Efficacies of alkaline protease, elastase and exotoxin A toxoid vaccines against gut-derived Pseudomonas aeruginosa sepsis in mice

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The protective efficacies of vaccines prepared from Pseudomonas aeruginosa alkaline protease, elastase and exotoxin A toxoids against gut-derived P. aeruginosa sepsis in mice were evaluated. Specific pathogen-free mice given P. aeruginosa strain D4 orally followed by cyclophosphamide (to promote translocation across the gut wall) died of bacteraemia. Mice immunised with one of the three individual toxoid vaccines were not significantly protected when compared to control mice immunised with bovine serum albumin. Combined immunisation with alkaline protease and elastase toxoids likewise showed no significant protective activity. However, combined immunisation with alkaline protease and exotoxin A toxoids significantly increased the survival rate, which reached 60% (compared with a 7.1% survival rate in the control group). These results show that alkaline protease and exotoxin A play important roles as pathogenic factors in gut-derived sepsis and that a combination of the two exoenzyme toxoids represents a logical candidate for vaccination against P. aeruginosa sepsis.

Introduction

Pseudomonas aeruginosa is a frequently isolated pathogen that causes septicaemia [1, 2] and exhibits a higher mortality rate than other gram-negative bacteria [1–3]. The need for effective immunotherapy is emphasised by the frequency of antibiotic resistance in this organism.

The cell surface of P. aeruginosa consists of lipopolysaccharide (LPS) and outer-membrane proteins (OMP). Some investigators have reported that active immunisation with LPS and OMP prevents infection [4–8]. However, LPS immunoprophylaxis is accompanied by a high incidence of toxic side-effects, and it is difficult to prepare OMP completely free of LPS contamination [6]. Furthermore, as there are several P. aeruginosa strains differing in immunotype, an effective vaccine requires the combination of numerous monovalent LPS antigens.

An alternative candidate vaccine against P. aeruginosa involves extracellular products such as alkaline protease, elastase and exotoxin A. These three proteins have been shown to be virulence factors in various animal models, including murine acute infection [9], the burned mouse model [10, 11], chronic pulmonary infection in rats [12] and murine corneal infection [13]. However, the efficacies of immunisation with toxoids of alkaline protease, elastase and exotoxin A against gut-derived sepsis are not well understood. This study investigated the efficacies of exoenzyme toxoid vaccines, either singly or in combination, against gut-derived sepsis in mice, a model representative of human infection.

Materials and methods

Animals

Specific pathogen-free male ddY mice (Japan Shizuoka Laboratory Center Co. Ltd, Shizuoka, Japan) weighing 20–24 g were used in the experiments. The animals were housed in sterile cages and received sterile distilled water except during the period when bacteria were being administered by the oral route. Experimental protocols were approved by the Institutional Animal Care and Use Committee at Toho University School of Medicine.
Bacterial strain

P. aeruginosa strain D4, isolated from the blood of a neutropenic mouse with bacteraemia [14], was used. The strain was maintained frozen at −80°C in Mueller-Hinton Broth (Difco Laboratories) containing glycerol 15%.

Vaccine preparations

Purified exotoxin A was purchased from List Biological Laboratories, Inc. (Campbell, CA, USA). Purified elastase and purified alkaline protease were purchased from Nagase and Co. Ltd, Tokyo, Japan. Bovine serum albumin (BSA) was purchased from Sigma. All protein determinations were performed by the Lowry method.

Toxoids of elastase and alkaline protease were prepared by a modification of the methods of Homma et al. [15]. Briefly, the reaction mixture containing protease (1 mg/ml, 0.1 M phosphate buffer, 0.2 M L-lysine and formalin 8% (pH 7.0) was kept at room temperature for 2 days. The reaction mixture containing elastase 2 mg/ml, formalin 4% and 0.2 M borate buffer (pH 9.0) was incubated overnight at 4°C. Each reaction mixture was dialysed against sufficient volume of phosphate-buffered saline (PBS) to remove the formalin.

A toxoid of exotoxin A was prepared by the method of Gilleland et al. [12]. Briefly, 1 mg of exotoxin A was dissolved in 1 ml of buffer containing 0.15 M NaCl, 0.01 M sodium phosphate and formalin 4% (pH 7.0). The solution was incubated at 37°C for 4 days and dialysed against a sufficient volume of PBS to remove the formalin. These toxoids were filtered through a 0.2-μm membrane filter and were stored at −80°C until required.

