The effect of humoral and cell-mediated immunity in resistance to systemic serratia infection

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Summary. Protection against experimental *Serratia marcescens* infection in mice was enhanced by prior injection of formalin-killed or viable bacteria of the same strain. From the first to the fourth week after vaccination, specific immunity was involved in the host defence against systemic serratia infection. The transfer of antiserum specific for *S. marcescens* increased bacterial clearance from the liver, but did not increase the survival of the mice. Bacterial clearance from the liver was also increased by the transfer of spleen cells from immunised mice, but, again, survival was not increased. However, the transfer of both antiserum and spleen cells from vaccinated mice increased both bacterial clearance from the liver and survival (*p* < 0.01). These results suggest an additive effect of humoral immunity and T-cell-mediated immunity in protection against systemic serratia infection.

Introduction

*Serratia marcescens* is one of the most important aerobic gram-negative rods (GNR) causing urinary tract infection, pneumonia, septicaemia and meningitis, especially in immuno-compromised hosts. However, host defence mechanisms against systemic serratia infection are not fully understood.

We have studied specific and non-specific immunity to systemic serratia infection in an experimental mouse model. In the early phase after vaccination (within 7 days), enhancement of host defence mechanisms depends on phagocytic function and is non-specifically activated by vaccination with dead bacteria. In the later phase (1–4 weeks after vaccination), specific immunity is thought to be involved. It has been suggested that immunity to *S. marcescens* infection is mediated by antibodies in that specific O-antiserum against *S. marcescens* provided protection against peritoneal infection by the same O-type strain. However, in other pyogenic infections, both T-cells and a combination of cell-mediated and humoral immunity have been implicated in protection. For example, Tsuda *et al.* reported that T-cell-mediated immunity (CMI) contributed to resistance against staphylococcal infection in nude mice, whereas Akeda *et al.* reported a synergic contribution of macrophages and antibodies in protection against *Salmonella typhimurium*.

To clarify the contribution of different host defence mechanisms against systemic serratia infection, we have examined the roles of humoral immunity, CMI and the combination of humoral immunity and CMI in resistance to systemic serratia infection.

Materials and methods

Bacterial strains

*S. marcescens* O-serotype 3 (O-3) was isolated from the blood of a patient suffering from septicaemia originating from urinary tract infection. *Escherichia coli* was also obtained from a clinical specimen. Each organism was passaged in ddY mice before use; briefly, organisms were inoculated intravenously (i.v.) into a ddY mouse, and fresh isolates were obtained from the spleen on the following day. The bacteria were then grown overnight on nutrient-agar plates at 37°C. Colonies were suspended in sterile phosphate-buffered saline (PBS, pH 7.2) and again inoculated i.v. to a ddY mouse. After three such passages, the bacteria were grown in Trypto-Soy Broth (Eiken Chemical Co., Tokyo, Japan) with shaking for 16 h at 37°C, washed three times with PBS and stored at -80°C. Formalin-killed bacteria were prepared by adding formalin to a final concentration of 0.5% v/v at room temperature. The killed bacteria were washed three times in sterile PBS and then suspended at a concentration of approximately 1 × 10^10/ml in sterile PBS.

Animals and experimental infection

Male ddY mice and BALB/c mice, aged 7–12 weeks, were purchased from the Kyudo Co. Ltd, Kumamoto, Japan. BALB/c mice were used for experiments involving spleen cell transfer. Mice were infected by i.v. injection of *S. marcescens*, and the LD50 and survival of mice were calculated by probit analysis. These experiments were repeated three to five times.
Immunisation procedure

Mice were immunised by i.v. injection of $1 \times 10^8$ formalin-killed *S. marcescens* or *E. coli* cells, or by i.v. injection of $5 \times 10^7$ viable *S. marcescens* cells.

Preparation of antiserum and passive immunisation

Mice immunised with formalin-killed *S. marcescens* were given a booster injection of the same dose 7 days later. After a further 7 days, the mice were bled and their sera pooled. The antibody titre of the serum was determined by bacterial agglutination with formalin-killed bacteria. Passive immunisation of mice against *S. marcescens* was by i.v. injection of 1 ml of this antiserum.

