

Neonatal Inoculation with the Protein-bound Polysaccharide PSK Increases Resistance of Adult Animals to Challenge with Syngeneic Tumor Cells and Reduces Azoxymethane-induced Precancerous Lesions in the Colon

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Abstract

We have investigated the results of neonatal inoculation with a protein-bound polysaccharide, PSK, as it affects the defense mechanism of animals against cancer. Male BALB/c mice received a single i.p. injection of 10 mg/kg PSK within 48 h of birth. When the mice were 8 weeks of age, colon adenocarcinoma 26 (C26 tumor) cells were transplanted s.c. Injection of PSK increased the number of tumor-rejecting mice from 10 to 50% compared with the control mice transplanted with 5×10^3 tumor cells and prolonged the median survival period to 174% of control mice with tumors. When the number of transplanted tumor cells was increased to 1×10^6 , PSK injection significantly prolonged the survival period, although tumors grew in all mice. The survival period was also significantly prolonged in male C57BL/6 mice that received an injection neonatally with PSK and were given a s.c. transplant of Lewis lung carcinoma or B16 melanoma at 8 weeks of age. The effect on survival was dependent on the PSK dose and the number of transplanted tumor cells. PSK was as effective for male mice 30 weeks of age as for mice 8 weeks of age treated with PSK during the neonatal period. However, prolongation of the survival period of tumor-bearing mice was not observed in the offspring (F₁). Neonatal injection of PSK also significantly reduced the number of metastatic foci in the liver of mice inoculated with 1×10^5 C26 tumor cells in the splenic vein after 8 weeks of age. In addition, neonatal injection of PSK significantly reduced the number of aberrant crypts and aberrant crypt foci, the precancerous lesions in the colon of F344 rats that received injections s.c. with azoxymethane after 7 weeks of age, to 47% of that of rats that received an injection with saline at the same age. The effect on precancerous lesions was dependent on the timing of PSK

injection and the dose. Regarding the mechanism, when animals thymectomized during the neonatal period or when congenitally athymic animals were used instead of healthy animals, the effect on survival or precancerous lesions did not appear. Neonatal injection of PSK significantly reduced the number of CD4⁺CD8⁺ T cells and significantly increased the number of CD4⁺CD8⁻ and CD4⁻CD8⁺ T cells in the thymus of healthy mice 10 weeks of age and C26 tumor-bearing mice. Furthermore, neonatal injection of PSK significantly elevated the T-cell differentiation induced by a mouse thymus extract 10 weeks of age. These findings suggest that neonatal injection of PSK induces resistance in adult mice to challenge by syngeneic tumor cells and reduces the azoxymethane-induced precancerous lesions in the colon of adult rats *via* the thymus functions.

Introduction

We have reported previously (1, 2) that rats inoculated with Friend or Gross virus during the neonatal period grow tolerant to virus-associated antigens, thus permitting the growth of Friend or Gross virus-induced tumors. In contrast, mice treated with an anti-idiotypic antibody against myeloma during the neonatal period showed increased resistance to the tumor after maturation, and the survival period was extended (3). Apart from these studies, however, no reports have been published to our knowledge describing attempts to prevent or treat cancer in animals treated neonatally with agents modifying the response of the host to tumors.

BRMs² have been used experimentally as well as clinically because their use enhances the antitumor effect through modifying the biological response of the host to cancer cells (4). Kobayashi *et al.* (5, 6) have reported that a protein-bound polysaccharide of mycelium of basidiomycetes origin, PSK, a representative BRM, exhibits various biological activities such as immunoregulatory and antioxidative activities, whereas other reports have shown that in randomized clinical studies the concomitant oral administration of PSK with chemotherapy prolonged the survival period of postoperative patients with gastric cancer (7) and colon cancer (8). PSK has almost no serious adverse side effects, and its characteristics permit long-term oral administration (7–9). The mean molecular weight of PSK is approximately 9.4×10^4 , and its major sugar moiety is a glucan with a main chain β 1–4 bond and a side chain β 1–3

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² The abbreviations used are: BRM, biological response modifier; AC, aberrant crypts; ACF, aberrant crypt foci; C26, colon 26; LLC, Lewis lung carcinoma; LN, Lymph node; mAb, monoclonal antibody; AOM, azoxymethane; R-PE, R-polycoerythrin.

bond, as well as β 1–6 bonds to a protein moiety through *O*- or *N*-glycoside bonds (9). This agent is now used clinically for the treatment of cancer patients in Japan.

In the present study, we investigated the effect of PSK injection during the neonatal period on the defense mechanisms of the host against tumors.

Materials and Methods

Animals. BALB/c mice, BALB/c *nu/nu* mice, BALB/c *nu/+* mice, and C57BL/6 mice at 8 weeks of age were purchased from Japan Charles River (Kanagawa, Japan), and F344 rats at 8 weeks of age were purchased from Japan SLC (Shizuoka, Japan). After acclimatization, two female animals and one male animal 11–13 weeks of age were placed together in a cage for mating. Pregnant animals were maintained individually after 17 days of pregnancy. In the case of the BALB/c mice, C57BL/6 mice, and F344 rats, the number of newborns was approximately three/pregnant mouse, six/pregnant mouse, and five/pregnant rat, respectively. On the 21st day after birth, newborn animals were divided into groups consisting of 8–10 animals/cage. Animals had free access to food: CE-2 (Oriental Yeast, Tokyo, Japan) and sterilized tap water. Animals were kept at temperatures of $24 \pm 2^\circ\text{C}$, humidity of $55 \pm 10\%$, a luminary air flow with about 5 lux of luminous intensity, and a lighting cycle from 8:00 a.m. to 8:00 p.m. Only people who changed cages and carried out experiments entered the animal room.

