*Acta Medica Okayama*

*Volume* 48, *Issue* 5 1994 *Article* 2

OCTOBER 1994

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**Abstract**

PSK (Krestin) is a protein-bound polysaccharide with antitumor and immunomodulatory activity. In this study, the effects of the oral administration of PSK were investigated on the natural killer (NK) activity of liver-associated lymphocytes and their subfractions separated by density gradient centrifugation, in WKAH rats with liver metastasis of KDA hepatoma. PSK was administered orally, at a dose of 500 mg/kg once a day for 3 weeks. The NK activity of nonparenchymal liver cells (NPLC) and their subfractions, including large granular lymphocytes (LGL), was markedly augmented by this treatment. The effects of oral PSK were also examined in CDF1 mice with liver metastases of Colon 26 adenocarcinoma; the survival of tumor-bearing mice was prolonged and both metastatic foci and liver weight were decreased. These results suggest that PSK may be effective for the suppression of liver metastasis through activation of liver-associated NK cells.

**KEYWORDS:** PSK, NK activity, liver, metastasis, cancer

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*PMID: 7863794 [PubMed - indexed for MEDLINE]

ACTA MED OKAYAMA 1994; 48(5): 237-242

# Suppression of Hepatic Natural Killer Activity by Liver Metastasis of Cancer and Restoration of Killer Activity by Oral Administration of a *Basidomycetes*-Derived Polysaccharide, PSK

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PSK (Krestin) is a protein-bound polysaccharide with antitumor and immunomodulatory activity. In this study, the effects of the oral administration of PSK were investigated on the natural killer (NK) activity of liver-associated lymphocytes and their subfractions separated by density gradient centrifugation, in WKAH rats with liver metatasis of KDA hepatoma. PSK was administered orally, at a dose of 500 mg/kg once a day for 3 weeks. The NK activity of nonparenchymal liver cells (NPLC) and their subfractions, including large granular lymphocytes (LGL), was markedly augmented by this treatment. The effects of oral PSK were also examined in CDF1 mice with liver metastases of Colon 26 adenocarcinoma; the survival of tumor-bearing mice was prolonged and both metastatic foci and liver weight were decreased. These results suggest that PSK may be effective for the suppression of liver metastasis through activation of liver-associated NK cells.

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PSK (Krestin), a protein-bound polysaccharide derived from *Coriolus versicolor* of the class *Basidiomycetes*, is a biological response modifier (BRM) currently used clinically in Japan (1). PSK has been reported to exert antitumor activity in animals by restoring depressed immune activity (2-4). It has also been shown that PSK stimulates the production of interferon, interleukin (IL)-1, IL-2, tumor necrosis factor (5) and IL-8 (6). In clinical studies, PSK has been reported to be effective against gastrointestinal cancers when used in combination with UFT (uracil and tegafur) (7-8). However, there have been few detailed studies on the mechanism of the antitumor action of orally administered PSK. We previously found that the NK activity of hepatic lymphocytes was enhanced by the oral administration of PSK (9). In the present study, we investigated the effects of PSK in rats and mice bearing liver metastasis of KDA hepatoma and Colon 26 adenocarcinoma, respectively.

## Materials and Methods

### *Animals*

Inbred male WKAH rats and CDF1 (BALB/c × DBA/2) mice were obtained from Japan SLC Inc. (Hamamatsu, Japan), and routinely used at 6-8 weeks of age.

### *Tumor Cell Line*

KDH-8, kindly donated by Prof. M. Hosokawa (Hokkaido University Medical School), is a transplantable hepatocellular carcinoma induced by 3'-methyl-4-dimethylaminoazobenzen; it was maintained in the abdominal cavity of WKAH (10). Colon 26 cancer, an undifferentiated colon adenocarcinoma generated from BALB/c mice (11), and YAC-1 lymphoma, induced by a Moloney virus in A/St mice (12), were maintained by *in vitro* cultures with RPMI-1640 medium (Nissui Seiyaku Co, Ltd., Tokyo, Japan) supplemented with 10 % fetal bovine serum (FBS; Grand Island Biological Co, Grand Island, NY, USA), 100 IU/ml of penicillin, and 100 $\mu$g/ml streptomycin. The YAC-1 tumor cells were used as the target cells in a $^{51}$Cr release assay.

