HOST RESPONSE TO INFECTION

Pre-treatment with Concanavalin-A increases resistance of mice to peritoneal infection by *Serratia marcescens*

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Mice pre-treated with Concanavalin-A largely survived an intra-peritoneal inoculum of 2×10^7 *Serratia marcescens*, whereas all control mice died within 15 h of inoculation. A subpopulation of peritoneal macrophages from Con-A pre-treated mice was able to phagocytose the bacteria *in vitro* (6.7 SEM 1.2% phagocytosing cells) and *in vivo* (16.9 SEM 2.1%), whereas control phagocytes did not phagocytose *S. marcescens*. The survival of Con-A pre-treated mice allowed their immunisation with living bacteria, and the antiserum thus produced increased the phagocytosis of *S. marcescens in vitro*. Control mice largely survived an inoculum of *S. marcescens* suspended in 50% immune serum, although the bacteria were resistant to the bactericidal activity of that serum. These results suggest that, in contrast to the delayed humoral protection afforded by immunisation, phagocytosis by phagocytes activated by Con-A conferred early protection to mice against experimental infection by *S. marcescens*.

Introduction

Resistance to the bactericidal activity of normal serum and to phagocytosis probably constitute important virulence factors of the gram-negative bacterium *Serratia marcescens*. The complement-mediated bactericidal activity of normal serum depends on the covalent fixation of C3b to the target cell surface. A strain of *S. marcescens* resistant to serum bound less C3b than did a sensitive strain [1]; this difference in serum sensitivity was attributed to the presence of long O antigen (LPS) chains on the surface of the resistant strain, that prevented complement binding. A similar correlation between the presence of O antigen and resistance to serum has been reported for several gram-negative bacteria [2–4]. On the other hand, the resistance to phagocytosis has often been correlated with the presence of K (capsular) antigens [5–7], whose role in *S. marcescens* resistance to phagocytosis is unclear. Nevertheless, an analysis of the immunogenic properties of *S. marcescens* outer membrane has demonstrated that most antigenic sites of the bacterial

surface are unable to react with antibodies and are possibly masked by the O antigen [8].

Mice challenged with a sublethal intraperitoneal inoculum of *Escherichia coli* later showed resistance to a lethal dose of the bacteria [9]; the observed increase in bacterial destruction during the early phase of infection was ascribed to non-specific activation of phagocytes. Similar results were observed following intravenous vaccination of mice with formalin-killed *S. marcescens* [10], also leading to the conclusion that enhancement of host defence in the early phase of infection depended on non-specific activation of phagocytic functions. However, those studies employing vaccination are inadequate in providing a clear distinction between early humoral immunity and non-specific activation of phagocytosis. A later study [11] showed that effective protection of mice vaccinated with formalin-killed *S. marcescens* was achieved only by the additive effect of humoral immunity and T-cell mediated immunity.

Previous studies demonstrated that the pre-treatment of mice with either concanavalin-A (Con-A) or jacalin increased the rate of clearance of *Candida albicans* from the peritoneal cavity, increased the phagocytosis of the yeast by peritoneal macrophages, and decreased

its dissemination to the viscera [12]; these effects were ascribed to non-specific activation of cell-mediated immunity (CMI) by Con-A and by jacalin. Because activation of CMI by Con-A avoids the exposure to bacterial antigens employed in vaccination studies, this investigation tested the hypothesis that Con-A could induce phagocytosis of *S. marcescens* in the early stage of infection and thus confer protection to mice challenged with these bacteria.

Materials and methods

Bacteria and growth conditions

Serratia marcescens strain O6, 14:H4 was isolated from blood of a patient with nosocomial infection at the University Hospital, Faculdade de Medicina de Ribeirão Preto, SP, Brazil, and was serotyped by Instituto Adolfo Lutz, São Paulo, Brazil. The bacteria were grown overnight in Tryptic Soy Broth (TSB; Difco) at 37°C. When the culture reached the required optical density (OD), the bacteria were harvested and transferred to the medium employed in the phagocytic assays. Three dilutions of each culture were plated on agar to determine the number of cfu, to confirm that it corresponded to the bacterial concentration expected from the OD measurement.

