The protective activity of immunostimulants against *Listeria monocytogenes* infection in mice

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Summary. The function of peritoneal macrophages induced by intraperitoneal (i.p.) injection of attenuated *Streptococcus pyogenes* (OK-432), Bacillus Calmette Guérin (BCG), protein-bound polysaccharide preparation isolated from *Coriolus vesicolor* (PSK) or *Lactobacillus casei* was examined. The PMA-triggered respiratory burst (production of O_2^- and H_2O_2) of macrophages induced by OK-432, BCG or *Lac. casei* was greater than that of resident or thioglycollate-stimulated macrophages and was similar to that of BCG-activated macrophages. PSK failed to enhance the production of O_2^- or H_2O_2 by macrophages. Alkaline phosphodiesterase (APD) activity was reduced in macrophages induced by OK-432, BCG or *Lac. casei* injection and in BCG-activated macrophages. The APD activity of macrophages obtained 7 and 13 days after i.p. injection of PSK was elevated, as with thioglycollate-stimulated macrophages. Listericidal activity *in vitro* was enhanced in macrophages obtained in 13 and 7 days, but suppressed in macrophages obtained 2 days after OK-432, BCG or *Lac. casei* injection. *Lac. casei* administered either 2 or 13 days before infection with *Listeria monocytogenes* was protective but OK-432, BCG (0.1 mg) and PSK were not, even though they were able to stimulate macrophage function.

Introduction

Attenuated *Streptococcus pyogenes* Su (OK-432), Bacillus Calmette Guérin (BCG), and a protein-bound polysaccharide preparation isolated from *Coriolus vesicolor* (PSK) have immunopotentiating properties such as enhancing host resistance to microbial infections or malignant tumours.^{1–5} *Lactobacillus casei* also enhances resistance to bacterial and viral infections^{6–8} and malignant tumours.⁹ We have reported previously that the anti-*Listeria monocytogenes* activity of *Lac. casei* in mice is most evident 2 and 13 days after administration, and resistance is associated with stimulation of macrophage functions and an increase in the number of macrophages.^{7,10} In the present study, the protective action of OK-432, BCG and PSK against *L. monocytogenes* infection in mice was compared to that of *Lac. casei*.

Materials and methods

Animals

Female 5-week-old ddY mice, purchased from the Japan SLC Inc., Shizuoka, Japan, were used.

Immunostimulants

BCG was obtained from Japan BCG Pharmaceutical Co., Tokyo, Japan. OK-432 and PSK were generous gifts from Chugai Pharmaceutical Co., Tokyo and Kureha Chemical Ind. Co., Tokyo, respectively. *Lac. casei* YIT 0003 was cultivated in Rogosa liquid medium¹¹ at 37°C for 18 h, washed and suspended in saline, and injected intraperitoneally (i.p.) or intravenously (i.v.) into mice at the appropriate dilution.

Bacteria

L. monocytogenes strain EGD was grown in Tryptic Soy Broth (Difco) at 37°C for 18 h, washed with saline, suspended in phosphate buffered saline (PBS, pH 7.2) containing glycerol 10%, and stored at –80°C. The bacterial suspensions were thawed and diluted in saline immediately before use.

Macrophage monolayers

Peritoneal exudate cells were obtained from mice at the appropriate time after i.p. injection of the various immunostimulants. Cells were harvested from five mice with phenol red-free Hanks's Balanced Salts Solution (HBSS, pH 7.2), pooled, centrifuged, and suspended in phenol red-free HBSS. For macrophage culture, the cells were suspended in Eagle's Minimal

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Essential Medium (MEM; Nissui Pharmaceutical Co., Tokyo) supplemented with glucose 0.4%, 1 mM pyruvate, 0.01 volumes of non-essential amino acids (Difco), and fetal bovine serum (M.A. Bioproducts, MD, USA) 10%, and poured into 35- or 50-mm plastic culture dishes (Nunc, Roskilde, Denmark) or on to plastic sheets (14 mm; Wako Pure Chemical, Osaka, Japan) in glass culture dishes (45 mm). After incubation at 37°C for 4–6 h in an atmosphere of CO₂ 5% in humidified air, the cultures were rinsed with PBS to remove non-adherent cells. Macrophages obtained 24 days after i.p. injection of 1 mg of BCG and 4 days after i.p. injection of 2 ml of thioglycollate served as activated and stimulated macrophages, respectively.