### *Tumor Inoculation and Treatment with PSK*

The spleens of WKAH rats were translocated under the skin. Three days after this operation, KDH-8 tumor

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cells ($5 \times 10^5$) in 0.1 ml of phosphate-buffered saline (PBS) were injected into the spleen. Colon 26 tumor cells ($1 \times 10^4$ in 0.5 ml) were injected into the portal veins of anesthetized CDF1 mice. From the next day, PSK 500 mg/kg (Kureha Chemical Industry Co., Ltd., Tokyo, Japan) was given orally once a day for 3 weeks.

***Preparation of Effector Cells***

***Isolation and purification of non-parenchymal liver cells (NPLC).*** NPLC were isolated from three rats in each treatment group, using the method described by Seglen (13) with some modification. General anesthesia was performed with Nembutal injected intravenously at a dose of 10 mg/0.2 ml/body, and the portal vein was exposed, cannulated with an 18G needle, and infused with $Ca^{2+}$- and $Mg^{2+}$-free Hanks balanced salt solution (HBSS) at the rate of 10 ml/min. The inferior vena cava was then ligated, and an 18G needle was inserted from the right atrium into the infrior vena cava as an outlet for the perfusion solution. Peripheral blood was expelled from the liver, and the perfusate was then changed to prewarmed HBSS containing 0.05 % (w/v) collagenase (Type 1, Sigma Chemical Co., St. Louis, MO, USA); the perfusion was continued for additional 15 min, using a peristaltic pump. The liver was then excised, minced with surgical scissors, and passed through a 150-gauge mesh. The single cell suspensions obtained were centrifuged at $50 \times g$ for 1 min at 4 °C to obtain the NPLC in the supernatant. This separation procedure was repeated three times. NPLC were cultured for 1 h in plastic Petri dishes coated with fetal calf serum, and passed through a nylon-wool column to eliminate adherent cells. The number of nonadherent NPLC was 50 %-70 % of the total. These nonadherent NPLC were fractionated by centrifugation on a discontinuous density gradient, by the method of Reynolds *et al.* (14) with some modification. The cells were loaded on a five-step Percoll (Sigma Chemical Co.) gradient at concentrations of 32.5 %, 37.5 %, 42.5 %, 47.5 % and 52.5 %, and centrifuged at $200 \times g$ for 25 min. The cells in each layer were harvested, washed three times with PBS, and resuspended in culture medium (CM: RPMI-1640 supplemented with 10 % heat-inactivated FBS). The recovery from fractions 1-5 was about 19 %-24 % of the total number of nonadherent NPLC.

***Spleen cells.*** Spleens obtained from a group of three mice were minced in CM, and passed through a 150-gauge mesh. The spleen cells were placed in 0.83 % $NH_4Cl$-Tris buffer for erythrocyte lysis. Isolated lymphocytes were washed three times, and resuspended in CM.

***Assay of NK Activity***

For the labeling of target cells, $10^6$ YAC-1 cells were incubated with 100 $\mu$Ci $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA, USA) at 37 °C for 1 h. After three washes, $5 \times 10^3$ target cells were mixed with lymphocytes, at an effector-to-target (E/T) ratio of 100-25, in a 96-well microculture plate (Nunc, Roskilde, Denmark) and cultured for 4 h at 37 °C in a humidified atmosphere containing 5 % $CO_2$. The amount of $^{51}Cr$ released in 0.1 ml of the culture supernatant, measured with a gamma-counter, was defined as the experimental release. Spontaneous release was obtained from wells containing medium only, with no effector cells, and total release was obtained from wells that contained 1 % Triton-X100. The percent cytotoxicity was calculated by the following formula:

Percent cytotoxicity = 100 × (test cpm - spontaneous cpm)/(total cpm - spontaneous cpm)

Results were also expressed as lytic units (LU)/$10^7$ cells, where one LU was the number of effector cells required to lyse 20 % of $5 \times 10^3$ target cells.

***Morphological Assessment***

Morphological analysis was made by observing effector cells centrifuged on a glass slide and stained with Giemsa according to Timonen's method (15). At least 200 cells were inspected for the determination of the LGL ratio.

***Assessment of Metastasis***

Tumor-bearing mice were sacrificed on day 21 of the protocol schedule, and the wet weight of the liver and the number of metastatic nodules were determined. Each treatment group consisted of five mice.

