IMMUNISATION AND IMMUNOTHERAPY

Effect of passive immunotherapy on murine gut-derived sepsis caused by *Pseudomonas aeruginosa*

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The effect of passive immunotherapy with antisera against heat-killed *Pseudomonas aeruginosa* and three of its exo-enzymes (elastase, alkaline protease and exotoxin A) in gut-derived *P. aeruginosa* sepsis was evaluated. Mice were given a suspension of *P. aeruginosa* strain D4 in their drinking water, together with ampicillin (200 mg/kg) to disrupt the normal bacterial flora. Cyclophosphamide was then administered to induce translocation of *P. aeruginosa* that had colonised the gastrointestinal tract so that gut-derived septicaemia was produced. In this model, intraperitoneal administration of antiserum against heat-killed bacteria, 100 µl/mouse, twice a day for 3 consecutive days significantly increased the survival rate over that of mice treated with normal rabbit serum. Antiserum against elastase, alkaline protease, or a combination of these two antisera, failed to provide significant protection. In contrast, antiserum against exotoxin A significantly increased the survival rate over that of mice treated with normal rabbit serum. These results indicate that passive immunisation with antiserum against heat-killed bacteria and exotoxin A, but not with antiserum against either elastase or alkaline protease, protects mice against gut-derived sepsis caused by *P. aeruginosa*.

Introduction

Pseudomonas aeruginosa is an important opportunist pathogen that causes severe infections such as septicaemia in immunocompromised patients. Although antibiotics are thought to be the most effective form of therapy against infections caused by this micro-organism, they are frequently ineffective due to its innate resistance to various antimicrobial agents. Therefore, effective immunotherapy may potentially represent a useful alternative therapy administered either alone or in combination with antibiotics.

Previous studies have shown that neutrophils [1], complement [2] and immunoglobulins [3] play important roles in host defence against *P. aeruginosa* infection. However, infection with *P. aeruginosa* is frequently identified in immunocompromised patients with neutropenia induced by antineoplastic chemotherapy [4]. Because normal neutrophil function is compromised in patients with neutropenia, humoral immune responses may play a more important role in the recovery of such patients from *P. aeruginosa*

infection. Vaccination with microbial antigens may be the most effective method for the induction of protective humoral immune responses [5].

The protective efficacy of immunisation with heat-killed *P. aeruginosa* has been reported previously and such immunisation has been found to provide complete protection of laboratory animals against fulminant sepsis [6]. Studies with vaccines prepared from *P. aeruginosa* alkaline protease, elastase and exotoxin A toxoids showed that a combination of alkaline protease and exotoxin A toxoids represents a potential candidate for vaccination against *P. aeruginosa* sepsis [7]. Although these studies established the efficacy of immunotherapy to develop antibodies in *P. aeruginosa* sepsis, vaccination of immunocompromised patients is frequently unsuccessful because of immunodeficiency [8]. Therefore, passive immunisation may be a more practical method of providing immunotherapy to protect individuals against *P. aeruginosa* infection.

These considerations led to the investigation of the effect of antiserum against heat-killed *P. aeruginosa* and exo-enzymes (elastase, alkaline protease and exotoxin A) on murine gut-derived *P. aeruginosa*

sepsis associated with neutropenia induced by anti-neoplastic chemotherapy.

Materials and methods

Bacterial strain

P. aeruginosa D4 isolated from the blood of a neutropenic mouse with bacteraemia [9] was used in the present study. The strain was maintained at -80°C in Mueller-Hinton Broth (Difco) containing glycerol 15%.

Preparation of antiserum against heat-killed P. aeruginosa and exo-enzymes

P. aeruginosa grown on Trypticase Soy Agar (BBL) at 37°C for 18 h was suspended in sterile saline to a concentration of 10^{10} cfu/ml. Heat-killed bacteria were prepared by heating the bacterial suspension at 60°C for 1 h. The protein concentration was determined with a Protein Assay (BioRad Laboratories, Hercules, CA, USA). For the preparation of rabbit antiserum against heat-killed bacteria, the rabbit was immunised twice at 14-day intervals by intravenous (i.v.) injection of heat-killed bacteria at a protein concentration of $100\text{ }\mu\text{g/dose}$.

Purified exotoxin A was purchased from List Biological Laboratories (Campbell, CA, USA). Purified elastase and purified alkaline protease were purchased from Nagase and Co. Ltd. (Tokyo, Japan). Rabbits were immunised twice at 14-day intervals by i.v. injection of each of these exo-enzymes at a protein concentration of $100\text{ }\mu\text{g/dose}$; thereafter rabbit antisera against these exo-enzymes were prepared.

Animals

Inbred, specific pathogen-free male ddY mice (Japan Shizuoka Laboratory Centre, Shizuoka, Japan) weighing 20–24 g were used in the experiments. The animals were housed in sterile cages and received sterile distilled water, except when *P. aeruginosa* was being administered orally. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Toho University School of Medicine.