Delayed foot-pad reaction

Mice were immunised with formalin-killed *S. marcescens* or *E. coli* cells. After 2 weeks, 0-05 ml of PBS containing $1 \times 10^7$ cells of formalin-killed *S. marcescens* was injected into their left hind foot-pads, and an equal amount of PBS into their right hind foot-pads. After 24 h, the thickness of the foot-pads was measured with a dial thickness gauge. The delayed foot-pad reaction (DFR) was calculated as the thickness of the left foot-pad minus the thickness of the right foot-pad; a difference of 0-1 mm was defined as 1 unit.

Spleen cell transfer

BALB/c mice were immunised with formalin-killed *S. marcescens*; spleens were removed under sterile conditions, and spleen cells were obtained by crushing between two sterile glass slides. After filtering the crushed splenic tissue through two pieces of sterile gauze, spleen cells were suspended in 3 ml of Eagle's Minimum Essential Medium (MEM, pH 7.4). The cells were washed twice with PBS, resuspended in MEM (without phenol red and containing 5 mM HEPES buffer, pH 7-4) and incubated in petri dishes (Falcon, Oxnard, CA, USA) for 1 h at 37°C in a humidified atmosphere of CO$_2$, 5% in air. Non-adherent cells were collected, and these procedures were repeated twice. One ml containing $1 \times 10^8$ non-adherent spleen cells was then injected i.v.; 1 h later, the animals were challenged by i.v. injection of viable *S. marcescens*, and bacterial counts on the liver were carried out 12 and 24 h after inoculation. In the second experiment, 0-5 ml of antiserum from BALB/c mice was injected i.v.; spleen cells were transferred 1 h later, and the animals were challenged with viable *S. marcescens* 1 h after the spleen cell transfer.

Bacterial counts on the livers of infected mice

At various intervals after injection, animals were bled by severing the carotid artery. The liver was immediately removed and homogenised in 10 ml of PBS. Serial 10-fold dilutions of the homogenate were made in PBS, and a 0-1-ml sample of each dilution was spread on nutrient agar. Colonies were counted after incubation for 20 h at 37°C.

Statistical analysis

The statistical significance of the data was determined by Student's $t$ test.

Results

The effect of vaccination on i.v. infection with *S. marcescens*

The LD$_{50}$ of *S. marcescens* at various time intervals after i.v. vaccination with viable or formalin-killed *S. marcescens*, or with formalin-killed *E. coli*, are shown in fig. 1. The LD$_{50}$ in animals vaccinated with viable *S. marcescens* was $3 \times 10^8$ bacteria/mouse (about eight times higher than the LD$_{50}$ in the control group) on day 7 after vaccination, decreasing to $9.5 \times 10^8$ bacteria on day 28. This LD$_{50}$ curve was not significantly different from that obtained after vaccination with formalin-killed *S. marcescens*. The LD$_{50}$ of *S. marcescens* in the groups vaccinated with formalin-killed *E. coli* was significantly lower than in the groups vaccinated with viable or formalin-killed *S. marcescens* on days 7, 14 and 28 after vaccination. Thus, a specific component in the immunity against *S. marcescens* infection was identified from 7 days after vaccination, and was still apparent after 4 weeks. In subsequent experiments, formalin-killed *S. marcescens* were used for immunisation because there was no significant difference between the LD$_{50}$ after vaccination with viable and formalin-killed organisms.

