BACTERIAL PATHOGENICITY

Role of bacterial capsule in local and systemic inflammatory responses of mice during pulmonary infection with *Klebsiella pneumoniae*

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The role of bacterial capsule in inflammatory responses in experimentally induced pneumonia caused by *Klebsiella pneumoniae* was evaluated by comparing the host immunological responses in mice infected with capsulate strain DT-S and non-capsulate mutant strain DT-X. Anaesthetised ICR mice were infected intranasally with inocula of strain DT-S or DT-X. Mice infected with strain DT-X survived significantly longer than those inoculated with strain DT-S. Viable bacterial counts in lungs and blood increased rapidly in mice infected with strain DT-S, in contrast to the gradual decrease in their density in lungs and intermittent bacteraemia in mice infected with strain DT-X. The number of broncho-alveolar lavage (BAL) cells in mice infected with strain DT-X at 24 h after inoculation was significantly higher than in those infected with strain DT-S. In the early stages of infection, the levels of tumour necrosis factor- α and interleukin-6 in BAL fluid of mice infected with strain DT-X were significantly higher than those of mice infected with strain DT-S. In contrast, in the late stage of infection, the levels of these cytokines in serum of mice infected with strain DT-S were significantly higher than in mice infected with strain DT-X. These results suggest that *K. pneumoniae* capsule may suppress the host immunological responses, thus allowing the bacteria to grow, causing pneumonia, septicemia and death.

Introduction

Klebsiella pneumoniae is one of the most important causative organisms of nosocomial infections, particularly in debilitated patients, and the pneumonia caused by this organism is often difficult to treat [1, 2]. Recent epidemiological studies indicate that the frequency of nosocomial infections caused by *Klebsiella* spp. has increased substantially over the last 20 years [3, 4].

Several bacterial factors are known to contribute to the pathogenic mechanisms of *klebsiella* infections. Current research into the pathogenicity of *Klebsiella* spp. focuses on five major bacterial factors: capsule, fimbriae (pili), serum resistance, lipopolysaccharide (LPS) and siderophores [5]. In particular, because the majority of clinical isolates of *K. pneumoniae* have a well-defined capsule, it seems that the presence of

capsule is important for the virulence of this organism [6–8].

Pulmonary host defences involve anatomical and mechanical barriers, humoral immune activity, cell-mediated immune function and phagocyte activity. Alveolar macrophages are resident phagocytic cells in the lower airways and consist of a morphologically and functionally diverse group of cells that play several roles in host defence. As phagocytic cells, they can eliminate certain organisms. However, when the number of organisms increases beyond the ability of the macrophages to handle them or when the organisms are particularly virulent, the macrophage becomes a mediator of an inflammatory response by producing cytokines that recruit neutrophils into the lung [9]. Various cytokines have been identified that appear to play an important role in this regard, especially tumour necrosis factor- α (TNF- α) and interleukin-1 (IL-1) [10–12].

Tateda *et al.* [13] reported that mice infected with *K. pneumoniae* strain DT-S, which has a thick capsule,

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develop a type of pneumonia different from that in mice infected with the mutant strain DT-X, which is non-capsulate. Inoculation of the DT-S strain into the lungs of mice caused an expansive, voluminous lethal pneumonia characterised by thickening of alveolar septa with infiltration of inflammatory cells and accumulation of bacteria within alveolar spaces. On the other hand, lungs of mice infected with strain DT-X showed infiltration of inflammatory cells into alveolar spaces. These investigators proposed that the difference in the pathological response was probably due to the capsular polysaccharide (CPS), which may alter the recruitment and activation of inflammatory cells. They also demonstrated in in-vitro studies that stimulation of alveolar macrophages with CPS induced weaker IL-1 activity than stimulation with LPS. However, they did not investigate in detail the cytokine kinetics in infected mice [13].

This study evaluated the role of the bacterial capsule in the inflammatory response in mice infected with *K. pneumoniae* by comparing the host immunological responses, including cytokine kinetics. Studies were conducted in mice infected with either capsulate *K. pneumoniae* strain DT-S or non-capsulate mutant strain DT-X.

Materials and methods

Animals

Specific pathogen-free (SPF) 4-week-old male Slc; ICR mice (Japan Shizuoka Laboratory Center, Shizuoka, Japan) 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 kind gift from Takeda Pharmaceutical, Osaka, Japan. Strain DT-S was derived from *K. pneumoniae* 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, then isolating the organism and repetitively infecting other mice with the harvested strain until cloning was complete. *K. pneumoniae* strain DT-X was a non-mucoid mutant which was isolated by subculture of strain DT-S. Strain DT-X was confirmed to lack capsule by Indian Ink staining. Both strains were maintained at -80°C in brain heart infusion (BHI) broth containing glycerol 15%.