Survival of mice with gut-derived P. aeruginosa sepsis

Gut-derived *P. aeruginosa* sepsis was produced as described previously in this laboratory [10–12]. Briefly, bacteria were grown on Trypticase Soy Agar (BBL) at 37°C for 18 h, suspended in sterile saline 0.45%, and adjusted to a concentration of 10^7 cfu/ml. The bacterial suspension was added to the drinking water on days 1–3. To facilitate in-vivo colonisation of the *P. aeruginosa* strain D4, that is insensitive to ampicillin, ampicillin 200 mg/kg/day was injected intraperiton-

cally (i.p.) on days 1–3 to disrupt the normal intestinal flora. Mice were then treated with cyclophosphamide $150\text{--}200\text{ mg/kg}$ by i.p. injection on days 5 and 8 to produce a state of neutropenia. Each experiment was repeated at least twice. Survival was recorded every 24 h until 7 days after the second administration of cyclophosphamide.

The effect of each antiserum was investigated by i.p. administration; each antiserum was administered twice daily at a dose of $100\text{ }\mu\text{l/mouse}$ for 3 days, treatment beginning after the second cyclophosphamide dose. Control mice received identical amounts of normal rabbit serum.

Statistical analysis

Differences in survival rates among the groups were evaluated by the χ^2 test. A p value <0.05 denoted a statistically significant difference.

Results

Effect of antiserum against heat-killed P. aeruginosa on the survival of mice after gut-derived sepsis

The protective effects of antisera against heat-killed *P. aeruginosa* in mice with gut-derived *P. aeruginosa* sepsis were first evaluated. As shown in Fig. 1, the survival rate of mice treated with the antiserum was significantly higher than that of control mice treated with normal rabbit serum ($p < 0.01$). This result suggests that administration of antiserum against heat-killed *P. aeruginosa* protects mice against gut-derived sepsis.

Effect of antisera against alkaline protease and elastase of P. aeruginosa on the survival of mice after gut-derived sepsis

The ability of *P. aeruginosa* to produce high levels of exo-enzymes may contribute to the development of fatal septicaemia [13]. Therefore, the protective efficacies of antisera against *P. aeruginosa* alkaline protease and elastase on the survival of mice with gut-derived sepsis were studied. The results demonstrated that administration of antiserum against elastase or alkaline protease alone did not produce a significant protective effect compared with normal rabbit serum (Fig. 2). The effect of using both antisera together was studied in this model; the combined use of the two antisera also failed to provide significant protection.

Effect of antiserum against exotoxin A on the survival of mice with gut-derived P. aeruginosa sepsis

Finally, the effect of antiserum against exotoxin A on the survival of mice with gut-derived sepsis was

Fig. 1. Effect of antiserum against heat-killed *P. aeruginosa* on the survival of mice with gut-derived septicaemia. Test mice ($n = 10$, $\circ-\circ$) each received $100\ \mu\text{l}$ of antiserum against heat-killed *P. aeruginosa* D4 i.p. twice a day for 3 days ($\blacktriangledown$), treatment beginning after the second cyclophosphamide dose (CY). Control mice ($n = 10$, $\bullet-\bullet$) received identical amounts of normal rabbit serum. ABPC, ampicillin; test versus control, $p < 0.01$.

Fig. 2. Effect of antiserum against alkaline protease and elastase of *P. aeruginosa* on the survival of mice with gut-derived sepsis. The mice ($n = 10$ in the group) each received $100\ \mu\text{l}$ of antiserum against either elastase (.....) or alkaline protease (---) i.p. twice a day for 3 days ($\blacktriangledown$), treatment beginning after the second cyclophosphamide dose (CY). Another group of mice ($n = 10$) each received $100\ \mu\text{l}$ of a combined antiserum against elastase and alkaline protease (---). Control mice ($n = 15$) received identical amounts of normal rabbit serum (—). ABPC, ampicillin.

studied. Fig. 3 shows the survival kinetics of mice inoculated with anti-exotoxin A antiserum or normal rabbit serum as a control. The results clearly showed a significant improvement of the survival of mice treated with anti-exotoxin A over that of mice treated with normal rabbit serum.

Discussion

Previous studies have demonstrated that gut bacteria can cross the gastrointestinal mucosal barrier and spread systemically, a process termed bacterial translocation [14, 15]. Tani *et al.* [16] reported two patients who developed septic shock that was probably caused

by bacterial translocation. Other clinical studies employing faecal surveillance cultures from neutropenic leukaemic patients have shown that $>80\%$ of patients with bacteraemia caused by *P. aeruginosa* were intestinal carriers of the same strain, suggesting that the gastrointestinal tract may be the primary reservoir of opportunist bacteria [17]. Therefore, translocation of micro-organisms from the gastrointestinal tract may play a role in the pathogenesis of septic complications, especially in immunocompromised patients, and *P. aeruginosa* may be the major pathogen in this type of infection.