Assay for superoxide (O_2^-) release

O_2^- release was assayed according to the method of Johnston *et al.*¹² with slight modifications. Macrophage monolayers, derived from 4×10^6 peritoneal exudate cells, were overlaid with 2.5 ml of HBSS (containing glucose 0.1%, without phenol red) and 200 μ mol of cytochrome C (Sigma). A 10- μ l volume of phorbol myristate acetate (PMA; Sigma) was added to the incubation mixture to give a final concentration of 10 ng/ml. The cultures were placed in a CO₂ incubator for 60 min. After incubation, the medium was removed and the reduction of cytochrome C was measured spectrophotometrically at 550 nm.

Assay for H₂O₂ release

Macrophage monolayers derived from 2×10^6 peritoneal exudate cells were overlaid with 2.5 ml of HBSS (containing 0.1% glucose, without phenol red) and horseradish peroxidase (type II; Sigma) 50 μ g/ml was added. A 10- μ l volume of PMA and 25 μ l of scopoletin (reduced type; Sigma) were added to the incubation mixture to give final concentrations of 10 ng/ml and 5 nmol/ml, respectively. The dishes were placed in a CO₂ incubator for 10–15 min. After incubation, the medium was removed, and the oxidation of scopoletin by H₂O₂ was measured by fluorescence (excitation at 350 nm, emission at 460 nm).¹³

Assay for alkaline phosphodiesterase (APD) activity

Cells from macrophage monolayers, prepared in 50-mm plastic culture dishes by seeding $(3-4) \times 10^6$ cells, were harvested into Triton X-100 0.1%. Cell lysates were stored at -80°C until assayed APD activity was measured by the method of Edelson and Erbs.¹⁴

Macrophage microbicidal activity

Macrophage monolayers were prepared on plastic sheets in glass culture dishes by seeding 9×10^6 cells. The monolayer was overlaid with MEM containing *L. monocytogenes* (10^6 bacteria/ml), incubated at 37°C

for 1 h, washed with PBS to remove the non-phagocytosed bacteria, and fresh MEM containing penicillin G 0.2 μ g/ml was added to inhibit growth of extracellular organisms. The infected cells were incubated at 37°C for 10 h in a CO₂ incubator, washed with PBS, and lysed with distilled water. The number of viable, intracellular bacteria was counted by plating serial 10-fold dilutions of cell lysate on Trypto Soy Agar (Eiken Chemical Co., Tokyo). In some cases, the sheet was fixed with methanol and stained with Giemsa solution, and the number of macrophages was counted by microscopy.

Mouse protection test

Mice were given 0.1 mg of *Lac. casei*, OK-432, BCG or PSK by i.v. injection 2, 7 or 13 days before i.v. infection with *L. monocytogenes* 10^6 cells. Mice given 1 mg of BCG by i.v. injection 24 days before infection were also used. The survival of mice was recorded 7 days after infection. Alternatively, animals were killed 6 h or 48 h after infection with *L. monocytogenes* 10^4 cells, and the number of viable *L. monocytogenes* in the liver was determined as previously described.⁷

Statistical analysis

Student's *t* and χ^2 tests were used to determine statistical significance.

Results

The respiratory burst of macrophages

The PMA-triggered respiratory burst (production of O_2^- and H₂O₂) of macrophages induced with various immunostimulants was measured (table I). The O_2^- and H₂O₂ production of macrophages harvested 2, 7 and 13 days after i.p. injection of *Lac. casei* was greater than that of resident or thioglycollate-stimulated macrophages. Production by *Lac. casei*-induced macrophages on days 13 and 7 was similar to that of BCG-activated macrophages, whereas macrophages on day 2 produced lower levels of O_2^- and H₂O₂ than BCG-activated macrophages. The PMA-triggered respiratory burst of OK-432 and BCG (0.1 mg)-induced macrophages was greater than that of resident or thioglycollate-stimulated macrophages and similar to that of BCG-activated macrophages and *Lac. casei*-induced macrophages at day 7 or 13, whereas production by PSK-induced macrophages was similar to that of resident macrophages. The increase in respiratory burst of macrophages induced by OK-432, BCG or PSK 2 days before cell harvest was intermediate between that of thioglycollate-stimulated macrophages and BCG-activated macrophages.

