PSK, a Polysaccharide from *Coriolus versicolor*, Enhances Oxygen Metabolism of Murine Peritoneal Macrophages and the Host Resistance to Listerial Infection

By HAJIME SAITO, HARUAKI TOMIOKA* AND KATSUMASA SATO

Department of Microbiology and Immunology, Shimane Medical University, Izumo 693, Japan

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PSK, a protein-bound polysaccharide isolated from the basidiomycete *Coriolus versicolor* (Fr.) Quél. was examined with regard to its effects of macrophage (M ϕ) oxygen metabolism in mice, a function important for the expression of M ϕ antimicrobial activity. The O $_2^-$ -producing ability and chemiluminescence (CL) of host peritoneal M ϕ s in response to phorbol myristate acetate were markedly elevated by preinjection of PSK (1 or 5 mg per mouse intraperitoneally) around 4–7 d before M ϕ harvest. The enhanced O $_2^-$ -producing ability due to PSK injection persisted much longer than the enhanced CL, indicating a discrepancy in regulation of generation of active oxygen species such as O $_2^-$, H $_2$ O $_2$, $\cdot$ OH, and 1 O $_2$. Daily injections of PSK (1 mg per injection) from 10 to 4 d before M ϕ harvest did not increase the efficacy of PSK over that given by a single 1 mg injection. When PSK (5 mg) was given intraperitoneally to mice in a single injection 10, 7 or 4 d before intravenous *Listeria monocytogenes* inoculation, a similar increase in the host resistance to the bacteria was noted regardless of the timing of the injection. Multiple PSK injections from 10 to 4 d before the infection also enhanced the host resistance, to the same degree. Therefore, PSK is thought to augment the host resistance to certain intracellular parasites including *L. monocytogenes* at least to some extent by enhancing oxygen metabolism of the host M ϕ s.

INTRODUCTION

Immunopotentiators, such as BCG (Borsos & Rapp, 1973), *Corynebacterium parvum* (Halpern *et al.*, 1973), OK-432 (a streptococcal preparation) (Shiraishi *et al.*, 1979), and *Lactobacillus casei* preparations (Kato *et al.*, 1981; Saito *et al.*, 1986*b*; Sato, 1984) can protect against experimental microbial infections due to opportunistic pathogens. PSK, a protein-bound polysaccharide preparation isolated from the basidiomycete *Coriolus versicolor* (Fr.) Quél., consisting mainly of glucan and acidic peptide (Tsukagoshi *et al.*, 1984), also enhances the host resistance to microbial infections such as those due to *Listeria monocytogenes* (Nomoto, 1981), *Pseudomonas aeruginosa* (Mayer & Drews, 1980) and *Candida* species (Uetsuka *et al.*, 1980), particularly in hosts with immunodeficiency due to malignant tumours or immunosuppressive treatment. PSK stimulates host macrophages (M ϕ s) and polymorphonuclear leucocytes (PMNs) causing an increase in chemotactic response, phagocytosis, pinocytosis and cell spreading, and this may be important for the PSK-mediated enhancement of host resistance to microbial infections (Nguyen & Stadtbaeder, 1980). Thus, it was of interest to determine whether this agent enhances the oxidative metabolism of phagocytic cells, which is closely related to their microbicidal function (Johnston, 1978; Klebanoff & Hamon, 1975; Nathan *et al.*, 1979), and to study the protective effect of PSK on murine infections induced by *L. monocytogenes*.

Abbreviations: CL, chemiluminescence; i.p., intraperitoneal(ly); i.v., intravenous(ly); M ϕ , macrophage; NBT, nitroblue tetrazolium; PEC, peritoneal exudate cell; PMA, phorbol myristate acetate; PMN, polymorphonuclear leucocyte.

METHODS

Mice. Female ddY mice (5 weeks old) were purchased from Shizuoka Laboratory Animal Center, Shizuoka, Japan.

Organisms. *Listeria monocytogenes* EGD (from Dr K. Takeya, Kyushu University, Fukuoka, Japan) was cultured statically in tryptic soy broth (Difco) at 37 °C for 24 h.

