VACCINES AND IMMUNISATION

Effect of immunisation with *Pseudomonas aeruginosa* on gut-derived sepsis in mice

T. MATSUMOTO, K. TATEDA, S. MIYAZAKI, N. FURUYA, A. OHNO, Y. ISHII, Y. HIRAKATA* and K. YAMAGUCHI

Department of Microbiology, Toho University School of Medicine, Omori-Nishi, Ota-ku, Tokyo and *Department of Laboratory Medicine, Nagasaki University School of Medicine, Sakamoto, Nagasaki, Japan

The protective efficacy of immunisation with heat-killed *Pseudomonas aeruginosa* on murine gut-derived sepsis was evaluated. Mice were immunised intraperitoneally six times with heat-killed bacteria. This induced mean (SEM) serum IgG and IgM antibodies of 1792 (374.7) and 37.3 (8.9) ELISA units, respectively. Specific pathogen-free mice given *P. aeruginosa* strain D4 orally died of bacteraemia after administration of cyclophosphamide. Immunisation with heat-killed bacteria significantly increased the survival rate compared with that of control mice immunised with bovine serum albumin. Macroscopic observation revealed marked production of liver abscesses in mice immunised with bovine serum albumin but not in those immunised with heat-killed bacteria. Only low titres of antibody against the exoenzymes alkaline protease, elastase and exotoxin A were observed, and no significant difference between antibody titres to boiled and unboiled suspensions of sonicated *P. aeruginosa* was detected. This suggests that the main protective antibodies might be those specific to the heat stable antigen (lipopolysaccharide). Immunisation with heat-killed bacteria provided complete protection against death from gut-derived *P. aeruginosa* sepsis.

Introduction

*Pseudomonas aeruginosa* sepsis carries a higher mortality rate than that due to other gram-negative bacteria [1–3]. Furthermore, treatment of *P. aeruginosa* infection is frequently hindered by antibiotic resistance, thus emphasising the need for effective immunotherapy. Some reports have shown that neutrophils [4], complement [5] and immunoglobulins [6] play important roles in host defence against *P. aeruginosa* infection. However, *P. aeruginosa* is identified frequently as a causative agent of sepsis in immunocompromised patients such as those with neutropenia due to anti-neoplastic chemotherapy [1, 2]. Because normal neutrophil function cannot be expected in patients with neutropenia, humoral immune responses may play a more important role in recovery from *P. aeruginosa* infection in such patients.

Clinical studies of the faeces from immunocompromised patients suggest that the gastrointestinal tract may be a primary reservoir for opportunistic bacteria [7]. The potential importance of this source of infection is underlined by the demonstration that bacteria contained within the gut can cross the gastrointestinal mucosal barrier and spread systematically, a process termed bacterial translocation [8, 9]. Furthermore, gut-derived *P. aeruginosa* sepsis has been induced by administering cyclophosphamide and ampicillin to specific pathogen-free mice fed this organism — thereby creating an animal model that closely mimics the pathophysiology of septicaemia in man [10].

Several lines of evidence support the potential effectiveness of vaccines in treating pseudomonal infection. For example, lipopolysaccharide (LPS) *pseudomonas* vaccine improved survival of guinea-pigs with pneumonia caused by *P. aeruginosa*; survival was correlated with the titre of type-specific, heat-stable opsonic antibody [11]. Furthermore, a clinical study has demonstrated the effectiveness of vaccinating patients with burns with heat-inactivated *P. aeruginosa* [12]. The effectiveness of heat-inactivated *P. aeruginosa* vaccine was further demonstrated in intraperitoneal infections of mice and in a wound sepsis model in rats [13]. However, it has been reported that prior immunisation with heat-killed *P. aeruginosa* did not improve the inflammatory response to homologous organisms injected intraperitoneally [14]. Therefore, the effectiveness of vaccination
against pseudomonal infections, especially gut-derived sepsis, is not clearly defined. The present study investigated the efficacy of immunisation with *P. aeruginosa* against gut-derived sepsis, a murine 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. They were housed in sterile cages and received sterile distilled water except during the period when bacteria were being administered orally. All 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 [15], was used. The strain was maintained at −80°C in Mueller-Hinton Broth (Difco Laboratories) containing glycerol 15%.

**Reagents**

Purified exotoxin A was purchased from List Biological Laboratories, Inc. (Campbell, CA, USA), purified elastase and purified alkaline protease from Nagase and Co. Ltd. (Tokyo, Japan), and bovine serum albumin (BSA) from Sigma.

**Vaccine preparation and immunisation protocol**

Bacteria grown on Trypticase Soy Agar (BBL Microbiology Systems, Cockeysville, MD, USA) at 37°C for 18 h were suspended in sterile saline to a concentration of 1 × 10^8 cfu/ml. Heat-killed bacteria were prepared by heating the bacterial suspension at 60°C for 1 h. The protein concentration was determined by Protein Assay (BioRad Laboratories, Hercules, CA, USA), and adjusted to a final concentration of 150 μg/ml.