Tumors. Syngeneic mouse tumor cell lines maintained in our laboratory were used in the study. C26 tumor cells, a colon adenocarcinoma of BALB/c mouse origin, and LLC cells were provided by Dr. M. Harada, Medical Institute of Bioregulation, Kyushu University (Fukuoka, Japan) and Riken Cell Bank (Ibaraki, Japan), respectively. These tumor cell lines were maintained *in vitro*. Tumor cells in *in vitro* log phase cultures were harvested and washed with HBSS, and a cell suspension in HBSS was used for *in vivo* transplantation. The cell line B16 melanoma was provided by the Cancer Institute (Tokyo, Japan) and maintained *in vivo* in C57BL/6 mice *s.c.* For transplantation, a cell suspension at an appropriate concentration with HBSS was prepared from the B16 tumor mass by treating it with collagenase, followed by washing with the medium and by passing it through a mesh with a $150\mu\text{m}$ sieve size.

Agents. PSK (Kureha Chemical Industrial Company, Limited, Tokyo, Japan) was dissolved in sterilized saline. Animals, within 48 h of birth or 7, 42, or 49 days after birth, received an injection *i.p.* with a fixed amount of PSK or 0.05 ml of saline. Litter mates from each animal were divided into a PSK-injected group and a saline-injected group. In some experiments, mice over 8 weeks of age received an *i.p.* injection of PSK at 10 mg/kg, 7 or 10 times every other day.

Tumor Transplantation. Mice 8 weeks of age that had received an injection with PSK or saline within 48 h of birth, or 7 or 49 days after birth, were given a *s.c.* transplant of 5×10^3 or 1×10^6 tumor cells, and their survival time was recorded. Tumor size was measured weekly using a caliper in two perpendicular directions (longest and shortest diameter), and the product of the two values was taken as the tumor size (mm^2). Mice were autopsied at death, and the cause of death and metastasis to other organs including the lungs and livers were examined. In some experiments, various numbers of tumor cells (5×10^4 , 5×10^5 , or 5×10^6) were *s.c.* transplanted into mice.

To set up a liver metastasis model, BALB/c mice were given transplants of 1×10^5 C26 tumor cells into the splenic portal vein. The liver was excised 14 days after transplantation. After the liver had been weighed, it was fixed in Bouin solution

for 2 h or longer, and the number of visible metastatic foci on the surface of the liver was counted.

Induction of Precancerous Lesions in the Colon of Rats.

Precancerous lesions were induced by the method reported by Kawamori *et al.* (10) and Bird (11) with modification. In each group, 10 male F344 rats, 7 weeks of age, received *s.c.* injections of 15 mg/kg AOM (Sigma Chemical Co., St. Louis, MO) once every week for 3 weeks. Eight weeks after the initiation of AOM treatment, five rats were selected at random from each experimental group and killed under ether anesthesia. The colon was excised, and the content was removed by washing with PBS (pH 7.5). The colon was sectioned lengthwise with scissors and pinned on a rubber plate with the mucous membrane face up. Then, the colon was fixed in 10% formalin in PBS for 24 h or longer. The fixed colon was washed by placing it in running tap water for 30 min or longer and soaked in a 0.2% methylene blue (Sigma) solution for 10 min for staining. Excess dye was washed out in running tap water, and the precancerous lesions in the colon were observed under a stereoscopic microscope. ACF and AC, which are precancerous lesions, were judged by increased size, thicker epithelial lining, and increased pericryptal zone (10, 11). Observers judged and counted ACF and AC in blinded experimental groups.

Neonatal Thymectomy. Thymectomy was performed within 48 h of birth, following the method of Reeves and Reeves (12). The animals were killed at the end of the experiment to ascertain the absence of the thymus, both macroscopically and microscopically.

Analysis of Thymus. To measure the number of dividing cells in the thymus, 3.7 MBq of 6- ^3H thymidine (Amersham Life Science, Buckinghamshire, United Kingdom) were injected *i.p.* into animals, and the thymus was excised after 1 h (13). The radioactivity incorporated into the thymus was measured using a liquid scintillation counter.

To measure the thymus cell subsets, the thymic cells were made to react with FITC or R-PE-conjugated mAb, after which the cells were counted using a FACScan and a software program (Becton Dickinson, Mountain View, CA). The following mAbs were used in the experiments: FITC-conjugated rat antimouse CD4 mAb (PharMingen International, San Diego, CA), R-PE-conjugated rat antimouse CD8a mAb (PharMingen International), and R-PE-conjugated rat antimouse CD90.2 (Thy-1.2) mAb (PharMingen International).

