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Influence of PSK (Krestin) on resistance to infection of *Pseudomonas aeruginosa* in tumor-bearing mice

Takao Ando[1], Isamu Motokawa[1], Katsuo Sakurai[1], Yoshio Ohmura[1], Takayoshi Fujii[1], Kenichi Matsunaga[1], Chikao Yoshikumi[1], and Kikuo Nomoto[2]

[1] Biomedical Research Laboratories, Kureha Chemical Industry Co., Ltd, 3-26-2 Hyakunin-cho, Shinjuku-ku, Tokyo 160, Japan
[2] Department of Immunology, Medical Institute for Bioregulation Research, Kyushu University, Higashi-ku, Fukuoka 812, Japan

Summary. C3H/He mice were inoculated with *Pseudomonas aeruginosa* by various routes 1 day after X5563 transplantation or 4 days after cyclophosphamide (CY) administration. Administration of PSK (Krestin) i.p. or p.o. to the tumor-bearing mice or CY-treated tumor-bearing mice resulted in an increase in survival rates. Viable *P. aeruginosa* were inoculated i.v. on day 0 into mice inoculated with tumor cells on day −12 and vaccinated with killed *P. aeruginosa* on day −10, or into mice inoculated with tumor cells on day −15, treated with CY on day −14 and vaccinated on day −10. Resistance to infection, which is enhanced by vaccination, was depressed by tumor burden or treatment with CY, but such depression was prevented by PSK administration.

Introduction

In patients with malignant tumors, the functions of phagocytes and immunocompetent cells are depressed [11], and the level of protection afforded against infections by such cell populations is further depressed by treatment with a variety of anticancer drugs [1, 11]. This suggests the need for augmentation of the host defense mechanism for successful prevention of infections.

Various microbial products have been shown to enhance nonspecific resistance to infections [4, 5, 14, 16] and suppress the growth of tumors [2, 3, 10] in experimental animals. PSK (Krestin), a protein-bound polysaccharide extracted from the cultured mycelium of *Basidiomycetes*, was reported to possess an activity to restore the depressed immunofunctions in tumor-bearing animals [12, 19, 21].

Accordingly, we investigated the protective action of PSK against *Pseudomonas aeruginosa* infection in tumor-bearing and anticancer drug-treated mice.

Materials and methods

Animals. Female C3H/He mice 6–8 weeks old were used (Charles River Japan Inc., Atsugi, Japan).

Tumor. X5563 plasmacytoma was maintained in ascites form. Mice were inoculated s.c. with $1-2 \times 10^6$ tumor cells.

Chemotherapy. Cyclophosphamide (CY, Endoxan, Shionogi & Co., Ltd, Osaka, Japan) was administered s.c. at a dose of 150 mg/kg 1 day after tumor inoculation.

Strain. Pseudomonas aeruginosa IAM1514 was used as the infectious organism. *P. aeruginosa* was cultured on heart infusion agar (Difco Laboratories, Detroit, Mich, USA) and suspended in sterile physiological saline.

Vaccine. Formalin-killed *P. aeruginosa* (2×10^8 cells) were s.c. injected into the foot pad of mice 2 days after tumor inoculation in an experiment without CY treatment or 4 days after CY administration in an experiment with CY treatment.

Infection. Mice were inoculated with *P. aeruginosa* i.p., i.v., or s.c. at various times after tumor inoculation. CY-treated tumor-bearing mice received the organisms i.p., i.v., s.c. or through the urinary tract, or were exposed to an aerosol containing bacteria at various times after CY treatment. For urinary tract infection, mice were not given water for a full day before infection. After injection of bacteria into the urethra, the orifice was closed with a small clip for 1 h, and mice were given water 1 h later. For respiratory tract infection, mice were exposed to an aerosol containing 10 ml bacterial suspension (2.5×10^9 cells/ml) for 30 min using a glass nebulizer. Mice were infected i.v. 10 days after vaccination. Deaths due to infection were recorded.

Determination of bacterial growth. Mice received injections i.v. or through the urinary tract of viable *P. aeruginosa.* At various times after such infection, they were bled from a femoral artery and their kidneys were removed. Individual kidneys were homogenized in 10 ml physiological saline using a Homoblender (Model 500, Sakuma Manufacturing Co., Ltd, Tokyo, Japan). The homogenates were diluted 10-fold, and 0.1 ml of each dilution was spread on heart infusion agar. After incubation at 37° C for 18 h colonies were counted.