Peritoneal phagocytic cells

Peritoneal phagocytes were collected from Swiss mice (weighing 25–30 g) that had received 500 µg of Con-A (Sigma) in 0.5 ml of phosphate buffered saline (PBS), or this vehicle only, *via* the intraperitoneal route 4 days earlier. The animals were killed with ether and the phagocytes were collected by washing the peritoneal cavity with 3 ml of RPMI medium (Roswell Park Memorial Institute medium for cell culture; Sigma) with albumin 1%. The phagocytes were allowed to adhere to coverslips for 1 h at 37°C, and the monolayers were then washed in RPMI medium and used in the phagocytic assays.

Phagocytosis assay

Monolayers of phagocytic cells were incubated with 2×10^7 cfu of *S. marcescens* for 1 h at 37°C in 1 ml of RPMI medium buffered to pH 7.0 with 12 mM Hepes. The phagocyte preparations were then washed with RPMI medium, incubated for 15 min with gentamicin 100 mg/L to remove non-ingested bacteria [13, 14], washed with PBS, fixed in absolute methanol for 15 min, and stained with haematoxylin eosin. For each preparation 200 cells were counted to evaluate both the percentage of cell types in the peritoneal exudate and the phagocytic index (PI); this index was calculated as the percentage of phagocytosing cells multiplied by the average number of internalised particles. As a control of the phagocytic activity of each sample of macrophages, in each experiment duplicate monolayers of

phagocytic cells were incubated with either 10^6 cfu of *C. albicans* or 0.5% sheep erythrocytes that had been opsonised with rabbit anti-sheep IgG [15, 16]. Results were reported as mean PI and SEM; Student's *t* test was employed for statistical analysis.

Assay of in-vivo phagocytosis and determination of the survival rate

Swiss mice, weighing 25–30 g and 6–8-weeks old, were used in these experiments. One group of mice received 500 µg of Con-A in 0.5 ml of PBS and the other 0.5 ml of PBS, administered in a single intraperitoneal dose 96 h before inoculation with 2×10^7 cfu of an exponential culture of *S. marcescens*; 1 h after the intraperitoneal inoculation of *S. marcescens*, the mice were killed and the peritoneal exudate cells were collected and processed for analysis of phagocytosis *in vivo*. The spleen, liver and kidneys were removed and plated on TSB-agar to evaluate the dissemination of the bacteria.

A group of 17 mice pre-treated with Con-A received an inoculum as above and was used for the determination of the survival rate; the control group of eight mice received an intraperitoneal inoculum of *S. marcescens* 96 h after an intraperitoneal injection of PBS 0.5 ml.

Immunisation procedure

The 13 mice pretreated with Con-A that survived the inoculation of *S. marcescens* were further immunised by two intraperitoneal injections of 2×10^7 cfu of viable *S. marcescens*, with an interval of 15 days between each inoculation. The mice were bled 7 days after the last boost and their sera were pooled; the antibody titre of the sera was determined by bacterial agglutination. O-antigen and H-antigen preparations of the *S. marcescens* strain [17] reacted with the antisera at a dilution of 1600. The sera of control mice did not cause agglutination of *S. marcescens* at 1 in 1 dilution.

Serum resistance assay

Blood collected from normal mice was allowed to clot at 4°C for 2 h, centrifuged at 4°C, and the separated serum was used immediately. The bacterial resistance to the lethal activity of complement was determined by a rapid turbidimetric assay in microtitration plates [18], with modification. Overnight bacterial cultures grown in TSB were diluted 1 in 100 in freshly prepared TSB medium, incubated at 37°C for 90 min with shaking, centrifuged (2500 g, 15 min), resuspended in cold PBS, pH 7.4 (10^7 cfu/ml), and kept in an ice bath for prompt use. The bacterial suspension was then pipetted into the wells of a microtitration plate, and either normal or immune mouse serum was added to a final concentration of 50%. The plates were shaken briefly and incubated at 37°C; absorbances of triplicates of the samples were read at 0, 60, 120 and 180 min in a

spectrophotometer (Titertek Multiskan, model 340) at 620 nm. The serum-sensitive strain *E. coli* K12-711 was used as control.