Apoptotic thymus cells were measured by the terminal deoxynucleotidyl transferase-mediated dUTP nick and translation method (14). Thymus cells at 1×10^7 cells/ml were washed twice with 1% BSA-supplemented PBS (pH 7.4), and 0.1 ml was added to each well of 96-well tissue culture plates (Becton Dickinson Labware, NJ). After the addition of 0.1 ml of 4% paraformaldehyde in PBS (pH 7.4) to each well, the plates were left at 25°C for 1 h, and the cells were fixed. Then, using an *in situ* cell death detection kit (Roche Diagnostics, Mannheim, Germany), fluorescence-labeled dUTP was reacted with apoptotic cell DNA at 37°C for 1 h, and the apoptotic cells were analyzed using a FACScan and a software program. Furthermore, using cell death detection ELISA (Roche Diagnostics), the amount of cytoplasmic histone-associated DNA fragments in the apoptotic thymus cells was measured by enzyme immunoassay.

To evaluate the biological activity of thymus extract, thymus extract was prepared following the method of Goldstein *et al.* (15) after modification. Briefly, the thymus was excised from mice 8 weeks of age and homogenized in the presence of 0.15 M sodium chloride at 0°C using a combination of a Waring

Fig. 1. Prolongation of the survival period of mice bearing syngeneic tumors after neonatal injection of PSK (1): effect of timing of injection. Male BALB/c mice or C57BL/6 mice ($n = 10$) within 48 h of birth, at 7 days of age, or at 49 days of age received a single i.p. injection of 10 mg/kg PSK or saline. C26 tumor cells (5×10^3 or 1×10^6), LLC tumor cells, or B16 tumor cells were then s.c. transplanted into the mice at 8 weeks of age, and the number of tumor-rejecting mice and the survival period were evaluated. †, MST, mean survival time (days); ††, (MST of the PSK-injected group/MST of the saline-injected groups) $\times 100$. Bars, SD. Significantly different at $* P < 0.01$ compared with that in the saline-injected group.

Time of PSK injection	No. of transplanted tumor cells	Expt. group	Tumor : Mouse								
			C26 : BALB/c			LLC : C57BL/6			B16 : C57BL/6		
			No. of mice with tumor / Total	MST†(days)		No. of mice with tumor / Total	MST†(days)		No. of mice with tumor / Total	MST†(days)	
Day +1 or +2	5×10^3	saline	9/10	33.3±3.4	H	8/10	32.4±4.3	H	9/10	32.9±4.1	H
			5/10	58.0±3.9			61.2±4.2			51.9±5.7	
	1×10^6	saline	10/10	22.1±3.4	H	10/10	21.7±8.1	H	10/10	22.6±6.1	H
			10/10	33.0±3.4		31.7±7.5	27.1±6.2				
Day +7	5×10^3	saline	8/10	31.5±3.4	H	9/10	31.6±3.5	H	8/10	32.9±3.4	H
			9/10	32.2±3.7		31.8±3.9	34.2±3.2				
	1×10^6	saline	10/10	21.5±3.0	H	10/10	22.6±5.8	H	10/10	23.0±5.0	H
			10/10	22.4±4.7		21.6±6.3	25.6±2.0				
Day +49	5×10^3	saline	9/10	33.7±4.2	H	8/10	33.0±4.1	H	8/10	31.2±3.4	H
			9/10	34.8±3.9		35.1±4.3	33.0±4.7				
	1×10^6	saline	10/10	23.3±5.1	H	10/10	23.3±4.0	H	10/10	23.2±4.9	H
			10/10	22.0±4.7		24.0±7.7	24.9±4.4				

Blender and an ultrasonic generator. The homogenate was centrifuged at $1200 \times g$ at 4°C for 15 min. The supernatant was centrifuged further at $105,000 \times g$ at 4°C for 1 h. The obtained supernatant was stored at -80°C until use. The protein content of the supernatant was quantitated using Lowry's method (16).

The *in vitro* T-cell differentiation-inducing activity of the thymus extracts was evaluated following the method of Komuro and Boyse (17) after modification. Briefly, after spleen cells from healthy BALB/c *nu/nu* mice were cultured *in vitro* in the presence or absence of thymus extracts at 37°C for 24 h, the cells were reacted with R-PE-conjugated rat antimouse CD90.2 mAb (PharMingen International). The number of positive cells was quantitated using a FACScan and a software program. The activity was expressed as an induction index (18).

Antitumor Activity of Axillary LN Cells. The antitumor activity of the axillary LN cells obtained from C26 tumor-bearing mice was assayed following the method of Winn (19) after modification. Briefly, the axillary LN cells were obtained from mice 14 days after C26 tumor transplantation. The LN cells (2×10^6) were mixed with C26 tumor cells (2×10^5) in a total volume of 0.2 ml with HBSS, and they were injected s.c. into the flanks of male BALB/c mice that were irradiated 2 h previously with 350 rad. Tumor size was measured every 3 or 4 days using a caliper in two perpendicular directions (longest and shortest diameter), and the product of the two values was taken as the tumor size (mm^2). In some experiments, the LN cells (1×10^7) were incubated with $15 \mu\text{g}$ of anti-CD4-mAb, $15 \mu\text{g}$ of anti-CD8-mAb, or mouse immunoglobulin (Seikagaku Kogyo Co. Ltd., Tokyo) at 4°C for 45 min, followed by incubation with rabbit complement (Cedarlane, Hornby, Ontario, Canada) at 37°C for 45 min.