Pulmonary inoculation of *K. pneumoniae*

Bacteria grown on BHI agar for 24 h at 37°C were suspended in sterile saline and adjusted to a concentra-

tion of 1×10^8 cfu/ml. Each mouse was anaesthetised with an intradermal injection of 0.1 ml of xylazine, ketamine HCl and saline mixture, then inoculated intranasally with 40 μl of bacterial suspension containing 4×10^6 cfu of *K. pneumoniae*. Animal survival was recorded every 24 h until 14 days after inoculation.

Determination of viable bacterial counts in blood and lung tissues

Mice were killed with ether and cardiac blood samples were collected under aseptic conditions. Lungs were removed aseptically and were homogenised with a tissue homogeniser in 5 ml of sterile saline. Blood and homogenised lung samples were serially diluted with sterile saline and plated on BHI agar. After incubation for 24 h at 37°C , bacterial colonies were counted and viable bacteria counts were calculated. The remaining blood samples were allowed to clot at 4°C and then centrifuged at 15 000 rpm for 1 min. Serum samples were preserved at -80°C until measurement of the cytokines.

Broncho-alveolar lavage

Broncho-alveolar lavage (BAL) was performed to obtain BAL cells and fluid. 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. Approximately 0.5–0.6 ml of lavage fluid was retrieved from each mouse. Next, 50 μl of lavage fluid was added to 50 μl of Turk solution and the total BAL cell count was determined. Lavage fluid was centrifuged at 10 000 rpm for 5 min. Supernates were collected and stored at -80°C for assessment of cytokine levels. Sediments were stained with Wrights-Giemsa solution to determine the differential cell count in BAL fluid.

Measurement of cytokines

The concentrations of murine TNF- α , IL-1 β and IL-6 in serum and BAL fluid (BALF) were determined with commercial enzyme-linked immunosorbent assay (ELISA) kits purchased from Genzyme (Cambridge, MA, USA) and Biosource International (Camarillo, CA, USA). Assays were performed according to the protocols recommended by the manufacturers.

Statistical analysis

Data were expressed as mean and SEM. Differences in survival rates were analysed by the χ^2 test, whereas differences in the number of bacteria, BAL cells and cytokine levels were analysed by the Mann-Whitney U-test. Data were considered statistically significant if p values were <0.05 .

Results

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

Intranasal inoculation of strain DT-S caused systemic toxicity within 24 h, including lethargy, reduced food intake and ruffled fur. Significant and progressive respiratory distress developed at 48 h. As shown in Fig. 1, all mice infected with strain DT-S died within 72 h of inoculation. In comparison, none of the mice infected with strain DT-X developed any symptoms and all were still alive at 14 days after inoculation.

Bacterial clearance

As shown in Fig. 2, the number of viable *K. pneumoniae* isolated from the lungs of mice infected

with strain DT-S increased in a time-dependent manner. *K. pneumoniae* was first detected in heart blood samples from mice infected with strain DT-S at 6 h after inoculation and the number of viable bacteria increased rapidly thereafter. On the other hand, the number of viable *K. pneumoniae* isolated from lungs of mice infected with strain DT-X diminished gradually until 12 h and showed a transient rise at 24 h. *K. pneumoniae* strain DT-X was detected in heart blood of mice only at 48 h.

Pathological findings

The study also examined the differences in pathological features in the lungs of mice infected with *K. pneumoniae* strain DT-S or DT-X. Inoculation of *K. pneumoniae* strain DT-S into the lungs resulted in a

Fig. 1. Survival of mice after pulmonary infection with *K. pneumoniae* strain DT-S (○) and strain DT-X (●) ($n = 17$ in each group). Mice were inoculated intranasally with $40 \mu\text{l}$ of bacterial suspension containing 4×10^6 cfu of *K. pneumoniae*. DT-S versus DT-X, $p < 0.01$.

Fig. 2. Viable bacterial counts in lungs (□) and serum (■) of mice after inoculation of *K. pneumoniae* strain DT-S (a) and DT-X (b). Values are mean (SEM) of five or six mice in each group.

partial infiltration of inflammatory cells at 24 h (Fig. 3a). Thereafter, expansive, voluminous pneumonia characterised by destruction of alveolar septa was induced by bacterial accumulation in alveolar spaces at 48 h (Fig. 3c). On the other hand, at 24 h and 48 h after inoculation with *K. pneumoniae* DT-X, the lungs showed infiltration of inflammatory cells into alveolar and interstitial spaces without destruction of alveolar septa during the course of this pneumonia (Fig. 3b and d).