Experimental infections. Mice were infected intravenously (i.v.) with 1×10^6 c.f.u. of *L. monocytogenes*. PSK (Kureha Chemical Co., Tokyo, Japan) was dissolved in physiological saline and given intraperitoneally (i.p.) to mice (0.2 ml) in a dose of 5 mg per mouse per injection in a single injection at 10, 7 or 4 d before infection or in daily injections from 10 to 4 d before the infection. Survival rates were observed for up to 7 d after the infection.

O₂⁻ production by Mφs. Peritoneal exudate cells (PECs) from mice given i.p. injections of PSK or 10% (w/v) peptone (2 ml) were harvested in Hanks' balanced salt solution (HBSS) and suspended (2.5×10^5 cells ml⁻¹) in Eagle's minimal essential medium (MEM) (Nissui Pharmaceutical Co.) containing 10% (v/v) foetal bovine serum (FBS) (M. A. Bioproducts Co.). The resultant cell suspension (1 ml) was poured onto 16 mm culture wells (Corning), each containing a 14 mm plastic culture sheet (Wako Pure Chemical), and incubated in a CO₂ incubator (5% CO₂, 95% humidified air) at 37 °C for 2–3 h. The culture sheet was then thoroughly rinsed with PBS to remove nonadherent cells. Mφ monolayer cultures were then incubated in a CO₂ incubator for 30 min, in 10% (v/v) FBS-MEM containing 0.1% (w/v) nitroblue tetrazolium (NBT), with or without the addition of 10 or 100 ng phorbol myristate acetate (PMA; Sigma) ml⁻¹. The Mφ monolayer sheet was then fixed with formaldehyde and the percentage of NBT-reducing cells was counted. O₂⁻ generated in Mφ reduces NBT, producing a blue pigment in the cytoplasm (Krueger *et al.*, 1976).

Chemiluminescence of Mφs. PECs induced with PSK or peptone were resuspended (10^6 cells ml⁻¹) in 1 ml phenol-red-free HBSS containing 0.1 mM-luminol. After warming at 37 °C for 1 min, the background photoemission was counted in a luminometer (Lumicounter ATP-237, Toyo Kagaku Sangyo, Tokyo) for 10 s and the PMA-triggered chemiluminescence (CL) was measured at intervals for up to 5 min after the addition of PMA dissolved in 10 µl dimethylsulphoxide, to give a final concentration of 100 ng ml⁻¹.

RESULTS

Alteration in oxidative metabolism of host Mφs by PSK. We studied the effects of PSK on oxygen metabolism of host Mφs, based on PMA-triggered O₂⁻ production and CL. Table 1 shows changes in the O₂⁻ production by host peritoneal Mφs in response to PMA after a single injection (i.p.) of PSK. A marked increase in O₂⁻-producing ability of host Mφs was seen around 4–7 d after injection of either 1 or 5 mg of PSK per mouse. The enhanced O₂⁻-generating state persisted for up to 21 d after the PSK injection and declined thereafter. At 4 d after the PSK injection, there was an increase in the spontaneous O₂⁻ production of host Mφs. A marked enhancement of CL was seen around 4 d after PSK treatment, a much higher response being observed in Mφs from mice given 5 mg PSK per mouse than those given only 1 mg (Fig. 1). It is possible that the peak of CL appeared earlier than day 4. The enhanced state of Mφ CL rapidly returned to normal thereafter, indicating that, on the basis of CL function of Mφs, PSK-induced activation of Mφ is transient.

To determine whether PSK primes Mφs systemically, we studied the effect of PSK given as a single i.v. injection on O₂⁻ generation by host peritoneal Mφs induced with peptone (Table 2). Although the peptone-induced Mφs from control mice showed a considerably more intense CL than did normal Mφs, the preceding PSK injection caused a more marked enhancement of CL of the peptone-elicited Mφs, and this effect was evident around 7–21 d after the PSK priming. In this case, no difference was noted between the priming effect of PSK given in a dose of 1 or 5 mg per mouse. PSK also stimulated or primed host PMNs systemically in respect of their CL response (data not shown).