Results

Effect of pre-treatment with Con-A on phagocytosis of S. marcescens

S. marcescens bacteria were not phagocytosed by resident peritoneal macrophages either in the absence or in the presence of mouse serum 10% (Table 1). Phagocytosis of *C. albicans* and of opsonised sheep erythrocytes was used as a control of the phagocytic activity of each sample of phagocytes, and it was observed that serum significantly increased the phagocytosis of these cells. On the other hand, macrophages from mice pre-treated with Con-A were able to phagocytose *S. marcescens* both *in vitro* and *in vivo* (Table 1). A subpopulation of macrophages capable of ingesting *S. marcescens* was increased in number by the pre-treatment of mice with Con-A (Table 2).

Effect of Con-A on mouse survival and immunisation

The *S. marcescens* strain was virulent for untreated mice; all eight died within 15 h of bacterial inoculation. However, 13 of the 17 mice pre-treated with Con-A survived the inoculation of *S. marcescens* and were subsequently immunised with two additional inocula of 2×10^7 viable bacteria. The survival rate of untreated mice was increased from 0 to 75% (6/8) by pre-incubating the bacterial inoculum for 2 h at 37°C with immune serum diluted 1 in 2.

The peritoneal fluid was intensely haemorrhagic in a second group of eight control mice killed 1 h after bacterial inoculation, and there was a reduction of c. 25 times in the number of cells collected from their peritoneal cavity compared with the mean of 2.6×10^6 cells/ml observed in the non-inoculated control mice and in previous studies [12]. Although the strain of *S. marcescens* used in this study was consistently non-haemolytic both on solid and in liquid assay media, the peritoneal fluid collected from

Table 2. Effect of pre-treatment with Con-A on the number of cells phagocytosing *S. marcescens*

Conditions	Mean (SEM) phagocytosing cells (%)	
	<i>in vitro</i>	<i>in vivo</i>
Control	0	0
Con-A	6.7 (1.2)	16.9 (2.1)

The phagocytic assays *in vitro* were carried out with phagocytes collected from mouse peritoneal cavity and incubated for 1 h with the bacteria. For the assays *in vivo*, the phagocytes were collected 1 h after inoculation of *S. marcescens* into the peritoneal cavity. Con-A: mice were treated with 500 µg of Con-A 4 days before the assays. The values are mean (SEM) of at least four assays.

these group of mice presented a large number of ruptured macrophages, cell nuclei and lysed erythrocytes. On the other hand, the mean number of phagocytes harvested from Con-A pre-treated mice was increased to a mean of 1.2×10^7 cells/ml 1 h after inoculation of the bacteria, as observed in the studies of phagocytosis *in vivo*.

Titres of the immune antiserum

The pooled serum from the immunised mice agglutinated *S. marcescens* to a titre of 800. The antiserum yielded a titre of 1600 against an O-antigen and H-antigen preparation of the *S. marcescens* strain. Neat serum from control mice did not agglutinate the *S. marcescens*.

Effect of immune serum on the phagocytosis of S. marcescens

Resident peritoneal macrophages phagocytosed *S. marcescens* in a medium containing the immune serum diluted in 1 in 1000, whereas in a medium containing control mouse serum they failed to phagocytose the bacteria (Fig. 1). Addition of control mouse serum to the medium containing the antiserum caused no further increase in phagocytosis ($p > 0.05$, Fig. 1).

Sensitivity of S. marcescens to the bactericidal effect of serum

S. marcescens was resistant to the lytic action of 50% normal or immune mouse serum, as shown in Fig. 2.

Table 1. Phagocytosis of *S. marcescens* by resident and by Con-A-activated phagocytes

Phagocytosis target	Mean (SEM) phagocytic index of macrophages			
	Resident <i>in vitro</i>	Resident* <i>in vitro</i>	Con-A treated	
			<i>in vitro</i>	<i>in vivo</i>
<i>S. marcescens</i>	0	0	12.7 (4.1)	36.0 (14.7)
<i>C. albicans</i>	19.9 (6.2)	144.7 (18.5)	97.7 (16.5)	41.2
ElgG	11.1 (3.4)	125.2 (29.4)	ND	ND

ND, not done. ElgG, sheep erythrocytes opsonised with rabbit anti-sheep IgG. Values are means (SEM) of at least four assays.

*After the addition of fresh normal mouse serum (10%) to the medium used in the assays of phagocytosis.