Cell counts and differentiation in BAL fluid

To assess the role of the capsule of *K. pneumoniae* in mediating lung leucocyte influx, BAL was performed 6, 12, 24 or 48 h after pulmonary inoculation of *K. pneumoniae* strain DT-S or DT-X. The results are depicted in Fig. 4. The number of BALF cells in mice infected with *K. pneumoniae* strain DT-X was significantly higher than in mice infected with strain DT-S at 24 h. On the other hand, the number of BALF cells

in mice infected with strain DT-S was significantly higher at 48 h than in mice infected with strain DT-X. The counts of neutrophils, lymphocytes and monocytes/macrophages in BALF were also determined, but no significant differences between the two groups of mice were observed at any time interval after inoculation of bacteria (data not shown). BAL was also done on control mice. The results obtained with normal mice indicated that alveolar macrophages were dominant (>80%) among BALF cells. The total numbers of BALF cells in mice infected with strain DT-S or DT-X were significantly higher than those found in normal control mice.

Kinetics of inflammatory cytokines in serum and BALF

In the early stage of infection (6 h after inoculation), BALF concentrations of TNF- α in mice infected with strain DT-X were significantly higher than in mice infected with strain DT-S. In the late stage of infection

Fig. 3. Histological sections of the lungs at 24 and 48 h after intranasal administration of *K. pneumoniae* strain DT-X and DT-S. (a) Lung tissue of a representative mouse 24 h after inoculation of *K. pneumoniae* DT-S showing partial infiltration of inflammatory cells (H&E stain, $\times 100$). (b) and (d) Lung tissues obtained from representative mice 24 h and 48 h after inoculation of *K. pneumoniae* DT-X. This strain induced pathological findings in the lungs that were different from those induced by strain DT-S, characterised by interstitial and intra-alveolar inflammatory cell infiltration (H&E stain, $\times 100$). (c) Representative lung tissue of a mouse 48 h after inoculation of *K. pneumoniae* DT-S showing expansive, voluminous pneumonia. Note thickening of alveolar septa with infiltration of inflammatory cells. Alveolar spaces show a ballooning pattern (H&E stain, $\times 100$).

Fig. 4. Total BALF cell counts of mice infected with *K. pneumoniae* strain DT-S (□) or DT-X (■). Values are mean (SEM) of 7–10 mice in each group; * $p < 0.05$.

(24 h after inoculation), the levels of TNF- α in the serum of mice infected with strain DT-X were significantly higher than in those infected with strain DT-S (Fig. 5). On the other hand, IL-1 β concentrations in the BALF of mice infected with *K. pneumoniae* strain DT-S were higher than in mice infected with strain DT-X at 24 h. Changes in serum IL-1 β levels in mice infected with *K. pneumoniae* DT-X were similar to those of TNF- α (Fig. 6). IL-6 levels in BALF of mice infected with *K. pneumoniae* strain DT-X were also determined; these were significantly higher than in mice infected with strain DT-S at any time point. In contrast, serum IL-6 levels in mice infected with strain DT-S were significantly higher than in those infected with strain DT-X at 48 h (Fig. 7).

Discussion

This study of experimentally induced *K. pneumoniae* pneumonia focused on differences in the virulence of DT-S and DT-X strains. The results showed that mice infected with strain DT-S were dying at 48 h after inoculation and all were dead at 72 h. *K. pneumoniae* strain DT-S was detected in the blood at the time when the density of infection increased in the lungs. Therefore, the cause of death was septicaemia, which followed severe pneumonia.

In all mice infected with strain DT-S, abscesses and hepatisation were observed just before death, and pleural cavities contained a viscous exudate [14]. Bacteria proliferated in the alveolar spaces soon after inoculation and these spaces were packed with the bacilli. Thus, inflammatory cells could not be recruited into alveolar spaces, although they could infiltrate the alveolar septa. Inflammatory cells in the alveolar septa may produce some enzymes and superoxide, which result in further tissue damage. At the same time, proliferation of bacteria was associated with destruction of alveolar septa and alveolar structure, resulting in a progressive expansion of the lesion [13].

With regard to infection caused by strain DT-X, the number of bacteria in the lungs decreased time-dependently, although there was a transient increase at one point. Pathological examination and BALF showed inflammatory cell recruitment to alveolar spaces and interstitia at 24 h after infection. Because strain DT-X is non-capsulate, LPS of these bacteria is not shielded by the capsule and, hence, inflammatory cells might be activated immediately by stimulation by the exposed LPS of strain DT-X. Thus, it seems that cells of *K. pneumoniae* DT-X were easily cleared from the alveolar spaces by recruited inflammatory cells whereas those of strain DT-S were capable of escaping

Fig. 5. TNF- α concentrations in (a) BALF and (b) serum of mice infected with *K. pneumoniae* strain DT-X (●, $n = 8$) or DT-S (○, $n = 8$). Values are mean (SEM); * $p < 0.05$.