Count of peritoneal exudate cells. One day after tumor inoculation, mice received formalin-killed *P. aeruginosa* (1×10^8 cells) by i.p. injection. Mice were sacrificed by cervical dislocation 6 and 24 h later, and the peritoneal cavities were exposed by abdominal incision. The cavity was washed with 10 ml Hanks' balanced salt solution (HBSS,

Offprint requests to: Takao Ando

Abbreviations used: CY, cyclophosphamide; i.p., intraperitoneally; s.c., subcutaneously; p.o., orally; i.v., intravenously

GIBCO Laboratories, Grand Island, USA). The peritoneal washings were centrifuged for 10 min at 300 *g*. The cells were then resuspended in 1.0 ml HBSS, and the total number of cells was counted using a hemacytometer.

Count of peripheral leukocytes. Blood specimens were collected by puncture of the retro-orbital venous plexus at various times after CY administration. Total numbers of leukocytes were determined by means of a Coulter counter (model ZBI, Coulter Electronics, Inc., Hialeah, Fla, USA), and differential cell counts were carried out after staining of smears with Giemsa's solution.

Determination of agglutinating antibody titers. Ten days after vaccination, sera were collected by puncture of the abdominal vein and diluted in serial 2-fold dilutions in physiological saline. Formalin-killed *P. aeruginosa* suspended to 1×10^8 cells/ml in physiological saline was used. Agglutination, scored from 1 to 4, was read macroscopically, and the agglutination titer was expressed as the reciprocal of the last antibody dilution giving 1 plus agglutination.

PSK. PSK was administered p.o. at a dose of 1000 mg/kg every day, or i.p. at 50 mg/kg, or i.v. at 5 mg/kg, every other day before and after infection.

Table 1. Survival rates of mice infected with *P. aeruginosa* at various times after tumor inoculation

Dose and route of *P. aeruginosa*	Survival (%) on day 7 after bacterial infection			
	Non-grafted control	Intervals between tumor inoculation and bacterial infection (days)		
		1	7	14
1×10^7 i.p.	40	0*	60	0*
4.5×10^7 i.v.	60	10*	80	0**
5×10^7 s.c.	50	0**	40	0**

Each group consisted of 10 mice
$*P < 0.05$, $**P < 0.01$ compared with the corresponding non-grafted controls

Table 2. Effects of PSK on the survival rates of tumor-bearing mice infected with *P. aeruginosa*

Dose and route of *P. aeruginosa*	Survival (%) on day 7 after bacterial infection[a]				
	Normal mice	Tumor-bearing mice	Tumor-bearing mice treated with PSK[b] by the following routes		
			i.p.	i.v.	p.o.
1.5×10^7 i.p.	90	10	100**	80**	60*
2×10^7 i.v.	90	0	80**	60**	30
8×10^7 s.c.	70	0	70**	30	40*

[a] Bacterial infection was carried out 1 day after tumor inoculation. Each group consisted of 10 mice
[b] PSK was given from 8 days before to 6 days after bacterial infection
$*P < 0.05$; $**P < 0.01$; for comparison with the corresponding tumor-bearing mice

Statistics. Student's *t*-test, the chi-square test or the Mann and Whitney test was used for the comparison of results.

Results

Protective effect of PSK against infection in tumor-bearing mice

Regardless of the route of challenge, survival rates were lower on days 1 and 14 after tumor inoculation in test animals than in normal mice. On day 7, however, the survival rate was not reduced (Table 1).

As shown in Table 2, the reduction of resistance to infection on day 1 after tumor inoculation was prevented by

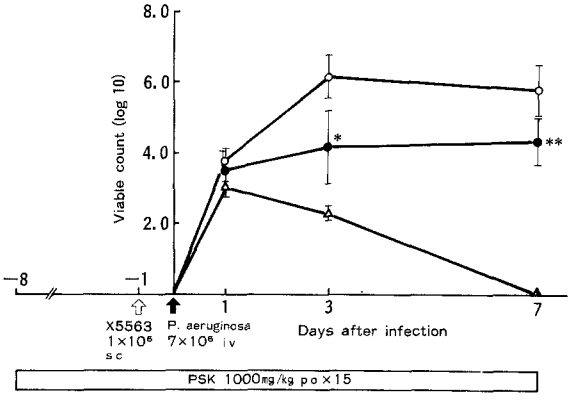

Fig. 1. Effect of PSK on bacterial growth in the kidneys of tumor-bearing mice infected i.v. with *P. aeruginosa*. Each group consisted of 5 mice. △, normal mice; ○, tumor-bearing mice; ●, PSK-treated tumor-bearing mice. $*P < 0.01$, $**P < 0.05$ compared with tumor-bearing mice