Fig. 1. Effect of antiserum against *S. marcescens* on its phagocytosis by non-activated peritoneal phagocytes. Phagocytic assays were carried out in: **a**, RPMI medium containing 2×10^7 cfu of *S. marcescens* (Phagocytic index = 0); or in the same medium supplemented with **b**, antiserum against *S. marcescens* (1:1000), **c**, antiserum plus fresh normal serum 10%, **d**, antiserum plus heat inactivated normal serum 10%. The differences between the means of b, c, or d were not statistically significant ($p > 0.05$). Bars represent SEM of four experiments.

Fig. 2. Resistance of *S. marcescens* to the bactericidal activity of normal (Δ) or immune ($\circ$) serum; serum-sensitive *E. coli* K12 was used as control ($\square$). Each symbol represents the mean of four experiments.

Escherichia coli K12, which is sensitive to lysis by complement, was used as a control.

Discussion

Peritoneal macrophages from mice pre-treated with Con-A were able to phagocytose *S. marcescens* both *in vivo* and *in vitro*, whereas resident phagocytes from control mice were not. However, these resident macrophages were capable of phagocytosing either opsonised sheep erythrocytes or *C. albicans*, which shows that they were capable both of opsonophagocytosis and lectinphagocytosis. Moreover, addition of

fresh mouse serum to the phagocytic assay did not cause complement-mediated phagocytosis of the bacteria by resident phagocytes, in agreement with a previous observation [1] that a strain of *S. marcescens* resistant to serum (as was the one used in this study) bound less C3b than a sensitive strain. Addition of cell-free exudate collected from the peritoneal cavity of mice pre-treated with Con-A to the *in-vitro* phagocytic assay was also ineffective in causing phagocytosis by control phagocytes (data not shown). As the macrophages used in the *in-vivo* assay were collected shortly (1 h) after inoculation of the bacteria, these results show that the early phagocytosis of *S. marcescens* can be attributed to an activation of phagocytes by Con-A and not to humoral immunity. Furthermore, macrophages collected from mice pre-treated with Con-A, but not inoculated with *S. marcescens*, phagocytosed the bacteria *in vitro*, corroborating the conclusion that a subpopulation of macrophages activated by Con-A was responsible for the phagocytosis of *S. marcescens*.

Mice pre-treated with Con-A survived an otherwise lethal inoculum of *S. marcescens*, whereas control mice died within 15 h of inoculation. This short survival period of the control mice suggests that the protection afforded by Con-A can be attributed to the early activation of phagocytosis discussed above. Con-A induces secretion of interferon gamma ($\text{IFN-}\gamma$) and other cytokines, and consequently causes activation of the bactericidal potential of macrophages [19,20]. Therefore, it seems likely that a particular subset of phagocytes (Table 2) was activated by Con-A-induced release of $\text{IFN-}\gamma$, and that this subset of phagocytes was responsible for the protection of mice against a lethal dose of *S. marcescens*.

The antiserum obtained from Con-A pre-treated mice that survived bacterial inoculation was effective in the opsonisation of *S. marcescens*, as shown by its induction of phagocytosis *in vitro* by resident macrophages. As immune complexes of IgG and IgM can bind to rabbit polymorphonuclear leucocytes [21], but only the IgG complexes are readily phagocytosed, these results suggest that IgG present in the immune serum mediated the phagocytosis of *S. marcescens* by non-activated phagocytes. Furthermore, pre-incubation of *S. marcescens* with the antiserum protected control mice against a lethal dose of the bacteria; because the bacteria were resistant to the lytic activity of 50% serum, either normal or immune, this protection afforded by the antiserum was possibly caused by induction of phagocytosis *in vivo*. Bacteria resistant to the lytic activity of normal serum and resistant to phagocytosis are often capsulated [5–7], and negative staining with India ink revealed that the strain used in this study was capsulate, corroborating the above results (picture not shown).

These findings demonstrate that the pre-treatment with Con-A protected mice against experimental infection

by *S. marcescens*, and support the hypothesis that activation of phagocytes constituted the major, if not the only, early mechanism of defence against *S. marcescens*.