The protocol for immunisation and induction of gut-derived sepsis is depicted in Fig. 1. Each mouse was immunised six times at 2- or 3-day intervals over the period day 0–day 11. Immunisation was by intraperitoneal injection of 0.2 ml of heat-killed bacteria at a concentration of 30 μg of protein dose. Control mice were inoculated intraperitoneally with the same dose of BSA.

**Monitoring the immune response**

On days 4, 7, 14 and 33, 20-μl samples of blood were obtained from the retro-orbital plexus with disposable heparinised capillary tubes. Normal sera were obtained by bleeding eight untreated normal mice. The titres of IgG and IgM antibodies against heat-killed bacteria, and of IgG antibodies against elastase, exotoxin A, and alkaline protease were determined by a modification of the ELISA method used to determine anti-alginate antibody [16]. ELISA units were defined as the serum dilution required to produce an absorbance at 405 nm (A405) equal to the mean absorbance value obtained for serum from untreated mice.

Bacteria suspended in phosphate-buffered saline (PBS, pH 7.2) at a concentration of 10^6 cfu/ml were sonicated at 0°C for 15 min with Insonator 201M (Kubota Corp., Tokyo, Japan). After filtration with 0.45 μm-pore filter units (Millex-HV Millipore Corp., MA, USA), the sonicated bacterial filtrate was used as an antigen for ELISA. A portion of the filtrate was boiled for 20 min. The protein concentrations of these two antigens were determined by Protein Assay (BioRad Laboratories, Hercules, CA, USA), and adjusted to a final concentration of 10 μg/ml with PBS. The antibody titres to boiled or unboiled suspensions of sonicated *P. aeruginosa* were also determined by a modification of the ELISA method of Cryz *et al.* [16].

**Survival studies**

Murine gut-derived sepsis was produced as described previously [10, 17, 18]. Bacteria were grown on trypticase soy agar 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 given in the drinking water during days 14–16. To aid colonisation by *P. aeruginosa*, the normal intestinal flora of the mice was disturbed by administering ampicillin (ABPC) intraperitoneally at a daily dose of 200 mg/kg body weight during 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 daily for mortality up to 7 days following the second cyclophosphamide administration.
**Statistical analysis**

Differences between group survival rates were evaluated by the $\chi^2$ test. Differences in antibody titres and numbers of viable bacteria in the liver were evaluated by Mann-Whitney's U-test. A level of 5% was considered to be significant.

**Results**

*Antibody response after immunisation with heat-killed bacteria*

Because heat-killed *P. aeruginosa* was used as an antigen for the immunisation of mice, the antibodies to whole bacteria were measured first. Immunisation of mice with heat-killed bacteria induced serum IgG and IgM antibodies to mean (SEM) levels of 1792 (374.7) and 37.3 (8.9) ELISA units, respectively (Fig. 2). This increase in antibody titres compared to BSA-immunised mice was significant ($p < 0.01$).

*Effect of immunisation on survival*

The effect of immunisation with heat-killed bacteria on the survival of mice with gut-derived sepsis was evaluated. Fig. 3 shows the survival kinetics of mice immunised with either heat-killed bacteria or BSA (control). Immunisation with heat-killed bacteria completely protected mice from death, whereas >90% of the control mice immunised with BSA died.

*Effect of immunisation on formation of macroscopic liver abscesses*

The livers of BSA-immunised control mice (A) and mice immunised with heat-killed bacteria (B) are

![Fig. 2. Antibody responses after immunisation with heat-killed bacteria. On days 4, 7, 14 and 33, blood samples were obtained from each group of mice (n = 6–9) to determine the titre of IgG and IgM antibodies against the immunising antigen. Serum IgG and IgM antibody levels were determined by ELISA as described in Materials and methods.](image)

![Fig. 3. Effect of immunisation with heat-killed bacteria on the survival of mice with gut-derived *P. aeruginosa* sepsis. The survival of mice (n = 12) immunised with heat-killed bacteria was significantly higher than that of BSA-immunised control mice (n = 15). ABPC, ampicillin; CY, cyclophosphamide treatment.](image)
shown in Fig. 4. Multiple abscesses were observed in the livers of the control mice, but abscess formation was completely inhibited by immunisation with heat-killed bacteria.

**Viable bacterial counts in liver after gut-derived sepsis**

Occurrence of liver abscesses was suspected to be influenced by the number of viable bacteria in the liver. Therefore, the number of viable bacteria in the liver of mice immunised with heat-killed bacteria or with BSA was determined. The results demonstrated a significant reduction in the number of viable bacteria in the liver following immunisation with heat-killed bacteria (Fig. 5).