***Statistics***

Statistical analyses were performed by Student's *t*-test.

## Results

***Effects of oral administration of PSK on NK activity in spleen cells of rats with liver metastasis.*** Liver metastasis was induced in the WKAH rats by the injection of KDH-hepatoma cells through the spleen that had been translocated subcutaneously. Each group consisting of three rats was then treated daily with PSK 500 mg or saline, given orally for

**Table 1** Effects of oral administration of PSK on NK activity of spleen cells in normal and tumor-bearing rats

| Rat | Treatment | Percent cytotoxicity Effector/Target 25/1 | 50/1 | 100/1 | Lytic unit[a] | |
|---|---|---|---|---|---|---|
| Normal | Saline | 4.5 | 11.5 | 15.4 | 18.8 ± 3.9 | |
| Normal | PSK | 6.1 | 14.5 | 19.3 | 24.3 ± 4.9 | * |
| Tumor-bearing | Saline | 3.0 | 5.2 | 7.6 | 10.0 ± 2.3 | |
| Tumor-bearing | PSK | 4.9 | 9.3 | 16.6 | 18.3 ± 1.5 | ** |

*a:* One lytic unit is the number of effector cells required to lyse 20 % of $50 \times 10^3$ target cells. Values are mean ± SD
* $P < 0.05$
** $P < 0.01$

**Table 2** Distribution of large granular lymphocytes (LGL) in subfractions of nonparenchymal liver cells separated by density gradient centrifugation

| | LGL (%) | Lytic units |
|---|---|---|
| NPLC | 4 | 17.5 |
| NANPLC | 7 | 32.2 |
| Fraction 1 | 13 | 55.5 |
| Fraction 2 | 44 | 199.5 |
| Fraction 3 | 49 | 234.5 |
| Fraction 4 | 11 | 53.5 |
| Fraction 5 | 6 | 27.0 |

NPLC: nonparenchymal liver cells, NANPLC: nonadherent NPLC
Lytic unit: See Table 1.

3 weeks. The NK activity of spleen cells examined on day 21 was not affected by this operative maneuver of splenic translocation only (sham-operated data not shown). However, tumor inoculation and the subsequent development of liver metastasis suppressed NK activity in the spleen, from 18.8 ± 3.9 LU in normal rats to 10.0 ± 2.3 LU in tumor-bearing rats on day 21 ($P < 0.05$). This suppressed NK activity recovered to the normal range following the oral administration of PSK 500 mg/kg every day for 3 weeks ($P < 0.01$), while in tumor-bearing rats given saline as a control, the NK activity remained at a low level (Table 1).

***Distribution of large granular lymphocytes (LGL) in nonparenchymal liver cell fractions and relationship with NK activity.*** The nylon wool-passed nonadherent fraction of whole NPLC was separated into five fractions by discontinuous density gradient centrifugation. In normal rats, the percentage of LGL was measured on the basis of the morphological characteristics. The LGL population in low-density fractions 2 and 3 was enriched at 44 % and 49 %, respectively (Table 2). The cytotoxic activity of the enriched LGL fractions was extremely elevated, being 234.5 LU in Fraction 3; this activity was only 27.0 LU in Fraction 5, in which the LGL population was 6 %.

***Effects of oral administration of PSK on cell population and NK activity of nonparenchymal liver cell fractions.*** Table 3 and Fig. 1 show the cell populations and NK activity of the NPLC fractions, respectively. In the normal rats, the numbers of cells in each fraction were not affected by the oral administration of PSK 500 mg/kg once a day for 3 weeks; however, the NK activity was augmented in fractions 2 and 3. The development of liver metastasis did not influence on the population of these NPLC fractons, but the NK activity was markedly suppressed, especially in fractions 2 and 3. The oral administration of PSK to these tumor-bearing rats increased the number of NPLC, and caused NK activity to return to the normal range (Fig. 1). These findings were repeatedly confirmed in the subsequent experiments.

