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,¹ pneumonia,² septicaemia³ and meningitis,⁴ 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.⁵ 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.⁶ 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,⁷ whereas Akeda *et al.* reported a synergic contribution of macrophages and antibodies in protection against *Salmonella typhimurium*.⁸

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 Tryptone-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.⁹ These experiments were repeated three to five times.

Immunisation procedure

Mice were immunised by i.v. injection of 1×10^8 formalin-killed *S. marcescens* or *E. coli* cells, or by i.v. injection of 5×10^7 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 1×10^7 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 CO₂ 5% in air. Non-adherent cells were collected, and these procedures were repeated twice. One ml containing 1×10^8 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 LD₅₀ 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 LD₅₀ in animals vaccinated with viable *S. marcescens* was 3×10^9 bacteria/mouse (about eight times higher than the LD₅₀ in the control group) on day 7 after vaccination, decreasing to 9.5×10^8 bacteria on day 28. This LD₅₀ curve was not significantly different from that obtained after vaccination with formalin-killed *S. marcescens*. The LD₅₀ 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 LD₅₀ after vaccination with viable and formalin-killed organisms.

Fig. 1. The LD₅₀ of *S. marcescens* after i.v. infection of mice vaccinated with 5×10^7 viable *S. marcescens* O-3 (○), 1×10^8 formalin-killed *S. marcescens* O-3 (●), or 1×10^8 formalin-killed *E. coli* (■). The shaded area shows the LD₅₀ value of unvaccinated controls. These experiments were repeated 3–5 times; bars represent SEM; #, *p* < 0.05 compared with *E. coli*-vaccinated group.

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.⁵ 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. Viable counts of bacteria in the livers of mice 12 and 24 h after i.v. infection with 7.6×10^8 viable *S. marcescens*. Mice received either (a) normal serum or (b) antiserum. Bars represent SEM of five mice.

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 *serratia* 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 6.2×10^8 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

Fig. 4. Viable counts of bacteria in the livers of mice 12 and 24 h after i.v. infection with 6.2×10^8 viable *S. marcescens*. (a) Control mice received non-immune serum and spleen cells. Experimental mice received either (b) immune spleen cells alone, or (c) immune antiserum and immune spleen cells. Bars represent SEM of five mice; # and *, $p < 0.05$ compared with non-immunised group.

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

Infecting dose (cells/mouse)	Spleen cells administered	Number of survivors /Number of mice	Survival (%)
9.4×10^8	Immune	0/10	0
	Non-immune	0/10	0
4.7×10^8	Immune	5/10	50
	Non-immune	4/10	40
2.3×10^8	Immune	10/10	100
	Non-immune	9/10	90

* Normal recipient mice were given 1 ml of 1×10^8 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*

Infecting dose (cells/mouse)	Serum and spleen cells administered	Number of survivors /Number of mice	Survival (%)
1.5×10^9	Immune	2/10	20
	Non-immune	0/10	0
7.3×10^8	Immune	10/10	100
	Non-immune	1/10	10
3.7×10^8	Immune	10/10	100
	Non-immune	5/10	50

* 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 1×10^8 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.

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.¹¹ 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*.¹²⁻¹⁵ *S. marcescens* was categorised as an extracellular bacterium by Hahn *et al.*¹⁰ 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.^{10, 16-18}

In our experiments, transfer of specific antibody did not increase the survival of mice with a systemic *S. marcescens* infection.⁵ 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.¹⁹⁻²² Generally, immunisation with live bacteria induces protective immunity and delayed hypersensitivity, whereas immunisation with killed organisms or culture filtrates containing surface antigens does not.²³⁻²⁶ 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.²⁷⁻³² Defective CMI is characterised by decreased delayed cutaneous hypersensitivity, decreased lymphocyte transformation by phytohaemagglutinin (PHA), and a decrease in the number of T-cells.^{20, 27, 32} Our data, showing that CMI is effective against systemic *Serratia* infection, support these clinical findings. However, we were unable to demon-