**Exoenzyme antibody responses to immunisation**

To characterise the antibodies responsible for the protection seen in this model, antibody titres against the exoenzymes alkaline protease, elastase and exotoxin A were analysed. Only low levels of antibodies against these exoenzymes were detected (Fig. 6).

![Fig. 4. Effect of immunisation with heat-killed bacteria on production of macroscopic liver abscesses. The livers of (A) BSA-immunised mice and (B) mice immunised with heat-killed bacteria were obtained 10 days after the second administration of cyclophosphamide.](image-url)
Fig. 5. The counts of viable bacteria expressed as the numbers of cfu/g of liver (wet weight) after gut-derived sepsis in the livers of mice immunised with BSA (□) or with heat-killed *P. aeruginosa* (■) 10 days after the second administration of cyclophosphamide. Values are means, bars are SEM (five mice in each group); *, p < 0.01.

Therefore, they appeared not to be responsible for protection.

**Antibody titres against boiled and unboiled antigens of filtrates of sonicated bacteria**

Antibody titres to boiled or unboiled filtrates of sonicated *P. aeruginosa* were also determined. No significant difference between the titres of antibody against these two antigens was found (Fig. 7). The main protective antibodies accordingly appeared to be specific to the antigen stable toward boiling (i.e. LPS).

**Discussion**

A protective effect of immunisation with heat-killed *P. aeruginosa* against murine gut-derived sepsis has been demonstrated. Immunisation with heat-killed bacteria induced a marked increase in IgG antibody titres against the bacteria. One of the most important points in evaluating the effectiveness of vaccination is the period during which antibody titres remain elevated. Although the IgG titre against *P. aeruginosa* gradually decreased, the level remained high enough to provide protection on day 33 of the study. Furthermore, the antibody levels did not decrease significantly with administration of cyclophosphamide (data not shown). Therefore, the immunisation protocol provided a good antibody response.

Fig. 6. Antibody responses to elastase, exotoxin A and alkaline protease after immunisation of mice with heat-killed *P. aeruginosa*. On days 7, 14 and 21, blood samples were obtained from mice immunised with heat-killed bacteria (n = 5–6). Serum IgG antibody levels against elastase, exotoxin A and alkaline protease were determined by ELISA.
The protective effect of immunisation with heat-killed bacteria was far greater than expected because complete protection against death from gut-derived sepsis was observed. This demonstrated protective effect was also supported by inhibition of the production of liver abscesses and by reduction in the number of viable bacteria in the liver.

This model incorporated oral inoculation of bacteria, subsequent bacterial colonisation, overgrowth in the intestinal tract and invasion into the bloodstream. This led us to suspect that the specific antibodies against P. aeruginosa would influence bacterial colonisation of the mouse gastrointestinal tract. However, a significant difference in bacterial colonisation between mice immunised with heat-killed bacteria and those immunised with BSA was not found (data not shown). Thus, the specific antibodies may play their primary protective role at the point where bacteria invade the bloodstream.

The virulence of P. aeruginosa is multifactorial, resulting from the action of several extracellular enzymes, especially alkaline protease, elastase and exotoxin A [19, 20]. Although at first it seemed plausible that antibodies against these exoenzymes might be responsible for the protective immunisation provided, the poor antibody response against these exoenzymes suggests that it is antibodies against other P. aeruginosa antigens that provide the protection.

Although analysis was not made of the antibody levels against all P. aeruginosa cellular antigens, the similarity of antibody titres against boiled and unboiled suspensions of sonicated P. aeruginosa suggests that the main protective antibodies are directed against LPS. Fomsgaard and Holder [21] reported that the range of specific antibody titres in normal human IgG against either whole live bacteria or heat-killed bacteria paralleled that of anti-LPS IgG. Therefore, we suggest that the antibodies produced by immunisation with heat-killed bacteria react mainly with LPS, and that these are most likely to be the ones protecting mice against gut-derived P. aeruginosa sepsis.

Because the bacterial surface is surrounded by LPS, this antigen is readily accessible to antibodies. Some investigators have shown that active immunisation with P. aeruginosa LPS prevents infection with this organism in animal models as well as in patients [22–25]. However, as there are several immunotype strains of P. aeruginosa, numerous monovalent serotype LPS antigens must be combined to produce an effective vaccine. To solve this problem, Wassermann et al. [12] used a vaccine containing 10 different strains of heat-inactivated bacteria, and were able to show that immunisation with these antigens did elicit a protective effect against P. aeruginosa infection in burned patients.

The other major problem in immunoprophylaxis with heat-killed bacteria is that LPS induces a high incidence of toxic side-effects due to the lipid A portion of the LPS molecule. Cryz et al. [25] overcome this problem by coupling lipid A-free oligosaccharide isolated from eight different P. aeruginosa immunotypes to exotoxin A of P. aeruginosa. Therefore, it seems that the most plausible candidate for creating a non-toxic vaccine reactive against a broad range of P. aeruginosa immunotypes might be polyvalent lipid A-free oligosaccharide.

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