***Antimetastatic effects of PSK.*** Each group consisting of five CDF1 mice with liver metastasis was treated with PSK 500 mg/kg or saline orally, once a day for 3 weeks, after injection of Colon 26 tumor cells into the portal vein. The effect of PSK on liver metastasis was assessed by measuring liver weight and counting the

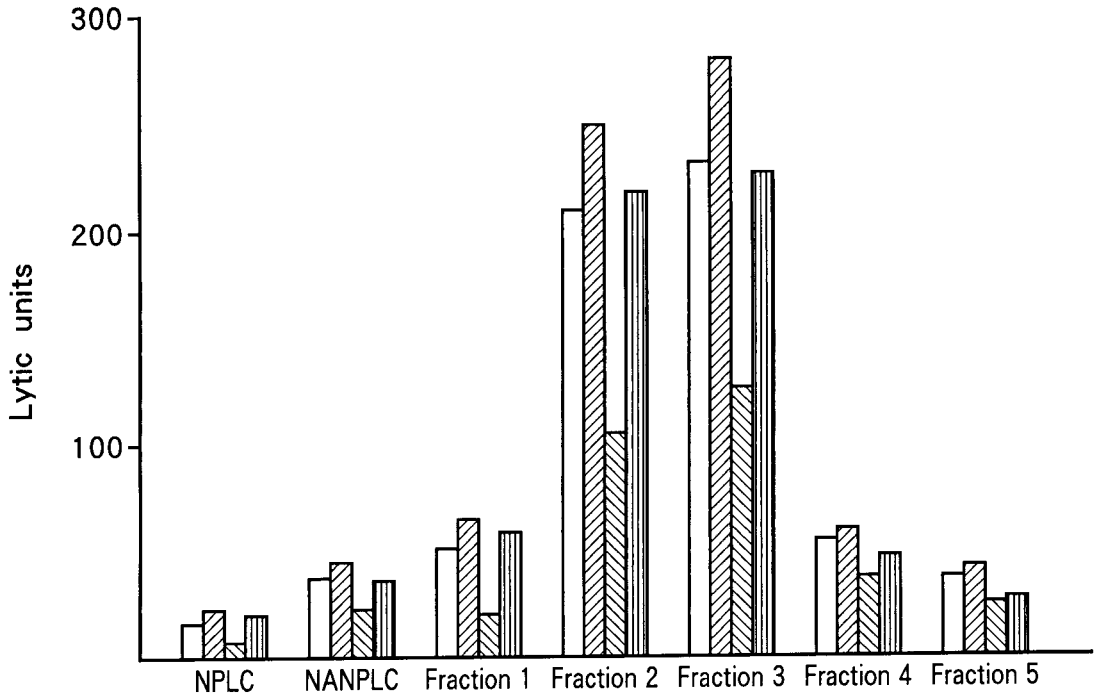


**Fig. 1** Effects of oral administration of PSK on natural killer (NK) activity of subfractions of nonparenchymal liver cells in rats with liver metastasis. NPLC: nonparenchymal liver cells, NANPLC: nonadherent NPLC, □: normal rats treated with saline, ▨: normal rats treated with PSK, ▧: tumor-bearing rats treated with saline, ▥: tumor-bearing rats treated with PSK

**Table 3** Effects of PSK on populations of nonparenchymal liver cell subfractions

| Rat | Treatment | Recovered cells per liver ($\times 10^{-6}$) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | NPLC | NANPLC | Fr. 1 | Fr. 2 | Fr. 3 | Fr. 4 | Fr. 5 |
| Normal | Saline | 101 | 56 | 3.2 | 2.5 | 2.0 | 3.3 | 2.5 |
| Normal | PSK | 117 | 75 | 2.5 | 3.7 | 3.1 | 4.4 | 3.1 |
| Tumor-bearing | Saline | 115 | 68 | 2.9 | 2.4 | 1.7 | 4.5 | 3.5 |
| Tumor-bearing | PSK | 154 | 99 | 3.3 | 4.5 | 4.9 | 3.5 | 3.0 |

NPLC: nonparenchymal liver cells, NANPLC: nonadherent NPLC, Fr. 1 - Fr. 5 (Fraction 1 - Fraction 5): fractionated by Percoll density centrifugation.

number of metastatic foci. In the group treated with PSK, the number of metastatic nodules per mouse was $89.5 \pm 43.8$, and the fresh liver weight was $3.5 \pm 1.2$ g. In the control group, the number of metastatic nodules was $125.0 \pm 35.2$ and the weight was $4.9 \pm 1.1$ g. The mean weight of metastatic fresh livers was significantly decreased by the PSK treatment ($P < 0.05$). As shown in Fig. 2, the NK activity in the spleen cells of these mice was augmented by oral PSK. The survival of tumor-bearing mice was $33.5 \pm 8.2$ days in the PSK group, and $28.5 \pm 4.8$ days in the control group, showing prolongation of survival by oral PSK.