Effect of PSK given in multiple injections on the Mφ oxidative burst. Figs 2 and 3 compare the effects of PSK injected i.p. to mice as a single dose or in multiple doses (four and seven injections) on O₂⁻ production and the CL response of host peritoneal Mφs. In both cases, no significant difference was noted in the level of Mφ stimulation whether single, four, or seven injections of the agent were given. Similarly, the Mφ-priming activity of PSK was not enhanced by multiple injections (data not shown).

Fig. 1. Effect of PSK on PMA-induced CL of host peritoneal M ϕ s. Mice were given PSK i.p. at a dose of 1 mg (○) or 5 mg (△) per mouse and the peritoneal M ϕ s were harvested and measured for CL in response to 100 ng PMA ml⁻¹ at the indicated times. The relative intensity of CL of a given M ϕ population was calculated as (cumulative counts from 1 to 5 min after PMA-triggering of test M ϕ s)/(cumulative counts of resident M ϕ s). The resident M ϕ s gave a CL reading of 13020 c.p.m. per 10⁶ cells in response to PMA as a peak value and the value for cumulative counts was 55290. Each symbol indicates the mean $\pm$ SEM of two incubation mixtures of pooled PECs.

Table 1. Enhancement of O₂⁻-producing ability of peritoneal M ϕ s after i.p. injection of PSK

Mice were given PSK i.p. at the indicated dose on day 0, and the O₂⁻-producing ability of host peritoneal M ϕ s in the presence or absence of 100 ng PMA ml⁻¹ was measured on the days indicated after PSK injection.

Time of M ϕ harvest after PSK injection (d)	PSK injection (mg per mouse)*	O ₂ ⁻ production of M ϕ s (% of M ϕ s reducing NBT)†		Increase in PMA-triggered O ₂ ⁻ production after PSK injection (%)
		Spontaneous	PMA-triggered	
4	-	0.2 $\pm$ 0.2	32 $\pm$ 1	
	1	13 $\pm$ 1	66 $\pm$ 1	106
	5	14 $\pm$ 1	67 $\pm$ 4	109
7	-	1.6 $\pm$ 0.2	22 $\pm$ 2	
	1	1.4 $\pm$ 0.3	41 $\pm$ 1	86
	5	1.4 $\pm$ 0.9	55 $\pm$ 1	150
14	-	0.3 $\pm$ 0.3	37 $\pm$ 2	
	1	0.8 $\pm$ 0.1	64 $\pm$ 1	73
	5	7.6 $\pm$ 4.6	67 $\pm$ 9	81
21	-	0.5 $\pm$ 0.5	40 $\pm$ 12	
	1	1.1 $\pm$ 0.1	76 $\pm$ 3	90
	5	1.1 $\pm$ 0.5	78 $\pm$ 3	95
28	-	0.8 $\pm$ 0.2	48 $\pm$ 2	
	1	1.1 $\pm$ 0.6	68 $\pm$ 7	42
	5	0.8 $\pm$ 0.7	60 $\pm$ 9	25

* -, Solute control.

† The results are means $\pm$ SEM of two incubations of pooled M ϕ s from four or five mice.

Effect of PSK on host resistance to listerial infection. As indicated above, PSK is thought to enhance the oxygen metabolism of host M ϕ s in a direct and systemic manner and by so doing to potentiate their antimicrobial activity, especially against intracellular parasites including *Listeria monocytogenes* (Klebanoff & Hamon, 1975; Nathan, 1983). We therefore examined the effect of PSK on host resistance to listerial infection. When mice were injected i.p. with PSK (5 mg) in a single dose at 10, 7 or 4 d before i.v. injection with *L. monocytogenes*, their survival at

Fig. 2

Fig. 2. Comparison of single and multiple injections of PSK for efficacy in enhancing O_2^- production of peritoneal Mφs in response to 10 (open bars) and 100 (shaded bars) ng PMA ml^{-1} . PSK (1 mg per injection) was given i.p. to mice in the indicated protocols. Each bar indicates the mean $\pm$ SEM of two incubation mixtures of pooled Mφs.