Table I. The effect of immunostimulants on the respiratory burst and enzyme activity of peritoneal macrophages

Immunostimulant administration			PMA-stimulated production of		APD activity (nmol/min/mg)
Agent	Dose	Day*	O ₂ ⁻ (pmol/min/10 ⁶ cells)	H ₂ O ₂ (pmol/min/10 ⁶ cells)	
None	...	...	110 (18)	180 (10)	13.4 (0.6)
Thioglycollate	2 ml	4	140 (16)	250 (25)†	18.6 (1.0)†
BCG	1 mg	24	720 (25)‡	2000 (110)‡	2.2 (0.3)‡
<i>Lac. casei</i>	0.1 mg	13	730 (38)‡	2000 (9)‡	2.3 (0.2)‡
		7	710 (11)‡	1900 (44)‡	3.6 (1.0)‡
		2	520 (34)‡	1400 (39)‡	4.1 (0.9)‡
OK-432	0.1 mg	13	540 (30)‡	1700 (77)‡	4.4 (0.2)‡
		7	720 (10)‡	1700 (120)‡	2.4 (0.1)‡
		2	330 (24)‡	1000 (25)‡	4.1 (0.8)‡
BCG	0.1 mg	13	640 (17)‡	1900 (87)‡	5.0 (0.2)‡
		7	570 (20)‡	1700 (200)‡	4.2 (0.2)‡
		2	280 (16)‡	400 (15)‡	5.3 (0.1)‡
PSK	0.1 mg	13	110 (3)	160 (20)	16.1 (0.4)‡
		7	100 (3)	150 (9)	18.2 (0.4)‡
		2	300 (9)‡	480 (12)‡	6.8 (0.1)‡

Results are presented as the mean (SEM) of three determinations.

* The number of days before macrophage harvest that the agents were administered to the mice.

† Significantly different from resident macrophages, $p < 0.05$.

‡ Significantly different from resident macrophages, $p < 0.01$.

Enzyme activity in macrophage lysates

As shown in table I, APD activity, which is known to increase in stimulated macrophages and to decrease in activated macrophages,¹⁵ was significantly elevated in thioglycollate-stimulated macrophages, but was markedly depressed in BCG-activated macrophages. The APD activity of OK-432-, BCG-, and *Lac. casei*-induced macrophages harvested 2, 7 or 13 days after i.p. injection and PSK-induced macrophages harvested 2 days after injection was depressed; the activity of macrophages obtained 7 or 13 days after i.p. injection of PSK was elevated.

In-vitro listericidal activity of macrophages

As shown in table II, the in-vitro listericidal activity of thioglycollate-stimulated and BCG-activated macrophages was enhanced compared to that of resident macrophages. The activity of OK-432- or BCG-induced macrophages was similar to that of *Lac. casei*-induced macrophages. The listericidal activity of PSK-induced macrophages was enhanced on day 13, but not on day 7 or 2 compared to that of resident or thioglycollate-stimulated macrophages.

Protective activity of immunostimulants against *L. monocytogenes* infection

Mice treated i.v. with the various immunostimulants were infected with *L. monocytogenes* 10⁶ cells, and

survival was determined 3 and 7 days after infection (table III). Prolongation of survival was significant in *Lac. casei*-treated mice, but not in mice pre-treated with OK-432, BCG (0.1 mg) or PSK. The increase in survival time in mice treated with 1 mg of BCG 24 days before infection was greater than that of *Lac. casei*-treated mice.

Inhibition of *L. monocytogenes* multiplication in vivo

Killing of *L. monocytogenes* in the liver during the first 6 h after infection was significantly enhanced by administration of *Lac. casei*, but not by administration of the other agents, when given 13 or 7 days before infection (table III). The early killing of organisms was greatest in mice given 1 mg of BCG 24 days before infection. The multiplication of the organisms between 6 and 48 h after infection was significantly suppressed by pre-treatment with all of the agents given 13, 7 or 2 days before infection. Inhibition of growth of the organisms during the middle phase was greatest in mice given 1 mg of BCG 24 days before infection. Inhibition of growth of *L. monocytogenes* was not seen in mice given PSK 13 or 7 days before infection.

Discussion

In this study we have used immunostimulants injected i.p. and i.v. to investigate the regulation of

Table II. The effect of immunostimulants on the listericidal activity of peritoneal macrophages *in vitro*

Immunostimulant administration			Listericidal activity— mean (SEM) percentage survival after 10 h
Agent	Dose	Day*	
None	...	...	136 (6)
Thioglycollate	2 ml	4	112 (6)
BCG	1 mg	24	38 (3)†
<i>Lac. casei</i>	0.1 mg	13	15 (1)†
		7	41 (3)†
		2	470 (49)‡
OK-432	0.1 mg	13	51 (15)†
		7	65 (8)†
		2	429 (45)‡
BCG	0.1 mg	13	78 (12)‡
		7	84 (8)‡
		2	198 (10)†
PSK	0.1 mg	13	120 (16)
		7	108 (13)
		2	92 (4)‡

* The number of days before macrophage harvest that the agents were administered to mice.

† Significantly different from resident macrophages, $p < 0.01$.