Immunisation protocol

The immunisation protocol and the induction of gut-derived sepsis is depicted in Fig. 1. Each mouse was immunised six times, at 2- or 3-day intervals over an 11-day period, by intraperitoneal injection of a single toxoid or a combination of toxoids. In individual or combination vaccines, the final concentration of each toxoid was 10 μg/dose. Control mice were inoculated intraperitoneally six times with bovine serum albumin (BSA) 10 μg/dose.

Monitoring the immune response

On day 14, 20-μl samples of blood were obtained from the retro-orbital plexus with disposable heparinised capillary tubes. Normal sera were obtained by bleeding eight BSA-immunised mice. The titre of IgG antibodies against each toxoid in the serum samples was determined by a modification of the ELISA method [14] used to study anti-alginate antibody. The absorbance at 405 nm (A405) was measured and the serum dilution that yielded a value of 0.2 in OD units was converted to ELISA units.

Survival of mice with gut-derived sepsis

Gut-derived sepsis was produced in mice as described previously [16, 17]. Bacteria were grown on Trypticase Soy Agar (BBL Microbiology Systems, Cockeysville, MD, USA) at 37°C for 18 h, suspended in sterile saline 0.45% and adjusted to a concentration of 10⁷ cfu/ml. Mice were allowed to take bacteria in the drinking water ad libitum during days 14–16. To aid P. aeruginosa colonisation, the normal intestinal flora of the mice was disturbed by administering ampicillin 200 mg/kg by intraperitoneal injection daily on days 1–3. Mice were then given cyclophosphamide 200 mg/kg by intraperitoneal injection on days 18 and 21. Each experiment was repeated at least twice. The animals were scored for mortality every 24 h up to 7 days following the second cyclophosphamide administration.

Statistics

The differences between the survival rates of groups were evaluated by the χ² tests. The differences between the antibody titres of toxoid-immunised mice and BSA-immunised mice were evaluated by the Mann-Whitney U test. A level of 5% was considered to be significant.

Results

Antibody responses after immunisation with various toxoids

Immunisation of mice with alkaline protease, elastase or exotoxin A toxoid induced serum IgG antibodies at mean (SD) titres of 914.3 (501.4), 971.4 (604.7), and 342.8 (222.5), respectively (Fig. 2). All antibody titres of BSA-immunised mice were <100. Immunised mice showed a significant increase in antibody titre against each exoenzyme toxoid when compared with titres in BSA-immunised mice (p < 0.01).
EXOENZYME VACCINATION AGAINST GUT-DERIVED P. AERUGINOSA SEPSIS

Fig. 2. Antibody responses after immunisation with various toxoids. On day 14, blood samples were obtained from each group of mice (n = 7) to determine the titre of IgG antibodies against each of the toxoids. Serum IgG antibody levels were determined by ELISA. All toxoid-immunised groups showed a significant increase in antibody titres compared with the BSA-immunised group.

**Effect on survival of single-toxoid immunisation**

The effect of immunisation with elastase, exotoxin A and alkaline protease toxoids individually on the survival of mice with gut-derived sepsis was evaluated. Fig. 3 shows the survival kinetics of mice immunised with the various toxoids or with BSA as a control. The results demonstrate that immunisation with alkaline protease, elastase or exotoxin A toxoid alone gave mice no statistically significant protection against death as a result of gut-derived P. aeruginosa sepsis.

**Effect on survival of immunisation with toxoid combinations**

The study also evaluated the effect of immunisation with a combination of alkaline protease toxoid and elastase toxoid, or a combination of alkaline protease toxoid and exotoxin A toxoid, on the survival of mice with gut-derived sepsis. Fig. 4 shows the survival kinetics of mice immunised with various toxoid combinations or with BSA as a control. It was found that immunisation with combined alkaline protease and exotoxin A toxoids reduced mortality significantly in comparison with controls, although immunisation with combined elastase and alkaline protease toxoids produced only a non-significant reduction.

