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 opportunistic 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 microorganism, 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 µg/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 µg/dose; thereafter rabbit antiserum 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-
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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
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, Wretlind 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 septicemia is established. Thus, elastase and alkaline protease may not contribute significantly to the pathogenicity of septicemia 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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