macrophages to enhance host defences in clinical situations [9]. In contrast, Held et al. [10] reported that subcutaneous injection of G-CSF before infection in an experimental model of murine klebsiella pneumonia increased mortality by promoting replication of bacteria in the liver and spleen, and worsened the outcome of the pneumonia. G-CSF specifically stimulates proliferation, differentiation and function of neutrophils, whereas GM-CSF exerts profound effects on the macrophage lineage [11]. Havill et al. [12] reported that mortality 72 h after endotoxin challenge in mice pretreated with GM-CSF was significantly higher than in control (saline-treated) mice. Others have reported that GM-CSF administered intravenously may impair the ability of neutrophils to infiltrate an inflammatory focus [13].

In the model used in the present study, BALF GM-CSF levels in strain DT-X-infected mice were higher than in strain DT-S-infected mice 48 h after inoculation, suggesting a protective role for GM-CSF. Serum GM-
CSF concentrations at 48 h after infection were significantly higher in strain DT-S-infected mice, reflecting the severity of infection. However, 24 h after infection, serum GM-CSF concentrations were significantly higher in DT-X-infected mice, probably due to transient bacteraemia.

Studies in intact infected animals have clarified the broad-spectrum activity of IFN-γ in vivo in both endogenous and exogenous forms. With respect to the exogenous effect of IFN-γ, in a K. pneumoniae-infected wound model, mice pretreated with IFN-γ followed by infection and then 2 days of additional IFN-γ treatment survived significantly longer than control mice [7]. With respect to the endogenous role of IFN-γ, mortality increased appreciably after pulmonary infection with Streptococcus pneumoniae in IFN-γ gene knockout mice [14]. These results suggest a protective role for IFN-γ in host responses to these pathogens. In contrast, it was reported that administration of a monoclonal antibody to IFN-γ before or after Escherichia coli challenge in an experimental gram-negative sepsis model protected mice from death [15]. In the present study, BALF IFN-γ levels in strain DT-X-infected mice were higher than those in strain DT-S-infected mice at all time points. This reaction may have been induced by the host response to the exposed LPS of the DT-X strain. We postulate that IFN-γ may also play a protective role in this model at the site of infection. In contrast to BALF, serum IFN-γ levels in this model were higher in strain DT-S-infected than in strain DT-X-infected mice at 24 h and 48 h after inoculation. This probably reflects the severe septicemia at the late stage of infection with strain DT-S.

IL-10 expression is detrimental to innate and cell-mediated immunity in the lung [8, 16–18]. However, IL-10 has been demonstrated to exert anti-inflammatory properties, in part by down-regulating the expression of TNF-α and IL-1 [19–21]. IL-10 has been shown to attenuate the overproduction of cytokines in states of systemic immune cell activation, as occurs in sepsis [22]. Greenberger et al. [8] reported that in the experimental model of murine klebsiella pneumonia, levels of TNF-α, MIP-2, MIP-1α and IL-10 mRNA and IL-10 protein in lung homogenates were maximal 48 h after inoculation. Administration of anti-IL-10 antibody resulted in enhancement of bacterial clearance, elevation of TNF, MIP-2 and MIP-1α levels, and prolonged survival. Based on this result, the investigators postulated that IL-10 regulated the expression of inflammatory cytokines [8]. On the other hand, van der Poll et al. [17] reported that in an experimental murine model of streptococcal pneumonia, levels of IL-10 in the lung were maximal 72 h after inoculation and IL-10 mRNA was detected from 12 h after inoculation; IL-10 was not detected in serum. When recombinant IL-10 was administered intratracheally with bacteria, lung levels of TNF-α and IFN-γ decreased and numbers of bacteria in the lungs and blood increased, as did mouse mortality; pretreatment of mice with anti-IL-10 antibody induced the opposite result. Based on these findings, the investigators postulated that IL-10 attenuated the effect of inflammatory cytokines in the lung [17].

Several studies have shown that IL-10 is released in experimental models of endotoxin- or staphylococcal enterotoxin B-induced shock, and that IL-10 (either exogenously administered or endogenously produced) confers a protective effect, most likely through down-regulation of pro-inflammatory cytokines such as TNF-α, IL-6 and IFN-γ [23–26]. Thus, it would appear that IL-10 plays two opposing roles in systemic infection: the anti-inflammatory properties of the molecule limit excess inflammation induced by microbial mediators such as endotoxin or superantigens; however, down-regulation of host defences by the anti-inflammatory actions of IL-10 interferes with clearance of invasive microbial pathogens. Maintaining a balance between the beneficial aspects of systemic inflammation and its deleterious effects is difficult in the face of ongoing infection.

IL-10 appears to be detrimental in models of infection where a vigorous immune/inflammatory response is crucial for effective clearance of the infective agent. Mohler et al. [27] studied cytokine kinetics in a murine model of pneumonia induced by virulent or avirulent strains of S. pneumoniae. Their results showed that IL-10 levels in lungs did not differ as a result of infection by virulent and avirulent strains. However, during the early stage of infection, the IL-10/TNF-α ratio in mouse lungs infected with the virulent strain was high, whereas it was low in mouse lungs infected with the avirulent strain. In the present study, the IL-10/TNF-α ratio in BALF in strain DT-S-infected mice was high during the early stage of infection, whereas it remained low at all times in strain DT-X-infected mice. However, the kinetics of IL-10 production differed from those documented by Mohler et al. [27]; BALF IL-10 levels in strain DT-S-infected mice were significantly higher than those in strain DT-X-infected mice during the early stage of infection. We speculate that the capsular polysaccharide alone of the DT-S strain may induce IL-10 production at the site of infection and that IL-10 serves to maintain immune homeostasis by down-regulating the expression of pro-inflammatory cytokines such as TNF-α, IL-6 and IFN-γ.

In summary, the capsule of K. pneumoniae induced IL-10 production from the early phase of infection and down-regulated the expression of TNF-α, IL-6 and IFN-γ at the site of infection. Therefore, the balance of pro- and anti-inflammatory cytokines may have an important effect on the host response in patients with bacterial pneumonia.

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