Statistics. Values are presented as mean \pm SD. Most of the analyses were carried out using Student's *t* test, and a *P* of 0.05 or lower was regarded as significant.

Results

Increased Resistance of Adult Mice to Challenge with Syngeneic Tumor Cells after Neonatal Injection of PSK. Within 48 h of birth, male BALB/c mice received a single i.p. injection of 10 mg/kg PSK. When the mice reached 8 weeks of age, they received s.c. transplant of C26 tumor cells, and the duration of their survival was observed. When 5×10^3 C26 tumor cells were transplanted, the tumor rejection rate in the group treated with PSK during the neonatal period was increased to 50% compared with 10% in the group treated with saline at the same age. In contrast, when 1×10^6 C26 tumor cells were transplanted, the tumor grew in all mice in both groups.

The mean survival time of the PSK-injected group transplanted with 5×10^3 and 1×10^6 tumor cells was 58.0 days (174%) and 33.0 days (149%), compared with the 33.3 days (100%) and 22.1 days (100%) of the saline-injected group, respectively (Fig. 1). Fig. 2 shows that tumor growth was also significantly suppressed in the PSK-injected group transplanted with 5×10^3 tumor cells. The autopsy of dead animals showed no death attributable to lung or hepatic metastasis.

In contrast, when PSK was injected i.p. into mice 7 days or 49 days after birth, the duration of the survival period after transplantation of 5×10^3 or 1×10^6 tumor cells was similar to the duration of survival time of mice that received an injection with saline at the same age. In these mice, the tumor growth was not affected by PSK injection (data not shown).

The same effect was observed in the C57BL/6 mice given transplants of LLC or B16. PSK treatment within 48 h of birth significantly extended the survival period and suppressed tumor growth after 5×10^3 tumor cells were transplanted into mature male C57BL/6 mice. In C57BL/6 mice transplanted with 1×10^6 LLC cells, PSK treatment within 48 h of birth significantly extended the survival period. A tendency toward extended survival was observed also in C57BL/6 mice transplanted with 1×10^6 melanoma B16, although the extension was not significant. No abnormalities attributable to neonatal PSK injection

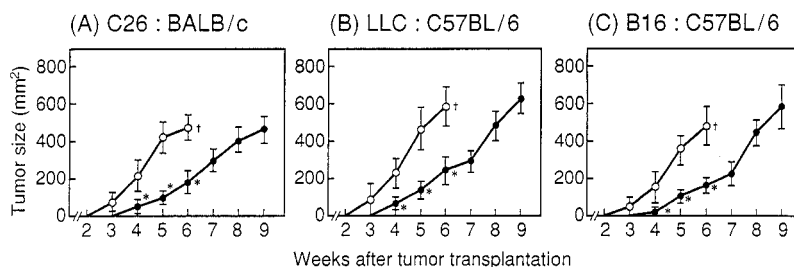


Fig. 2. Suppression of tumor growth by neonatal injection of PSK in mice bearing syngeneic tumors. Male BALB/c or C57BL/6 mice received a single i.p. injection of 10 mg/kg PSK or saline within 48 h of birth, and C26 tumor cells (5×10^3), LLC tumor cells, or B16 tumor cells were s.c. transplanted when the mice reached 8 weeks of age. The tumor was measured once a week with a caliper in two directions (the longest and shortest diameter), and tumor size was determined by multiplying the longest diameter by the shortest diameter. †, all mice died of disease; ●, mean tumor size of PSK-injected mice with tumor; ○, mean tumor size of saline-injected mice with tumor. Bars, SD. Significantly different at * $P < 0.01$ compared with the saline-injected group.

tion were observed during the experimental period (data not shown).

These findings suggest that the effect of PSK seen is dependent on the timing of the injection after birth and that an injection within 48 h of birth enhances resistance to the tumor after maturation.

Dependence of the Effect upon PSK Dose and the Number of Transplanted Tumor Cells. With regard to the effect of PSK treatment given within 48 h of birth, we investigated its dose dependency and the transplanted tumor cells number dependency. Fig. 3A shows the PSK dose-dependence effect on survival after transplantation of 5×10^3 tumor cells. The groups that received an injection neonatally with 5 mg/kg or 10 mg/kg PSK showed a significant difference in the extension of survival time compared with that of the group that received injections with saline.

Next, we transplanted varying numbers of tumor cells s.c. into mice that received an injection neonatally with 10 mg/kg of PSK i.p., and the survival period was evaluated. Fig. 3B shows that a significant extension of the survival period was seen when $< 5 \times 10^6$ tumor cells had been transplanted.

As Fig. 3C shows, a significant extension of the survival period was observed when 1×10^6 C26 tumor cells had been transplanted at 30 weeks of age into mice that received an injection neonatally with PSK. However, we observed almost no prolongation of survival when tumor cells were injected into F₁ mice 8 weeks of age generated by mating mice that had been treated with PSK during the neonatal period.

In addition, we investigated whether there was a gender difference in the effects of PSK. The mean duration of survival after 1×10^6 C26 tumor transplantation was 22.3 days in female BALB/c mice that received injections with saline within 48 h of birth and 19.3 days in male mice that received the same treatment; however, no gender difference was observed in terms of the PSK effect, and the duration of survival was extended equally in both male and female mice after PSK was injected within 48 h of birth (data not shown).