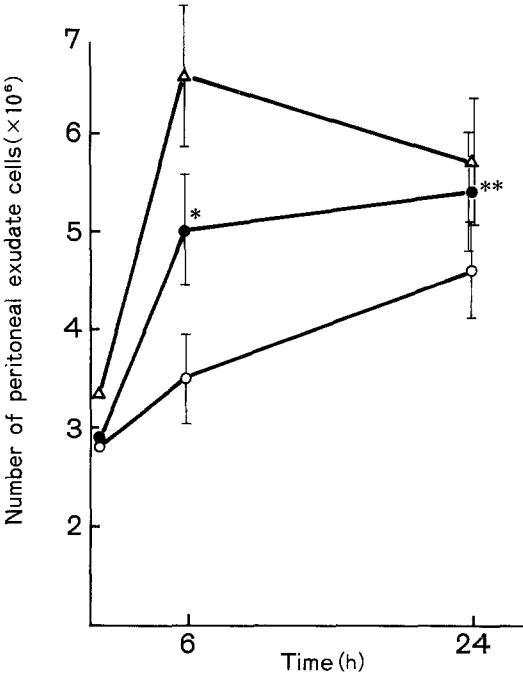

Fig. 2. Effect of PSK on peritoneal exudate cell counts in tumor-bearing mice. Killed bacteria were injected i.p. 1 day after tumor inoculation. PSK was given p.o. for 8 days before injection of killed bacteria. Each group consisted of 5 mice. △, normal mice; ○, tumor-bearing mice; ●, PSK-treated tumor-bearing mice. $*P < 0.01$, $**P < 0.05$ compared with tumor-bearing mice

PSK administration (i.p., i.v. or p.o.). The number of bacteria in the kidneys of mice inoculated with bacteria at such a time was markedly increased over that in normal mice at 3 days after infection, but was decreased by oral administration of PSK (Fig. 1).

The peritoneal exudate cell count at 6 and 24 h after an i.p. injection of killed bacteria was lower in mice inoculated with tumor 1 day before than in controls that had received injections of killed bacteria but been restored by oral administration of PSK (Fig. 2).

Protective effect of PSK against infection in CY-treated tumor-bearing mice

When mice were given CY 1 day after tumor inoculation and infected with *P. aeruginosa* after another 4 days, a further reduction in resistance to infection was noted regardless of the route of infection (Table 3).

When PSK was given i.p., the survival rates were higher in the animals infected through the i.v., i.p., s.c., urinary tract, or respiratory tract route than in mice infected through such routes but not given PSK. After urinary tract infection, bacteria in the kidneys disappeared more rapidly in PSK-treated mice than in controls (Table 4).

Table 3. Effects of PSK on the survival rates of CY-treated tumor-bearing mice infected with *P. aeruginosa*

Dose and route of *P. aeruginosa*	Survival (%) on day 7 after bacterial infection[a]		
	Normal mice	CY-treated tumor-bearing mice	
		Control	PSK[b]
4×10^6 i.p.	100	20	90*
5×10^6 i.v.	100	20	80*
5×10^6 s.c.	100	0	80*
1×10^7 urinary tract	100	50	100*
Aerosol	100	30	100*

[a] Bacterial infection was carried out on day 0 after tumor inoculation on day −5 and CY-treatment on day −4. Each group consisted of 10 mice

[b] PSK was given i.p. from 8 days before to 6 days after bacterial infection

*$P < 0.01$ compared with the corresponding controls

Table 4. Effect of PSK on bacterial growth in the kidneys of CY-treated tumor-bearing mice infected by the urinary tract route

Days after bacterial infection[a]	Number of bacteria in the kidneys (log 10)[b]		
	Normal mice	CY-treated tumor-bearing mice	
		Control	PSK[c]
5	5.0±0.6	7.7±0.4	7.2±0.5
12	2.9±0.6	7.0±1.2	5.4±0.9*

[a] Bacterial infection (1×10^7 cells) was carried out on day 0 after tumor inoculation on day −5 and CY-treatment on day −4. Each group consisted of 5 mice

[b] Mean ± SD

[c] PSK was given i.p. from 8 days before to 6 days after bacterial infection

*$P < 0.05$ compared with the corresponding controls

On day 4 after CY treatment in normal mice, the granulocyte count was decreased. Recovery started on day 7, and a transient increase was observed on day 9. PSK administration led to a rapid recovery and a remarkable increase in the granulocyte count (Fig. 3).