Fig. 3

Fig. 3. Comparison of single and multiple injections of PSK for efficacy in enhancing CL of peritoneal Mφs in response to 100 ng PMA ml^{-1} . PSK (1 mg per mouse per injection) was given i.p. to mice in a single dose 4 d before Mφ harvest (●), in four doses at 10, 8, 6, and 4 d before harvest (Δ), or daily from 10 to 4 d (seven doses) before harvest (▲). ○, CL of normal Mφs. Each symbol indicates the mean $\pm$ SEM of two incubation mixtures of pooled PECs.

Table 2. *Priming activity of PSK on O_2^- -producing ability of peritoneal Mφs*

Mice were given PSK i.v. at the indicated dose on day 0, and peptone-induced peritoneal Mφs were harvested 4–28 d later (peptone was given i.p. 4 d before Mφ harvest) and assayed for their O_2^- -producing ability in the presence or absence of 100 ng PMA ml^{-1} .

Time of Mφ harvest after PSK injection (d)	PSK injection (mg per mouse)*	O_2^- production of Mφs (% of Mφs reducing NBT)†		Increase in PMA-triggered O_2^- production after PSK injection (%)
		Spontaneous	PMA-triggered	
4	–	5.8 ± 2.1	58 ± 12	
	1	6.2 ± 1.3	70 ± 3	21
	5	11 ± 2	69 ± 2	19
7	–	1.9 ± 0.4	50 ± 4	
	1	1.8 ± 0.2	74 ± 4	48
	5	2.2 ± 0.6	76 ± 12	52
14	–	1.0 ± 0.4	46 ± 2	
	1	1.9 ± 0.5	61 ± 2	33
	5	2.8 ± 0.7	66 ± 6	43
21	–	2.8 ± 2.7	45 ± 0	
	1	5.4 ± 5.4	68 ± 3	51
	5	2.8 ± 0.6	62 ± 13	38
28	–	2.1 ± 0.0	41 ± 4	
	1	2.3 ± 0.3	49 ± 1	20
	5	4.1 ± 4.0	48 ± 2	17

* –, Solute control.

† The results are means $\pm$ SEM of two incubations of pooled Mφs.

3.5 and 7 d was increased from 0% (control mice) to 50% and 20–30%, respectively. A similar increase in the host resistance was noted regardless of the timing of the injection. A similar efficacy was also noted when PSK was given to mice in multiple injections from 10 to 4 d before the infection. In such cases, it may be noteworthy that the protective effect of PSK is systemic because the agent injected i.p. increased the host resistance against *L. monocytogenes* injected i.v.

DISCUSSION

As indicated in Tables 1 and 2, the oxidative burst of host Mφs in response to PMA was enhanced following PSK injection, especially when PSK was administered 4–7 d before Mφ harvest. This indicates the functional activation of Mφs by PSK, because 'activated' Mφs possess higher responsiveness to PMA-triggering, in terms of respiratory burst, than do normal Mφs (Johnston *et al.*, 1978; Nathan & Root, 1977; Tomioka & Saito, 1980). About a twofold increase in the number of Mφs in the peritoneal cavity of host mice was observed from 4 to 14 d after the injection (i.p.) of PSK, while the number of PMNs was increased only within 4 d of the PSK injection. Therefore, the Mφ populations newly accumulated in the peritoneal cavity of host mice after PSK injection may be closely related to the functionally activated state of the host peritoneal Mφs.