Enhanced Resistance of Adult Mice to Challenge with Syngeneic Tumor Cells by Combination of PSK Injection within 48 h of Birth and after Maturation. We investigated whether the combination of PSK injection within 48 h of birth and PSK injection after maturation would enhance the effect. A preliminary study confirmed that the dosing schedule of 10 i.p. injections of 10 mg/kg PSK every other day starting on the day after tumor transplantation is the optimal schedule for extend-

ing the duration of survival in tumor-bearing mature animals.³ The mean duration of survival of the group that received an injection with PSK within 48 h of birth was 35.6 days, whereas the mean duration of survival in the group that received injections repeatedly with PSK after maturation was 31.0 days (Table 1). In contrast, the mean duration of survival of the group given the sequential combination was 40.0 days, which showed a significant prolongation. Thus, the combination of PSK injection within 48 h of birth and repeated PSK injection after tumor transplantation significantly extended the duration of survival in tumor-bearing mice beyond that obtained with either treatment alone.

We also investigated the effect of PSK injection within 48 h of birth in a tumor metastasis model. Male BALB/c mice received a single i.p. injection of 10 mg/kg PSK within 48 h of birth, and 1×10^5 C26 tumor cells were transplanted into the splenic portal vein of these mice at 8 weeks of age. Fourteen days after inoculation the metastatic foci in the liver were counted. Table 2 shows that the number of liver metastases in the group that received an injection with PSK within 48 h of birth was reduced significantly compared with that in the group that received saline injection. Furthermore, the number of metastatic foci was reduced significantly by a single PSK injection within 48 h of birth combined with repeated PSK injection after tumor transplantation. This suggests that a single injection of PSK during the neonatal period suppresses tumor metastasis in the liver and that the effect is enhanced by repeated injection of PSK after tumor transplantation.

Inhibition of AOM-induced Precancerous Lesions in the Colon of Adult Rats after Neonatal Injection of PSK.

Whether PSK injection during the neonatal period negatively affects precancerous lesions was investigated using the colon carcinogenesis model system. Within 48 h of birth, male F344 rats received i.p. injection of 10 mg/kg of PSK. When these rats reached 7 weeks of age, 15 mg/kg of AOM was s.c. injected once every week for 3 weeks, and 8 weeks after the initiation of AOM treatment, the number of precancerous lesions, AC and ACF, in the colon was measured. The mean number of AC and ACF in the neonatal PSK injection group was 145 and 82, respectively, which was decreased significantly compared with the 337 and 176 in the control saline injection group, although the mean number of AC/focus was similar in both groups (Fig. 4).

³ Unpublished findings.

(A) Dose-dependency of the effect

Dose of PSK	Tumor : Mouse											
	No. of mice with tumors /Total	C26 : BALB/c			No. of mice with tumors /Total	LLC : C57BL/6			No. of mice with tumors /Total	B16 : C57LB/6		
		MST†(days)	20	40		60	MST†(days)	20		40	60	MST†(days)
saline	9/10	36.8±4.8			9/10	34.7±4.1			9/10	35.5±4.2		
PSK 1 mg/kg	9/10	40.4±4.1			9/10	39.8±4.0			8/10	37.1±3.7		
PSK 5 mg/kg	8/10	48.9±3.5			9/10	48.2±3.6			8/10	45.4±3.9		
PSK 10 mg/kg	6/10	62.5±4.7			7/10	55.5±3.4			7/10	54.9±4.3		

Fig. 3. Prolongation of the survival period of BALB/c mice bearing C26 tumors after neonatal injection of PSK (2); A, dose dependency of the effect: male BALB/c mice or C57BL/6 mice ($n = 10$) received a single i.p. injection of saline or a fixed amount of PSK within 48 h of birth. C26 tumor cells (5×10^3), LLC tumor cells, or B16 tumor cells were s.c. transplanted at 8 weeks of age, and the number of tumor-rejecting mice and the survival period were evaluated. Significantly different at * $P < 0.01$ and ** $P < 0.05$ compared with that of the saline-injected group. Other symbols are the same as those in Fig. 1; B, dependency of the effect on the number of transplanted tumor cells: male BALB/c mice or C57BL/6 mice ($n = 10$) received a single i.p. injection of 10 mg/kg PSK or saline within 48 h of birth. Different numbers of C26 tumor cells, LLC tumor cells, or B16 tumor cells were s.c. transplanted at 8 weeks of age, and the survival period was evaluated. Symbols are the same as those in Fig. 1; C, duration of the effect: male BALB/c mice or C57BL/6 mice ($n = 10$) received a single i.p. injection of 10 mg/kg PSK or saline within 48 h of birth. C26 tumor cells (1×10^6), LLC tumor cells, or B16 tumor cells were s.c. transplanted at 8 or 30 weeks of age, and the survival period was evaluated. NT, not tested. Other symbols are the same as those in Fig. 1.