Berg *et al.* [18] reported that gram-negative enteric bacilli translocated systemically in mice treated with

Fig. 3. Effect of antiserum against exotoxin A on the survival of mice with gut-derived *P. aeruginosa* sepsis. Test mice ($n = 10$, ○-○) each received 100 μ l of antiserum against exotoxin A i.p. twice a day for 3 days (▼), treatment beginning after the second cyclophosphamide dose (CY). Control mice ($n = 10$, ●-●) received identical amounts of normal rabbit serum. ABPC, ampicillin; test versus control, $p < 0.05$.

antibiotics combined with an immunosuppressive drug, such as penicillin G and cyclophosphamide. In the present study, gut-derived *P. aeruginosa* sepsis was induced by administering ampicillin and cyclophosphamide to specific pathogen-free mice fed this organism. In this model, *P. aeruginosa* colonises the intestinal tract and invades body tissues after induction of immunosuppression or disruption of the intestinal mucosal barrier by administration of cyclophosphamide. Once the bacteria pass through the trap of Kupffer cells in the liver, systemic bacteraemia develops, followed by death in the majority of animals. This model resembles the septic infection caused in man by pathogens derived from the intestinal tract, especially in immunocompromised patients [19].

The virulence of *P. aeruginosa* is multifactorial and depends on several extracellular enzymes and other substances [20]. As experimental data suggest that lipopolysaccharide (LPS) is an important virulence factor in *P. aeruginosa* infection, most studies have focused on the protective activities of anti-LPS antibody or polysaccharide vaccines [5, 21–24]. The protective effects of immunisation with heat-killed *P. aeruginosa* have been shown recently in this laboratory and the data suggested that the main protective antibodies induced by vaccination might be those specific to LPS [6]. On the basis of these early results and the present findings, the hypothesis seems likely that the protective activity of antiserum against heat-killed *P. aeruginosa* was mainly induced by antibodies against LPS included in the antiserum.

Although immunotherapy with antibodies against LPS may be the most protective in *P. aeruginosa* infection, the presence of a wide variety of LPS serotypes of *P.*

aeruginosa makes it difficult to produce protective antibodies that have a wide spectrum of efficacy against *P. aeruginosa* [21–23, 25–27]. On the other hand, there may be common antigenicities in the exoenzymes (elastase, alkaline protease and exotoxin A) of *P. aeruginosa*, which have also been reported to be important virulence factors in *P. aeruginosa* infection [28–30]. These findings suggest that antibodies against these exo-enzymes might be useful for immunotherapy and protection against a wide range of *P. aeruginosa* strains.

Elastase interferes with the host immune system by cleaving IgG and IgA [31, 32], by inhibiting the activity of various cytokines [33] and by interfering with the function of neutrophils [34], T cells [35] and natural killer cells [36]. Immunisation with elastase toxoid is effective in certain experimental models of *P. aeruginosa* infection [37–39]. However, passively transferred IgG of anti-elastase serum showed no protective effect against *P. aeruginosa* in a murine burn wound sepsis model [23] or in an experimental leukopenic mouse model [40]. The results of the present study also failed to show any protective effect of antiserum against elastase; therefore, elastase is probably a less important virulence factor in gut-derived sepsis.

Alkaline protease is reported to cleave IgG [31], to degrade interferon- γ [41] and to inhibit neutrophil function [34]. The effectiveness of immunisation with alkaline protease toxoid has been described in haemorrhagic pneumonia in mink [37] and burns in mice [39]. These studies confirmed the importance of alkaline protease as a virulence factor and showed the effectiveness of immunisation with alkaline protease in the prevention of infection with *P. aeruginosa*.

However, the present study did not demonstrate a protective effect for antiserum against alkaline protease in murine gut-derived sepsis; and a combination treatment consisting of antisera against elastase and alkaline protease also failed to provide significant protection. In this regard, Wretling *et al.* [42] argued that although elastase and alkaline protease might play a part in localised *P. aeruginosa* infections, these enzymes are probably less important after septicaemia is established. Thus, elastase and alkaline protease may not contribute significantly to the pathogenicity of septicaemia in this mouse model.

Exotoxin A is believed to be the most toxic virulence factor produced by *P. aeruginosa* [43] and its cytotoxic activity extends to a wide variety of mammalian cells [44]. The importance of exotoxin A as a principal lethal factor has been demonstrated in a few studies of experimental *P. aeruginosa* infection [45, 46]. In this laboratory, infection of mice with an exotoxin-deficient mutant of *P. aeruginosa* (PA0-PR1) was associated with a significantly lower mortality than that associated with the parent strain [28]. This present study has also demonstrated the protective action of antiserum against exotoxin A. Taken together, data from these experiments support the role of exotoxin A as an important factor in the pathogenesis of gut-derived sepsis.

Previous studies have indicated that the importance of each of the above exo-enzymes as a virulence factor may vary from one model to another [1, 13, 47]. Therefore, the importance of these exo-enzymes in the pathogenesis of *P. aeruginosa* infections may depend on the type of infection. Furthermore, it is concluded that these exo-enzymes are not necessarily equally significant in *P. aeruginosa* infection and that exotoxin A may contribute to the development of gut-derived sepsis caused by *P. aeruginosa*.

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