strate 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

1. Maki DG, Hennekens CG, Phillips CW, Shaw WV, Bennett JV. Nosocomial urinary tract infection with *Serratia marcescens*: an epidemiologic study. *J Infect Dis* 1973; **128**: 579-587.
2. Balikian JP, Herman PG, Godleski JJ. *Serratia pneumonia*. *Radiology* 1980; **137**: 309-311.
3. Bosch JA, Vilaseca M, Fernandez F *et al*. Sepsis caused by *Serratia marcescens*. Report of 19 cases. *Rev Clin Esp* 1979; **155**: 93-96.
4. Sautter RL, Mattman LH, Legaspi RC. *Serratia marcescens* meningitis associated with a contaminated benzalkonium chloride solution. *Infect Control* 1984; **5**: 223-225.
5. Kumagai Y, Okada K, Ishimaru T, Sawae Y, Kuroiwa A, Nomoto K. Effects of vaccination against systemic *Serratia* infection. *J Clin Lab Immunol* 1989; **29**: 125-132.
6. Simberkoff MS, Moldover NH, Rahal JJ. Specific and nonspecific immunity to *Serratia marcescens* infection. *J Infect Dis* 1976; **134**: 348-353.
7. Tsuda S, Sasai Y, Minami K, Nomoto K. Contribution of T cell-mediated immunity to the resistance to staphylococcal infection. *J Invest Dermatol* 1978; **70**: 345-347.
8. Akeda H, Mitsuyama M, Tatsukawa K, Nomoto K, Takeya K. The synergistic contribution of macrophages and antibody to protection against *Salmonella typhimurium* during the early phase of infection. *J Gen Microbiol* 1981; **123**: 209-214.
9. Finney DJ. Probit analysis. A statistical treatment of the sigmoid response curve. Cambridge, Cambridge University Press. 1952.
10. Hahn H, Kaufmann SHE. The role of cell-mediated immunity in bacterial infections. *Rev Infect Dis* 1981; **3**: 1221-1250.
11. Klastersky J. Infections in immuno-compromised patients. I. Pathogenesis, etiology, and diagnosis. *Clin Ther* 1985; **8**: 90-99.
12. Carter PB. Pathogenicity of *Yersinia enterocolitica* for mice. *Infect Immun* 1975; **11**: 164-170.
13. Horwitz MA, Silverstein SC. Legionnaires' disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. *J Clin Invest* 1980; **66**: 441-450.
14. World Health Organization Scientific Group. Cell-mediated immunity and resistance to infection. WHO Technical Report Series 1973; **519**: 6-64.
15. Zinkernagel RM. Review: Cellular immune responses to intracellular parasites: role of the major histocompatibility gene complex and thymus in determining immune responsiveness and susceptibility to disease. *Parasite Immunol* 1979; **1**: 91-109.
16. Fukutome T, Mitsuyama M, Takeya K, Nomoto K. Importance of antiserum and phagocytic cells in the protection of mice against infection by *Klebsiella pneumoniae*. *J Gen Microbiol* 1980; **119**: 225-229.
17. Ogata N. Analysis of protective mechanisms against infection by *Pseudomonas aeruginosa*: the synergistic contribution of polymorphonuclear leucocytes and antibody. *Fukuoka Igaku Zasshi* 1983; **74**: 335-350 (in Japanese).
18. Tsuru S, Nomoto K, Mitsuyama M, Zinnaka Y, Takeya K. Importance of polymorphonuclear leucocytes in protection of mice against *Escherichia coli*. *J Gen Microbiol* 1981; **122**: 335-338.
19. Kearn RJ, Hinrichs DJ. Kinetics and maintenance of acquired resistance in mice to *Listeria monocytogenes*. *Infect Immun* 1977; **16**: 923-927.
20. Lane FC, Unanue ER. Requirement of thymus (T) lymphocytes for resistance to listeriosis. *J Exp Med* 1972; **135**: 1104-1112.
21. Mackaness GB. The influence of immunologically committed lymphoid cells on macrophage activity in vivo. *J Exp Med* 1969; **129**: 973-992.
22. Mitsuyama M, Nomoto K, Akeda H, Takeya K. Enhanced elimination of *Listeria monocytogenes* at the site of delayed foot-pad reaction. *Infect Immun* 1980; **30**: 1-4.
23. Hobson D. Resistance to reinfection in experimental mouse typhoid. *J Hyg* 1957; **55**: 334-343.
24. Holland JJ, Pickett MJ. A cellular basis of immunity in experimental *Brucella* infection. *J Exp Med* 1958; **108**: 343-360.
25. Mackaness GB. Cellular resistance to infection. *J Exp Med* 1962; **116**: 381-406.
26. Stableforth AW. Brucellosis. In: Stableforth AW, Galloway IA (eds) Infectious disease of animals, vol 1. London, Butterworths Scientific Publications. 1959: 53-159.
27. Carney JM, Warne MS, Borut T *et al*. Cell-mediated immune defects and infection. *Am J Dis Child* 1980; **134**: 824-827.
28. George CD, Morello PJ. Immunologic effects of blood transfusion upon renal transplantation, tumor operations, and bacterial infections. *Am J Surg* 1986; **152**: 329-337.
29. Clumeck N, George C. Immunological aspects of severe bacterial sepsis. *Intensive Care Med* 1981; **7**: 109-114.
30. Miedema F, Tetteroo PAT, Terpstra FG *et al*. Immunologic studies with LFA-1- and Mol-deficient lymphocytes from a patient with recurrent bacterial infections. *J Immunol* 1985; **134**: 3075-3081.
31. Petersen PK. Host defense abnormalities predisposing the patient to infection. *Am J Med* 1984; **76** (5A): 2-10.
32. Sorensen RU, Stern RC, Chase P, Polmar SH. Defective cellular immunity to gram-negative bacteria in cystic fibrosis patients. *Infect Immun* 1979; **23**: 398-402.