(B) Dependency of the effect on the number of transplanted tumor cells

No. of transplanted tumor cells	Expt. group	Tumor : Mouse											
		No. of mice with tumors /Total	C26 : BALB/c			No. of mice with tumors /Total	LLC : C57BL/6			No. of mice with tumors /Total	B16 : C57LB/6		
			MST†(days)	20	40		60	MST†(days)	20		40	60	MST†(days)
5×10^4	saline	10/10	28.0±3.9			10/10	26.6±2.5			10/10	27.1±2.5		
	PSK	10/10	42.0±3.2			10/10	37.5±3.1			10/10	38.8±3.3		
5×10^5	saline	10/10	22.7±2.8			10/10	21.3±2.2			10/10	22.4±2.1		
	PSK	10/10	33.8±3.7			10/10	28.9±3.0			10/10	31.4±2.2		
5×10^6	saline	10/10	19.7±2.0			10/10	18.6±2.1			10/10	17.3±1.9		
	PSK	10/10	27.2±3.4			10/10	24.0±2.2			10/10	22.7±2.6		

(C) Duration of the effect

Mouse age when tumor was transplanted	Expt. group	Tumor : Mouse											
		No. of mice with tumors /Total	C26 : BALB/c			No. of mice with tumors /Total	LLC : C57BL/6			No. of mice with tumors /Total	B16 : C57LB/6		
			MST†(days)	20	40		60	MST†(days)	20		40	60	MST†(days)
8-weeks-old	saline	10/10	21.0±3.6			10/10	22.5±2.9			10/10	22.6±2.3		
	PSK	10/10	29.4±4.7			10/10	30.9±3.5			10/10	30.1±3.1		
30-weeks-old	saline	10/10	35.8±1.0			10/10	34.5±1.8			10/10	32.9±2.1		
	PSK	10/10	47.3±2.7			10/10	44.5±2.6			10/10	42.1±3.9		
F1 mouse of 8-weeks-old ♀	saline	10/10	23.1±3.8			NT			NT				
	PSK	10/10	22.8±3.1			NT			NT				

In contrast, the mean number of AC and ACF was 308 and 162, respectively, in the group that received an injection with PSK 6 weeks after birth, which was similar to that in the saline-injected group, which was 320 and 168, respectively. There were almost no differences in changes in body weight, food intake, and liver weight at autopsy between the neonatal PSK injection group and the saline injection group (data not shown).

Fig. 5 shows the dose-dependent effect of neonatal PSK treatment. In the group that received an injection with 5 mg/kg or higher PSK within 48 h of birth, the mean number of ACF and AC was decreased significantly compared with that in the saline injection group.

Analysis of the Mechanism. We investigated the possible involvement of the thymus in the exertion of the PSK effect. When tumor cells were transplanted s.c. to male BALB/c *nu/nu* mice 8 weeks of age or neonatally thymectomized BALB/c mice that received an injection with PSK within 48 h of birth, there was almost no effect on the duration of the survival period (Fig. 6A). Similarly, in rats thymectomized during the neonatal period, inhibition of precancerous lesions by neonatal PSK treatment disappeared (Fig. 6B). These findings suggest that the presence of the thymus is essential for the exertion of the PSK effect.

Therefore, we investigated the effect of neonatal injection of PSK on the mouse thymus. The thymus was excised at 3 or

Table 1 Prolongation of the survival period of BALB/c mice bearing C26 tumor cells after combination of neonatal and after-maturation injection of PSK

Group	Time of PSK injection ^a		Mean survival days	% control ^b
	Within 48 h of birth	Day +57 ~ +75		
1	PSK	PSK	40.0 ± 3.9 ^{c,d}	159
2	PSK	Saline	35.6 ± 2.1 ^c	142
3	Saline	PSK	31.0 ± 3.4 ^c	124
4	Saline	Saline	25.1 ± 3.4	100

^a Male BALB/c mice ($n = 10$) received a single i.p. injection of 10 mg/kg PSK or saline within 48 h of birth. Mice were s.c. transplanted with 1×10^6 C26 tumor cells at 8 weeks of age. The mice were given 10 injections of 10 mg/kg PSK or saline every other day, starting the day after the tumor transplantation, and the survival period was evaluated.

^b % control = (mean survival days in groups 1, 2, or 3/mean survival days in group 4) $\times 100$.

^c The mean survival period was significantly different at $P < 0.01$ compared with that in group 4.

^d The mean survival period was significantly different at $P < 0.05$ compared with that between group 1 and group 2.

Table 2 Reduction of the liver metastasis by combination of neonatal and after-maturation injection of PSK in BALB/c mice after C26 tumor cells had been transplanted into the splenic portal vein

Group	Time of PSK injection ^a		No. of metastatic foci in the liver	% control ^b
	Within 48 h of birth	Day +57 ~ +69		
1	PSK	PSK	50 ± 23 ^{c,d}	23
2	PSK	Saline	103 ± 44 ^c	46
3	Saline	PSK	161 ± 50 ^c	73
4	Saline	Saline	222 ± 95	100

^a Male BALB/c mice ($n = 10$) received a single i.p. injection of 10 mg/kg PSK or saline within 48 h of birth. C26 tumor cells (1×10^5) were transplanted into the splenic portal vein at 8 weeks of age. The mice were given seven injections of 10 mg/kg PSK or saline every other day, starting the day after tumor transplantation, and the number of metastases in the liver was counted after 14 days.

^b % control = (mean no. of metastatic foci in groups 1, 2, or 3/mean no. of metastatic foci in group 4) $\times 100$.

^c Significantly different at $P < 0.01$ compared with that in group 4.

^d Significantly different at $P < 0.05$ between group 1 and group 2 in the metastatic foci of the liver.