Combined effect of PSK and vaccine

The vaccine showed a marked protective action against *P. aeruginosa* infection in normal mice. This protection could be transferred by serum from vaccinated mice (Table 5).

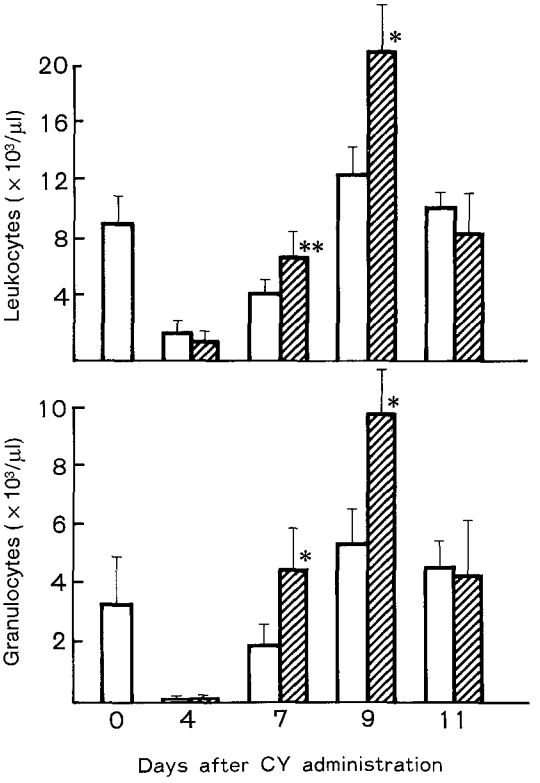

Fig. 3. Leukocyte and granulocyte counts in mice treated with CY and PSK. CY (150 mg/kg) was given s.c. to all mice. PSK (50 mg/kg) was given i.p. every other day from 4 days before CY administration. Each group consisted of 5 mice. *Open columns*, control mice; *shaded columns*, PSK-treated mice. * $P < 0.01$, ** $P < 0.05$ compared with corresponding controls

Table 5. Effects of vaccine and serum transfer on the survival rates of normal mice infected with *P. aeruginosa*

Dose and route of *P. aeruginosa*	Survival (%) on day 7 after bacterial infection		
	Non-treated control	Vaccination[a]	Serum transfer[b]
6×10^8 i.v.	0	0	–
1.5×10^8 i.v.	0	100*	60*
2×10^7 i.v.	90	100	–

Each group consisted of 10 mice

[a] Vaccination was carried out 10 days before bacterial infection

[b] Two hours before bacterial infection mice were given 0.5 ml serum i.p., which was obtained from mice 10 days after vaccination

*$P < 0.01$ compared with the corresponding non-treated controls

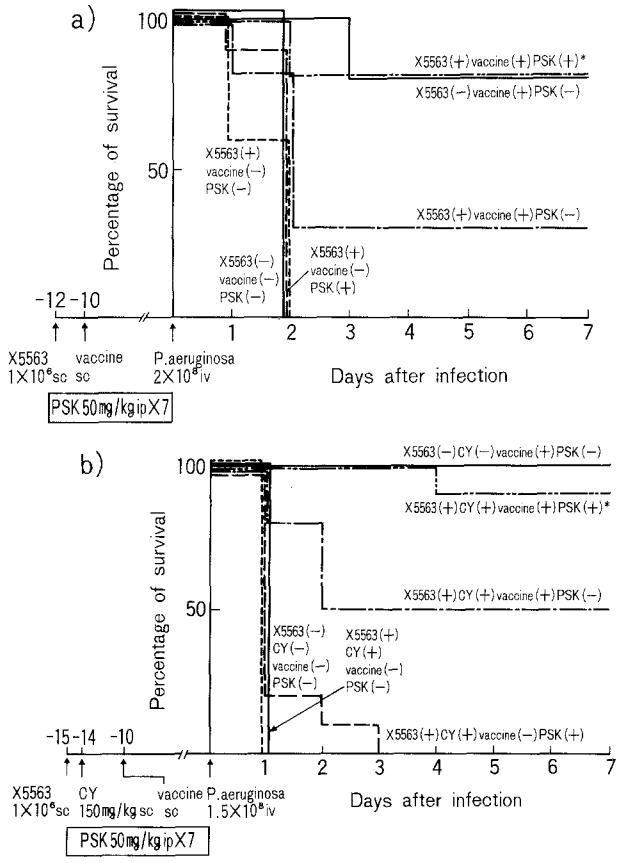

Fig. 4a, b. Synergism of PSK and vaccine in protection against *P. aeruginosa* infection in tumor-bearing mice **(a)** and CY-treated tumor-bearing mice **(b)**. Each group consisted of 10 mice. * $P < 0.05$ compared with corresponding non-PSK-treated groups