Fig. 6. IL-1 β concentrations in (a) BALF and (b) serum of mice infected with *K. pneumoniae* strain DT-X (●, n = 8) or DT-S (○, n = 8). Values are mean (SEM); *p < 0.05.

Fig. 7. IL-6 concentrations in (a) BALF and (b) serum of mice infected with *K. pneumoniae* strain DT-X (●, n = 8) or DT-S (○, n = 8). Values are mean (SEM); *p < 0.05, #p < 0.01.

host defence systems by suppressing the immune response due to the bacterial capsule.

Domenico *et al.* [15] reported that bismuth-dimercaprol inhibited capsular expression, thus promoting phagocytosis, enhancing the reactivity of specific antibodies for LPS O antigen, LPS core epitopes or outer-membrane proteins and stimulating complement interaction with otherwise capsulate *K. pneumoniae* by unmasking bacterial surface structures. The results of the present study also demonstrated that the absence of the bacterial capsule enhances the immune system reactivity to bacteria. Therefore, the results are consistent with those of Domenico *et al.*

Laichalk *et al.* [12] demonstrated that administration of

TNF antagonist resulted in a significant reduction of BALF neutrophils, increased *K. pneumoniae* counts and shortened the survival time of mice. On the other hand, administration of TNF agonist resulted in a significant increase in BALF neutrophils, reduction of *K. pneumoniae* counts and prolongation of survival time [11]. An earlier study reported that serum concentrations of TNF- α were significantly higher in mice with bacteraemia than in those without bacteraemia and that treatment with murine anti-TNF- α monoclonal antibody (MAb) significantly reduced the mortality from septic infection [16]. However, clinical trials in which anti-TNF- α MAbs were given to patients with sepsis produced unfavourable results [17]. In any case, TNF is probably a key mediator of innate immunity against gram-negative bacillary infec-

tions including sepsis. In the present study, these findings were extended by examining the dynamics of TNF- α production in the lungs of mice during development of pneumonia by inoculation of two different strains of *K. pneumoniae*. The results showed significantly higher levels of TNF- α in mice infected with strain DT-X relative to those infected with strain DT-S during the early stage of infection. It may be speculated that this response was probably due to stimulation by exposed LPS of the non-capsulate DT-X strain. A further speculation is that the significantly greater production of TNF- α in the lungs of mice infected with strain DT-X might induce marked inflammatory cell recruitment into the lungs.

Laichalk *et al.* [12] studied an experimental model of murine klebsiella pneumonia and reported a bimodal pattern of TNF mRNA and protein in BALF with two peaks noted during the course of infection. The results of immunohistochemical analyses in the same study demonstrated that the first peak was derived from macrophages and the second peak was derived from neutrophils. The present study was also able to demonstrate a bimodal pattern of TNF- α production in BALF from mice infected with strain DT-X. However, the type of leucocytes that induced the rise in TNF- α production in this model could not be determined. Serum TNF- α concentrations were also significantly higher in mice infected with strain DT-X at the late phase of infection, probably because of transient bacteraemia.

IL-6 levels in BALF of mice infected with strain DT-X were higher than those in mice infected with strain DT-S at all time intervals. This reaction may also reflect the host response to the exposed LPS of strain DT-X. In contrast to BALF, serum IL-6 levels were higher in mice infected with strain DT-S than in mice infected with strain DT-X at 48 h. These changes may reflect local or systemic inflammation, or both, and therefore, discrimination can be made between pneumonia and sepsis by evaluating IL-6 levels in BALF and serum samples. Furthermore, these results suggest that serum IL-6 might be a suitable marker for the severity of sepsis.

The kinetics of IL-1 β production in BALF were different from those of TNF- α and IL-6, i.e., IL-1 β levels in BALF from mice infected with strain DT-S were significantly higher than those in mice infected with strain DT-X at 24 h after inoculation. The exact mechanism of this observation is not clear at present and further studies are necessary to explore the mechanisms of different cytokine production patterns. In this regard, Mohler *et al.* [18] studied cytokine kinetics in a murine model of pneumonia induced by virulent or avirulent strains of *Streptococcus pneumoniae*. Their results showed an initial local expression of pro-inflammatory cytokines (TNF- α and IL-6) with the avirulent strain, whereas these cytokines were released

after a delay in infections caused by the virulent strain. The results of the present study were consistent with those of Mohler *et al.* [18], especially with regard to acute cytokine reaction in mice infected with the avirulent strain.

In summary, in a murine model of pneumonia with a capsulate strain of *K. pneumoniae* or a non-capsulate mutant strain, the present study demonstrated that the capsule of this organism alters the immunological host responses. The results showed that *K. pneumoniae* bacterial capsule may play a key role in the suppression of host responses at an early phase of infection with resulting pneumonia followed by severe septicaemia and death. The effects of the capsule of *K. pneumoniae* on the expression of other cytokines are currently under study.

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