References

- Jessop HL, Lambert PA. The role of surface polysaccharide in determining the resistance of *Serratia marcescens* to serum killing. *J Gen Microbiol* 1986; **132**: 2505–2514.
- Joiner KA, Schmetz MA, Goldman RC, Leive L, Frank MM. Mechanism of bacterial resistance to complement-mediated killing: inserted C5b-9 correlates with killing for *Escherichia coli* 0111B4 varying in O-antigen capsule and O-polysaccharide coverage of lipid A core oligosaccharide. *Infect Immun* 1984; **45**: 113–117.
- Taylor PW. Bacterial and bacteriolytic activity of serum against gram-negative bacteria. *Microbiol Rev* 1983; **47**: 46–83.
- Muschel LH, Larsen LJ. The sensitivity of smooth and rough gram-negative bacteria to the immune bactericidal reaction. *Proc Soc Exp Biol Med* 1970; **133**: 345–348.
- Domenico P, Salo RJ, Cross AS, Cunha BA. Polysaccharide capsule-mediated resistance to opsonophagocytosis in *Klebsiella pneumoniae*. *Infect Immun* 1994; **62**: 4495–4499.
- Allen PM, Fisher D, Saunders JR, Hart CA. The role of capsular polysaccharide K21b of *Klebsiella* and of the structurally related colanic-acid polysaccharide of *Escherichia coli* in resistance to phagocytosis and serum killing. *J Med Microbiol* 1987; **24**: 363–370.
- Lee C-J. Bacterial capsular polysaccharides – biochemistry, immunity and vaccine. *Mol Immunol* 1987; **24**: 1005–1019.
- Jessop HL, Lambert PA. Immunochemical characterization of the outer membrane complex of *Serratia marcescens* and identification of the antigens accessible to antibodies on the cell surface. *J Gen Microbiol* 1985; **131**: 2343–2348.
- Vuopio-Varkila J, Mäkelä PH. Killing of *Escherichia coli* in the peritoneal cavity of convalescent mice; role of specific and non-specific immune mechanisms. *J Med Microbiol* 1988; **25**: 205–211.
- 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.
- Kumagai Y, Okada K, Sawae Y. The effect of humoral and cell-mediated immunity in resistance to systemic *Serratia* infection. *J Med Microbiol* 1992; **36**: 245–249.
- Felipe I, Bim S, Somensi CC. Increased clearance of *Candida albicans* from the peritoneal cavity of mice pretreated with concanavalin A or jacalin. *Braz J Med Biol Res* 1995; **28**: 477–483.
- Vaudaux P, Waldvogel FA. Gentamicin antibacterial activity in the presence of human polymorphonuclear leukocytes. *Antimicrob Agents Chemother* 1979; **16**: 743–749.
- Tabrizi SN, Robins-Browne RM. Elimination of extracellular bacteria by antibiotics in quantitative assays of bacterial ingestion and killing by phagocytes. *J Immunol Methods* 1993; **158**: 201–206.
- Felipe I, Bim S, Loyola W. Participation of mannose receptor on the surface of stimulated macrophages in the phagocytosis of glutaraldehyde-fixed *Candida albicans*, *in vitro*. *Braz J Med Biol Res* 1989; **22**: 1251–1254.
- Pacheco-Soares C, Gaziri LCJ, Loyola W, Felipe I. Phagocytosis of enteropathogenic *Escherichia coli* and *Candida albicans* by lectin-like receptors. *Braz J Med Biol Res* 1993; **25**: 1015–1024.
- Edwards PR, Ewing WH. Identification of Enterobacteriaceae, 3rd edn. Minneapolis, Burgess, 1972; 311–316.
- Pelkonen S, Fine JA. Rapid turbidimetric assay for the study of serum sensitivity of *Escherichia coli*. *FEMS Microbiol Lett* 1987; **42**: 53–57.
- Ferrante A, Staugas RE, Rowan-Kelly B *et al.* Production of tumor necrosis factor alpha and beta by human mononuclear leukocytes stimulated with mitogens, bacteria, and malarial parasites. *Infect Immun* 1990; **58**: 3996–4003.
- Moscardi-Bacchi M, Brummer E, Stevens DA. Support of *Paracoccidioides brasiliensis* multiplication by human monocytes or macrophages: inhibition by activated phagocytes. *J Med Microbiol* 1994; **40**: 159–164.
- Furriel RPM, Lucisano YM, Mantovani B. Precipitated immune complexes of IgM as well as of IgG can bind to rabbit polymorphonuclear leucocytes but only the immune complexes of IgG are readily phagocytosed. *Immunology* 1992; **75**: 528–534.