We noted a marked difference between the patterns of PMA-triggered O_2^- production and CL response of Mφs, after a single injection (i.p.) of PSK (Table 1 and Fig. 1). An elevated state of O_2^- -generating ability of host Mφs due to PSK injection persisted, even for up to 28 d after the injection, whereas the PSK-induced enhancement of Mφ CL was seen only around 4–7 d after the injection. Similar discrepancies were noted between the H_2O_2 -releasing ability and CL response of host Mφs during the course of *Mycobacterium intracellulare* and *Listeria monocytogenes* infections (Saito *et al.*, 1986a) and between the O_2^- -producing ability and CL of host Mφs after *Lactobacillus casei* injection (Saito *et al.*, 1987). Mφ CL has been reported to originate from 1O_2 and 1OH (Sagone *et al.*, 1977) rather than O_2^- and H_2O_2 . In Mφs, 1O_2 and 1OH are generated mainly through the Haber–Weiss reaction, catalysed by iron and tryptophan (Halliwell, 1978) and through the peroxidase/ H_2O_2 -mediated halogenation reaction (Rosen & Klebanoff, 1977), and these reactions are thought to be regulated by different cellular mechanisms from that related to regulation of PMA-triggering of NADPH oxidase (Johnston & Kitagawa, 1985), which generates O_2^- (Tsunawaki & Nathan, 1984). Therefore, in the later period after PSK injection, the peritoneal Mφs are probably still enhanced in the NADPH oxidase system and in the signal transfer system from the PMA-receptor to the enzyme (Johnston & Kitagawa, 1985) but the 1O_2 and 1OH generating functions are reduced to a normal level. However, the profile of PMA-triggered O_2^- production differs too much from that of PMA-triggered CL to be fully explained by the above reasoning. Because HOCl, which is generated in a H_2O_2 -mediated halogenation reaction catalysed by peroxidases such as myeloperoxidase and lactoperoxidase or by Fe^{2+} bound to certain chelating substances (Harrison & Schultz, 1976), oxidizes luminol resulting in intense photoemission (Seitz, 1975), the PMA-triggered Mφ CL might partly reflect the activity of the halogenation system in a given Mφ cell. If so, the observed discrepancy between PMA-triggered O_2^- production and CL may be given an additional explanation, based on the situation that the duration of the elevated state in the halogenation system differs from that in the NADPH oxidase system. The O_2^- (and H_2O_2)-producing ability and CL, in response to PMA and other stimuli (Pick & Keisari, 1981), serve as parameters representing the activated state of Mφs (Johnston *et al.*, 1978; Karnovsky & Lazdins, 1978; Nathan & Root, 1977; Schleupner & Glasgow, 1978). Our present observations indicate that the two parameters are not always in concert, particularly with respect to the timing of their alteration patterns after Mφ stimulation. It remains to be determined which parameter more adequately represents the 'activated state of Mφs', including differentiation of the 'immunologically activated state' (Karnovsky & Lazdins, 1978) from the merely 'stimulated state' (Karnovsky & Lazdins, 1978; Tomioka & Saito, 1980).

We also found that PSK to some extent enhanced the host resistance to *Listeria monocytogenes* infection in mice. Regardless of the timing of PSK injection(s) in a period of 10–4 d before the infection, the agent enhanced the host resistance to *L. monocytogenes* in a similar fashion. This suggests the importance of the priming action of the agent on M ϕ functions, as revealed in experiments shown in Table 2, or by an action which accelerates emigration of blood monocytes to the site of infection (North, 1970). We observed that *Lactobacillus casei*, a bacterial immunopotentiator (Kato *et al.*, 1981), stimulated not only the cellular functions of fixed M ϕ s in the reticuloendothelial system but also the accumulation of free M ϕ s (blood monocytes) at the site of listerial infection. The latter is important for expression of the protective activity of *Lactobacillus casei* against *Listeria monocytogenes* infection (Sato, 1984).

Multiple injections of PSK did not increase the protective efficacy of the agent as compared to a single injection. This may be explained by the finding that both the O₂-producing ability and the CL of host M ϕ s were similarly enhanced by either multiple PSK injections or a single injection (Figs 2 and 3). Although the reason for this phenomenon is unknown, it suggests that multiple injections of PSK would probably be of little benefit in controlling infection in a clinical situation. It may be noteworthy that some immunopotentiators, such as OK-432 (streptococcal preparation) and LC 9018 (*Lactobacillus casei* preparation), induced potent suppressor M ϕ s in the spleen of host animals when administered in multiple injections (Tomioka & Saito, 1985; unpublished results). In preliminary experiments, we also found that PSK given in a large dose elicited suppressor cells among mouse splenocytes (unpublished observations).

Our study has shown that PSK enhances the resistance of mice to infection by *Listeria monocytogenes*. Since PSK has few side effects (Tsukagoshi *et al.*, 1984), it may be useful for immunotherapy of immunocompromised hosts.

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