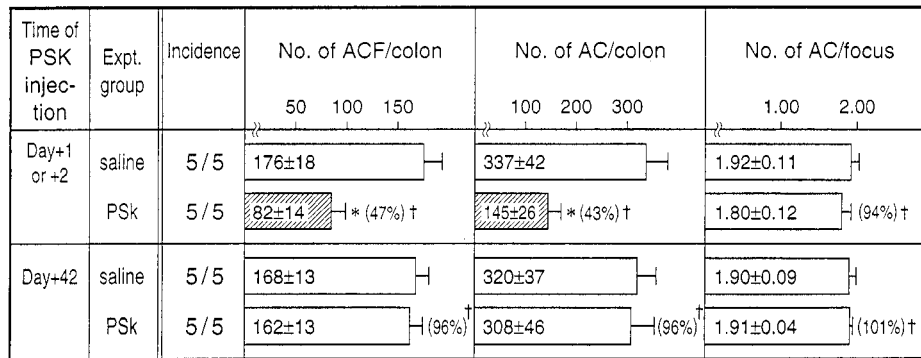


Fig. 4. Reduction of the precancerous lesions in the colon of AOM-injected F344 rats after neonatal injection of PSK (1): time of injection. Male F344 rats ($n = 10$) within 48 h of birth or at 42 days of age received a single i.p. injection of 10 mg/kg PSK or saline. s.c. injection of 15 mg/kg AOM once every week for 3 weeks was initiated 7 weeks after birth. Eight weeks after the initiation of AOM treatment, five rats were selected at random from each group and killed under ether anesthesia. The colon was excised, fixed with formalin, and stained with methylene blue, and precancerous lesions were observed under a stereoscopic microscope. Bars, SD. †, [No. of AC (or ACF) of the PSK-injected group/No. of AC (or ACF) of the saline-injected groups] $\times 100$. Significantly different at * $P < 0.01$ compared with that of the saline-injected group.

10 weeks of age from mice that received an injection with PSK within 48 h of birth, and the total cell number, cell subsets, and [^3H]thymidine uptake were investigated. The thymus cell counts within 48 h of birth, 3 weeks after birth, and 10 weeks after birth in the saline-injected mice were 3×10^7 , 1.9×10^8 , and 0.9×10^7 , respectively, and the ratios of thymus cell subsets in mice 10 weeks of age were 63% of $\text{CD4}^+\text{CD8}^+$ cells (double-positive cells), 7% of $\text{CD4}^-\text{CD8}^+$ cells (double-negative cells), 23% of $\text{CD4}^+\text{CD8}^-$ cells (single-positive cells), and 7% of $\text{CD8}^+\text{CD4}^-$ cells (single-positive cells). Although there were no significant differences in the total cell number in the thymus from mice 10 weeks of age that received an injection with PSK within 48 h of birth, the number of $\text{CD4}^+\text{CD8}^+$ T cells was reduced significantly, and the number of $\text{CD8}^+\text{CD4}^-$ T cells and $\text{CD4}^+\text{CD8}^-$ T cells was increased significantly in both C26 tumor-bearing mice and mice without tumor transplantation (Fig. 7). A similar tendency was observed in mice 3 weeks of age that had been injected with PSK within 48 h of birth (data not shown).

When 3.7 MBq of [^3H]thymidine was injected i.p. into mice at 10 weeks of age, the radioactivity incorporated into the thymus 1 h after injection was 9.5% in the group that received

an injection with PSK within 48 h of birth and 10.1% in the group that received an injection with saline during the same period, showing no significant difference in thymic cell proliferation between the two groups.

In addition, we transplanted s.c. C26 tumor cells into mature mice that had been treated with PSK during the neonatal period, obtained the axillary LN cells 14 days after the transplantation, and evaluated their antitumor activities by transplanting a mixture of the LN cells and C26 tumor cells into irradiated mice and by measuring the tumor growth. The tumor size 21 days after the transplantation in PSK-treated group was 110 mm^2 , which was decreased significantly compared with 310 mm^2 in the saline-treated group, suggesting the enhanced antitumor activity of axillary LN cells by neonatal PSK treatment (Fig. 8). This PSK effect was lost when the assay was performed after the LN cells were treated with anti-CD8 mAb and complement. These findings indicate that CD8^+ T cells are the effector cells involved in the expression of the PSK effect.

We investigated whether the changes in the number of single-positive T cells or double-positive T cells in the thymus could be attributable to increased apoptosis of double-positive T cells or increased rates of differentiation of double-positive

Fig. 5. Reduction of the precancerous lesions in the colon of AOM-injected F344 rats after neonatal injection of PSK (2): dose dependency of the effect. Male F344 rats ($n = 10$) within 48 h of birth received a single i.p. injection of saline or a fixed amount of PSK. s.c. injection of 15 mg/kg AOM once every week for 3 weeks was initiated 7 weeks after birth. Eight weeks after the initiation of AOM treatment, precancerous lesions were evaluated. Symbols are the same as those in Fig. 4.