![Fig. 1. The LD$_{50}$ of *S. marcescens* after i.v. infection of mice vaccinated with $5 \times 10^7$ viable *S. marcescens* O-3 (○), $1 \times 10^8$ formalin-killed *S. marcescens* O-3 (●), or $1 \times 10^9$ formalin-killed *E. coli* (■). The shaded area shows the LD$_{50}$ value of unvaccinated controls. These experiments were repeated 3–5 times; bars represent SEM; #, p < 0.05 compared with *E. coli*-vaccinated group.](image-url)
The effect of transfer of antiserum on bacterial counts in the liver

In previous studies, survival of mice infected with *S. marcescens* was not increased by transfer of specific antiserum. Therefore, bacterial counts in the livers of mice which had received specific antiserum were analysed. High-titre mouse antiserum (512 in the agglutination test) against *S. marcescens* was administered i.v. (1 ml) to normal mice 1 h before i.v. infection with the same strain. After injection, the serum of the recipient mice had an anti-*S. marcescens* antibody titre of 64. Fig. 2 shows that there was no significant difference in bacterial counts between mice that received antiserum and control mice that had received normal mouse serum.

Induction of CMI as assessed by the foot-pad swelling test

The foot-pad reaction of mice immunised with viable or killed *S. marcescens*, or with killed *E. coli*, was measured 24 h after inoculation of *S. marcescens* into the mouse foot-pad (fig. 3). Foot-pad swelling was significantly greater in mice immunised with *S. marcescens* than in those immunised with killed *E. coli* or non-immunised control mice, suggesting that a specific CMI response had been induced.

![Fig. 2](image)

Fig. 2. Viable counts of bacteria in the livers of mice 12 and 24 h after i.v. infection with 7.6 × 10⁸ viable *S. marcescens*. Mice received either (a) normal serum or (b) antiserum. Bars represent SEM of five mice.

![Fig. 3](image)

Fig. 3. Delayed foot-pad reaction in mice vaccinated with (a) viable *S. marcescens*, (b) formalin-killed *S. marcescens* or (c) formalin-killed *E. coli*, or (d) in unvaccinated control mice. Bars represent SEM of 10 mice.

The effect of transfer of antiserum and spleen cells on bacterial counts in the liver and on mouse survival

To evaluate the contribution of CMI and humoral immunity against systemic serrata infection, bacterial growth in the liver of unvaccinated BALB/c mice was compared following transfer of non-adherent spleen cells and antiserum from vaccinated BALB/c mice. Fig. 4 shows the bacterial counts in the liver of recipients of both serum and spleen cells from unvaccinated or vaccinated mice after i.v. infection with 6.2 × 10⁸ viable *S. marcescens* cells (c. 1.5 LD₅₀ in control mice); 12 h after infection, counts in the liver of the group receiving immune spleen cells were significantly lower than in those receiving control spleen cells. However, 24 h after infection no significant difference was observed. The bacterial counts in the livers of mice that had received both antiserum and immune spleen cells were lower than in those that had received immune spleen cells alone; however, the differences at both 12 and 24 h were not significant. The percentages of mice surviving after infection with various doses of viable bacteria, and transfer of either immune or non-immune spleen cells, are shown in table I. There was no difference between the group that received immune cells and the group that received non-immune cells. However, the survival of mice that received both antiserum and immune spleen cells was greater than those that received control (non-immune) serum and spleen cells (table II). These results suggest that transfer of both antiserum and immune spleen cells to normal mice was more effective in conferring resistance to i.v. infection with *S. marcescens* than transfer of antiserum or immune spleen cells alone.

Discussion

Bacterial infections have been categorised into two types on the basis of the host's response to infection.¹⁰
Bacteria that cause purulent infections or cause infections in neutropenic patients are categorised as facultative extracellular bacteria; this category includes cocci as well as the gram-negative rods found in many kinds of infections. Bacteria that cause granulomatous infections or infections in CMI-impaired patients are categorised as facultative intracellular bacteria; this category includes *Mycobacterium* spp., *Brucella* spp., *Listeria monocytogenes*, *Yersinia enterocolitica* and *Legionella pneumophila*. *S. marcescens* was categorised as an extracellular bacterium by Hahn et al. In extracellular bacterial infections, opsonising antibodies exert a protective effect, and usually the disease subsides after polymorphonuclear leucocytes and mononuclear phagocytes have phagocytosed most of the infecting bacteria.