**Discussion**

Clinical studies employing faecal surveillance cultures from immunocompromised patients suggest that the gastrointestinal tract may be a primary reservoir for opportunistic bacteria [18]. Berg and Deitch demonstrated that bacteria contained within the gut can cross the gastrointestinal mucosal barrier and spread systematically, a process termed bacterial translocation [19, 20]. In the present study, gut-derived P. aeruginosa sepsis was induced by feeding the organism to specific pathogen-free mice concurrently treated with ampicillin followed by cyclophosphamide [16, 17, 21, 22], and this animal model may closely mimic the pathophysiology of septicaemia in man [21].

The virulence of P. aeruginosa is multifactorial and caused by several extracellular enzymes and other substances [23, 24] whose importance depends on the type of infection [25]. For acute, systemic infections in immunocompromised patients, exotoxin A, alkaline protease and elastase are essential virulence factors. Elastase plays a major role in interfering with the host immune system by cleaving IgG and IgA [26, 27].
inhibiting the activity of various cytokines [28], and interfering with the function of neutrophils [29], T cells [30] and natural killer cells [31]. Immunisation with elastase toxoid has been found to be effective in haemorrhagic pneumonia of mink [15], in a rat model of chronic pulmonary infection [12] and in burned mice infected with *P. aeruginosa* [11].

Alkaline protease has also been reported to cleave IgG [26], degrade γ-interferon [32] and inhibit neutrophil function [29]. As with elastase toxoid immunisation, immunisation with alkaline protease toxoid has been found to be effective in mink haemorrhagic pneumonia [15] and in burned mice [11].

Exotoxin A is thought to be the principal lethal factor in experimental *P. aeruginosa* infection. This belief is founded on the demonstrable toxin in burn infections [33, 34], and endogenous septicaemia with *P. aeruginosa* in mice [22].

The above-cited reports reveal the importance of these exoenzymes as virulence factors and the effectiveness of immunisation with elastase and alkaline protease in preventing infection with *P. aeruginosa*. However, some authors have reported that passively transferred anti-elastase IgG does not protect against *P. aeruginosa* in a murine burn wound sepsis model [10] or in an experimental leukopenic mouse model [35]. Likewise, strains producing high levels of exotoxin A *in vitro* are not significantly more virulent when enclosed in vinyl diffusion chambers than strains producing little exotoxin when the chambers are implanted intraperitoneally into mice [36]. Furthermore, Wretlind *et al.* examined production of elastase and exotoxin A as *P. aeruginosa* virulence factors in an experimental mouse burn infection model and found no evidence that either exoenzyme plays any role in such infections [37].

The present study could not demonstrate the effectiveness of immunisation with any of the individual exoenzyme toxoids. Any protection that antibodies against a single exoenzyme may have provided was insufficient to produce a statistically significant reduction in mortality from gut-derived sepsis. Pavlovskis *et al.* likewise found that active immunisation with exotoxin A toxoid alone did not elicit a significant increase in survival rate following *P. aeruginosa* infection in burned mice [38]. This led us to study the effectiveness of combination vaccines, demonstrating that immunisation with a combination of alkaline protease and exotoxin A toxoids protected mice against mortality in this model. Therefore, it is clear that combined immunisation with these two toxoids has an additive or synergic effect in protecting against *P. aeruginosa* sepsis. On the other hand, these results revealed no significant protection following immunisation with a combination of elastase and alkaline protease toxoids. Thus, elastase may not contribute to pathogenicity in this model.

There may be differences in the importance of the various exoenzymes as virulence factors in different tissues or types of infection. For example, exotoxin A and elastase may be responsible for much of the destruction of respiratory tract tissue seen in pseudomonas infections, whereas alkaline protease appears to have no detectable effect [39]. Similarly, elastase, but not alkaline protease, contributes to the invasiveness of *P. aeruginosa* after intramuscular injection in mice [9]. On the other hand, a previous report showed that all but one of the lethal strains isolated from blood produced large quantities of exotoxin A or alkaline protease.
protease or both [40]. Thus, the ability of *P. aeruginosa* to produce high levels of exotoxin A and alkaline protease may contribute to the development of fatal septicaemia [40]. We conclude that, although these exoenzymes may be important factors in the pathogenesis of *P. aeruginosa* infection, it does not necessarily follow that all are equally significant in different types of infection.

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