Table 6. Effects of PSK on serum agglutinating antibody titers of tumor-bearing mice and CY-treated tumor-bearing mice given vaccine

Sera[a]	Agglutinating antibody titers (log 2)[e]
Normal mice	5.0±0.6
Tumor-bearing mice[b]	2.6±0.5
PSK[c]-treated tumor-bearing mice	3.8±0.4**
CY-treated tumor-bearing mice[d]	1.8±0.8
CY- and PSK[c]-treated tumor-bearing mice	3.0±0.8*

[a] Sera were obtained from 5 mice in each group 10 days after vaccination
[b] Vaccination was carried out on day 0 after tumor inoculation on day −2
[c] PSK was given i.p. from 4 days before to 10 days after vaccination
[d] Vaccination was carried out on day 0 after tumor inoculation on day −5 and CY-treatment on day −4
[e] Mean ± SD
*$P < 0.05$; **$P < 0.01$ compared with corresponding non-PSK-treated mice

Bacterial infection was carried out on day 0 after tumor inoculation on day −12 and vaccination on day −10 (Fig. 4a). In an experiment with CY treatment, bacterial infection was carried out on day 0 after tumor inoculation on day −15, CY treatment on day −14 and vaccination on day −10 (Fig. 4b).

In tumor-bearing mice or those treated with CY, the protection afforded by the vaccine was less pronounced and agglutinating antibody titers were lower. However, these phenomena were attenuated by PSK administration (Fig. 4, Table 6).

Discussion

It has been reported that neutrophils play an important role in resistance to infection with *P. aeruginosa* [18], and when serious infection occurs antibodies have been shown to induce aggregation and to promote phagocytosis of neutrophils [6].

The resistance to *P. aeruginosa* infection was decreased in the initial and terminal stages of tumor development in mice. On the other hand, resistance was slightly higher in the intermediate stage.

Chemotaxis of neutrophils and macrophages is reported to decrease in cancer petients [9] or tumor-bearing mice [17]. We also noted a decrease in the number of peritoneal exudate cells in response to killed *P. aeruginosa* in tumor-bearing mice. This suggests that phagocyte accumulation at the infected lesion is inhibited by the tumor, decreasing the host's resistance to infection. An increase in the resistance to infection in the intermediate stage of tumor development seems to have resulted from activation of phagocytes by lymphokine-producing sensitized lymphocytes, which may be greater in degree than a decrease in accumulation. PSK was effective in restoring resistance to infection by *P. aeruginosa* in the initial stage of tumor development.

Cyclophosphamide reduces resistance to infection by its cytotoxic effect on leukocytes. Significant decreases in both the number of granulocytes and the resistance to infection were noted in our experiment. The administration of PSK prevented such decreases in resistance. Satoh et al. [15] reported that PSK increased colony-stimulating factors in murine serum. PSK was considered to prevent the decrease in resistance to infection by increasing the number of granulocytes after CY administration.

Immunodeficient patients are given various types of bacterial vaccines to prevent infectious diseases [20, 22]. However, vaccines are not effective in patients with a decreased ability to produce antibody, as observed in leukemia [13, 22].

In this study, protection by the vaccine was reduced in mice treated with CY and or inoculated with tumor. With the administration of PSK, however, resistance recovered nearly to the level of non-tumor-bearing mice. PSK was reported to prevent the tumor-induced decrease in the antibody production against sheep red blood cells [12, 21]. A similar mechanism may work to prevent the decrease in the antibody production against *P. aeruginosa.*

The incidence of complications related to infectious disease is high in cancer patients [7, 8]. Such complications not only have an adverse effect on the treatment of cancer, but are often the direct cause of death. *P. aeruginosa* is considered to be one of the most serious opportunistic bacterial pathogens causing terminal infection and superinfection. Antimicrobial agents can have only inadequate effects in the absence of host defense against infection.

PSK has been demonstrated to be effective in preventing infectious diseases in tumor-bearing hosts or in hosts whose immunological functions are suppressed by carcinostatic agents.

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