In our experiments, transfer of specific antibody did not increase the survival of mice with a systemic *S. marcescens* infection. The numbers of viable bacteria in the livers of mice that received specific antibodies were lower 12 and 24 h after i.v. infection with *S. marcescens*, but the differences were not significant (p > 0.1) (fig. 2). Because antiserum was not very effective in protecting against systemic serratia infection, we examined the protective effect of cell-mediated mechanisms. The induction of a cell-mediated response was demonstrated by the delayed foot-pad response test. Generally, immunisation with live bacteria induces protective immunity and delayed hypersensitivity, whereas immunisation with killed organisms or culture filtrates containing surface antigens does not. Our results (fig. 1) suggest that immunisation with either viable or killed *S. marcescens* generated specific CMI, as assessed by the delayed foot-pad response.

Growth of *S. marcescens* in the liver 12 h after i.v. infection was significantly (p < 0.01) suppressed by the transfer of non-adherent spleen cells from mice immunised with *S. marcescens* (fig. 4). This suggests that activated T-cells produce lymphokines that activate tissue macrophages, resulting in enhanced host resistance. Patients with impaired CMI frequently develop infections with gram-negative rods such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *E. coli*, *S. marcescens*, *Proteus mirabilis*, *Enterobacter cloacae* etc. Defective CMI is characterised by decreased delayed cutaneous hypersensitivity, decreased lymphocyte transformation by phytohaemagglutinin (PHA), and a decrease in the number of T-cells. Our data, showing that CMI is effective against systemic serratia infection, support these clinical findings. However, we were unable to demon-

### Table I. The effect of spleen cell transfer on resistance to i.v. infection by *S. marcescens* 0-3*

<table>
<thead>
<tr>
<th>Infecting dose</th>
<th>Spleen cells administered</th>
<th>Number of survivors /Number of mice</th>
<th>Survival (%)</th>
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<td>9.4 x 10⁸</td>
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<td>Non-immune</td>
<td>4/10</td>
<td>40</td>
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<tr>
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<td>10/10</td>
<td>100</td>
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<tr>
<td></td>
<td>Non-immune</td>
<td>9/10</td>
<td>90</td>
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* Normal recipient mice were given 1 ml of 1 x 10⁸ spleen cells from immunised or non-immunised mice by i.v. injection 1 h before i.v. infection with *S. marcescens* O-3. The number of survivors was counted 7 days after infection; the data represent three experiments.

### Table II. The effect of antiserum and spleen cell transfer on resistance to i.v. infection by *S. marcescens* O-3*

<table>
<thead>
<tr>
<th>Infecting dose</th>
<th>Serum and spleen cells administered</th>
<th>Number of survivors /Number of mice</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(cells/mouse)</td>
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<td>Non-immune</td>
<td>5/10</td>
<td>50</td>
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</table>

* Normal recipient mice were given 0.5 ml of mouse antiserum (titre 32 in agglutination test) against *S. marcescens* O-3 or normal mouse serum by i.v. injection 2 h before i.v. infection with *S. marcescens* O-3, and 1 ml of 1 x 10⁸ spleen cells from immunised or non-immunised mice 1 h before i.v. infection of the same strain. The number of survivors was counted 7 days after infection. These data represent three experiments.
strat any increase in survival in recipients of immune spleen cells (table I). Therefore, we tested the simultaneous transfer of both antiserum and immune spleen cells to recipient mice. Bacterial growth in the livers of these mice was lower than in mice that received immune spleen cells alone (fig. 4). Moreover, the survival of mice that received both antiserum and immune spleen cells was clearly greater than that of mice that received immune spleen cells alone.

Our results suggest that specific antibody serves as an opsonin, facilitating the uptake of invading microorganisms by circulating or tissue phagocytes, and that immune T-cells activate the phagocytic and microbicidal functions of macrophages in the liver and other reticulo-endothelial organs. Enhanced protection against bacterial growth in the liver and the increase in survival seen following the transfer of both antiserum and immune spleen cells suggest an additive effect of humoral and cell-mediated immunity in conferring protection against systemic serratia infection.

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References