‡ Significantly different from resident macrophages, $p < 0.05$.

macrophage functions and the induction of protective immunity against *L. monocytogenes* infection in mice. We have shown previously that activation of macrophages, e.g., by *Lac. casei*, is most readily seen in newly migrated macrophages, rather than in resident macrophages such as Kupffer cells of the liver.⁷ Therefore, we used the two routes of immunisation to probe the changes which occur in newly migrating macrophages, as exemplified by the activity of peritoneal exudate cells following i.p. stimulation, and the ability to control infection in the liver.

Peritoneal macrophages harvested 13 and 7 days after i.p. injection of OK-432, BCG (0.1 mg) or *Lac. casei* showed an increase in the PMA-triggered respiratory burst, which is a correlate of macrophage activation.^{12,13,16} They also showed a significantly lowered APD activity, which is known to decrease in immunologically-activated macrophages but to increase in chemically-elicited macrophages.¹⁵ The listericidal activity of peritoneal macrophages induced 13 or 7 days after injection of OK-432, BCG and *Lac. casei* was significantly increased, but was decreased in macrophages induced 2 days after injection. Thus, administration of OK-432, BCG (0.1 mg) and *Lac. casei* appears to result in activation of macrophages 13 and 7 days after injection. However, neither OK-432 nor BCG (0.1 mg) induced protection against *L. monocytogenes* infection in mice. Increased host resistance against *L. monocytogenes* 2 and 13 days after *Lac. casei* injection is largely mediated by macrophages newly migrating from the blood stream to the site of infection, although the increased resistance seen around 13 days after injection is also partly mediated by fixed macrophages which accumulate in the liver in response to *Lac. casei* treatment.⁷ Killing of *L. monocytogenes* 6 h after infection was not enhanced by i.v. injection of OK-432 or BCG (0.1 mg), but growth of the organisms between 6 and 48 h was inhibited, although the inhibition was lower than that caused by *Lac. casei* injection. In the early phase of infection (0–6 h), organisms are killed mainly by fixed macrophages in the liver, whereas during 6–48 h after infection blood monocyte-derived macrophages, newly accumulating at the site of infection, play a major role in preventing further multiplication of organisms.^{17,18} Thus, it appears that the functional enhancement of fixed macrophages in the liver by

Table III. The effect of immunostimulants on host resistance to *L. monocytogenes*

Immunostimulant administration			Protective activity evident (days after infection)		Viable <i>L. monocytogenes</i> harvested from livers—mean (SEM) log cfu/ liver (time after infection)			
Agent	Day*	Dose	3	7	6 h		48 h	
None	...	...	0/10	0/10	2.25	(0.05)	6.03	(0.13)
BCG	24	1 mg	10/10†	8/10†	0.60	(0.30)‡	1.73	(0.39)†
<i>Lac. casei</i>	13	0.1 mg	7/10†	5/10	0.96	(0.27)‡	3.90	(0.17)†
	7		NT	NT	1.97	(0.41)	4.15	(0.22)
	2		10/10†	4/10	2.39	(0.15)	4.11	(0.17)†
OK-432	13	0.1 mg	0/10	0/10	2.35	(0.12)	5.21	(0.23)‡
	7		NT	NT	2.35	(0.12)	5.23	(0.24)
	2		2/10	0/10	2.65	(0.15)	5.08	(0.28)‡
BCG	13	0.1 mg	3/10	0/10	2.12	(0.15)	4.51	(0.46)‡
	7		NT	NT	2.30	(0.13)	5.12	(0.28)‡
	2		0/10	0/10	2.36	(0.15)	4.79	(0.23)†
PSK	13	0.1 mg	0/10	0/10	2.28	(0.08)	5.85	(0.29)
	7		NT	NT	2.37	(0.14)	5.52	(0.37)
	2		0/10	0/10	2.12	(0.04)	5.74	(0.20)†

NT, not tested.

* The number of days before infection that the agents were administered to the mice.

† Significantly different from resident macrophages, $p < 0.01$.

‡ Significantly different from resident macrophages, $p < 0.05$.

OK-432 or BCG was not accompanied by an increase in the protective activity against *L. monocytogenes* infection. Killing of *L. monocytogenes* during the early (up to 6 h) stage of infection, as is seen with *Lac. casei*,⁷ is probably due to an increase in the number of fixed macrophages already present in the liver in response to the immunostimulating agent. Accumula-

tion of macrophages at the site of infection 6–48 h after *L. monocytogenes* infection is important for expression of protection in mice.

Thus, the lack of protective activity seen with OK-432, BCG or PSK may be due to the inability to adequately induce recruitment of macrophages to the site of infection.

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