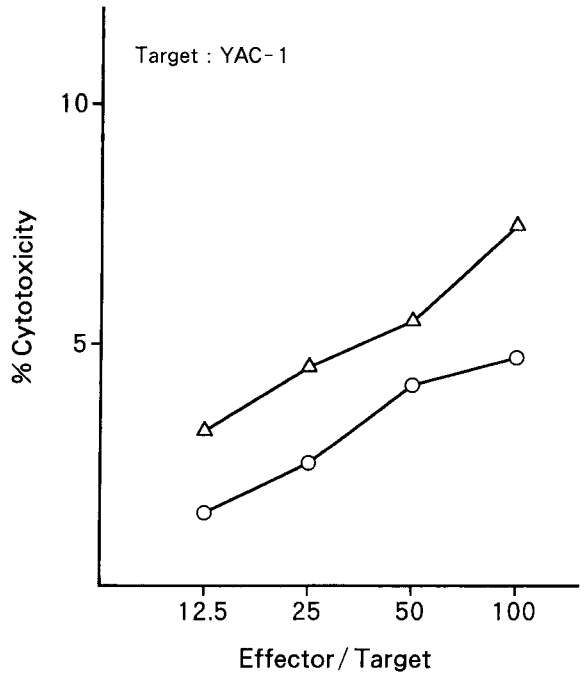


**Fig. 2** Effect of PSK given orally on NK activity of spleen cells in mice with liver metastasis, △: PSK, ○: saline

## Discussion

PSK, usually administered orally, is widely used in Japan, but its antitumor mechanism has not been clearly elucidated, although there have been some reports on its effects on antibody production (4), cellular immunity (16) and cytokine secretion (17). A notable characteristic of PSK is that, unlike other BRMs, it restores the reduced immunity in tumor-bearing animals to normal levels (4, 16, 18, 19). It has been reported that the oral administration of PSK in mice enhanced the NK activity of the spleen and lymph nodes, with the enhancing effect on NK cells being greater in tumor-bearing animals than in healthy ones (20). In a clinical study, we found that intratumoral injection of PSK enhanced the NK activity of regional lymph nodes of gastric cancer patients (21), whereas the oral administration of PSK did not augment the NK activity of peripheral blood lymphocytes (unpublished data).

NK cells, predominantly distributed in the blood, spleen, tonsils, lymph nodes and bone marrow, are also present in the liver and lung, and in the mucosal tissues of the digestive tract (22). The morphology of large granular lymphocytes in the liver was revealed to be coincident with that of pit cells in the liver (23-24). These NK cells in the non-lymphoid organs have been shown to act as a host defense mechanism both against tumor cells (25) and against virally infected cells (26).

It has been reported that the NK activity of nonparenchymal liver cells was significantly increased after the administration of certain BRMs, *i.e.*, *Corynebacterium parvum* (23), MVE-2, a pyran copolymer (23), and flavone acetic acid (27). This augmentation coincided with a 10- to 50-fold increase in the number of LGL. In our previous study, we found that the oral administration of PSK did not significantly affect the number of nonparenchymal liver cells in any fraction obtained by discontinuous density gradient centrifugation, whereas it markedly increased the NK activity in the low density fraction (9). In this study, the NK activity in the NPLC of tumor-bearing rats was obviously suppressed in all fractions of NPLC, although the cell number was not significantly changed. Oral administration of PSK caused the suppressed NK activity to return to the normal range. Since the cell number of the low-density fraction was increased, NK function of the liver of tumor-bearing mice was estimated to be much greater than normal.

Liver metastasis induced in the CDF1 mice with Colon 26 was also significantly suppressed by oral PSK. It has already been reported that increased resistance to metastasis confered by PSK is related to augmented NK activity localized in various organs (28). Although the NK activity of the liver was not analyzed in that tumor model, the NK activity of the spleen was observed to be augmented by PSK.

The above results suggest that the oral administration of PSK may be effective for suppressoin of liver metastasis through activation of liver NK cells.

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Received May 13, 1994; accepted June 21, 1994.