Dose of PSK	Incidence	No. of ACF/colon		No. of AC/colon		No. of AC/focus	
		100	200	200	400	1.00	2.00
saline	5/5	216±23		430±37		2.00±0.09	
PSK 1 mg/kg	5/5	201±24	(93%)†	379±49	(88%)†	1.94±0.07	(97%)†
PSK 5 mg/kg	5/5	164±26	(76%)†	324±58	(75%)†	1.97±0.09	(99%)†
PSK 10 mg/kg	5/5	98±17	*(45%)†	188±26	*(44%)†	1.89±0.07	(95%)†
PSK 50 mg/kg	5/5	87±19	*(40%)†	167±34	*(39%)†	1.91±0.06	(96%)†

(A) Effects on survival of mice after transplantation of C26 tumor cells

Expt. No.	Mice	Expt. group	No. of mice with tumors	MST [†] (days)			
				10	20	30	40
1	BALB/c nu/nu	saline	10/10	20.5±2.1			
		PSK	10/10	21.4±1.7			(104%)††
	BALB/c nu/+	saline	10/10	22.8±2.0			
		PSK	10/10	29.9±2.5			*(131%)††
2	neonatally-thymectomized BALB/c	saline	10/10	21.7±3.8			
		PSK	10/10	22.4±3.8			(103%)††
	sham-operated BALB/c	saline	10/10	23.8±2.9			
		PSK	10/10	32.9±3.1			*(136%)††

(B) Effects on precancerous lesions in colon of rat

Rats	Expt. group	Incidence	No. of ACF/colon		No. of AC/colon	
			50	100	100	200
neonatally-thymectomized F344	saline	5/5	101±18		194±47	
	PSK	5/5	107±23	(106%)††	199±32	(103%)††
sham-operated F344	saline	5/5	126±47		214±53	
	PSK	5/5	86±15	*(68%)††	133±30	*(62%)††

cells to single-positive T cells. On analysis of flow cytometry, there were almost no differences in the pattern of fluorescence-labeled apoptotic cells between the neonatal PSK treatment and saline treatment groups (data not shown). Furthermore, as shown in Table 3, there was no difference in the amount of cytoplasmic histone-associated DNA fragments between the neonatal PSK treatment and saline treatment groups.

We prepared thymus extracts from mice 10 weeks of age that had been treated with PSK within 48 h of birth following the method of Goldstein *et al.* (15) as modified; its biological activity was examined by T-cell differentiation induction assay (17). Fig. 9 shows that thymus extracted from mice treated with PSK within 48 h of birth significantly promoted differentiation of CD90.2 positive T cells *in vitro* among spleen cells from BALB/c nu/nu mice.

Discussion

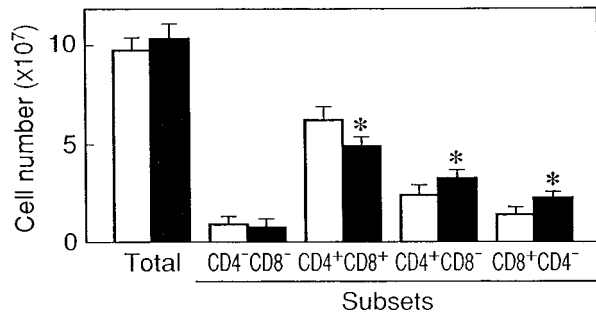
Very few studies have reported the effect of drug administration during the neonatal period on the growth and progression of cancer. It has only been reported that splenic natural killer cell activity is very low in mature female BALB/c mice treated with

estrogen during the neonatal period (20), whereas the incidence of mammary gland tumor rises (21). Although one study has reported that certain BRMs are effective in preventing infectious diseases when given during the neonatal period (22), no reports exist on the application of neonatal BRM treatments to the field of cancer treatment or prevention.

The findings of the present study are as follows: PSK treatment given within 48 h of birth was effective in prolonging the survival period of mature mice given transplants of syngeneic tumors, reducing the number of liver metastatic foci in a mouse metastasis model, and reducing the number of precancerous lesions in a model of AOM-induced rat colon carcinogenesis. The effects were dependent on the PSK dose and the number of transplanted tumor cells and persisted until at least 30 weeks of age. According to a study (9, 23) of the *in vivo* fate of PSK, 85–90% of the molecules were excreted within 72 h after the administration, and almost no accumulation was observed. Therefore, it is unlikely that PSK remains in the neonatal body for a long time or exerts its effect after maturation. Furthermore, in BALB/c mice transplanted with C26 tumor cells, the life prolongation effect and inhibition of metastasis

Fig. 6. Effect of neonatal injection of PSK on athymic animals; A, male BALB/c nu/nu mice ($n = 10$) received a single i.p. injection of 10 mg/kg PSK or saline within 48 h of birth. Male BALB/c mice ($n = 8$) were thymectomized or sham-operated within 48 h of birth and received a single i.p. injection of 10 mg/kg PSK or saline. C26 tumor cells (1×10^6) were s.c. transplanted at 8 weeks of age, and the survival period was evaluated; B, male F344 rats ($n = 10$) were thymectomized or sham-operated within 48 h of birth and received a single i.p. injection of 10 mg/kg PSK or saline. s.c. injection of 15 mg/kg AOM once every week for 3 weeks was initiated 7 weeks after birth. Eight weeks after the initiation of AOM treatment, precancerous lesions were evaluated. Symbols are the same as those in Fig. 4.

(A) BALB/c mice without tumor transplantation



(B) BALB/c mice transplanted with C26

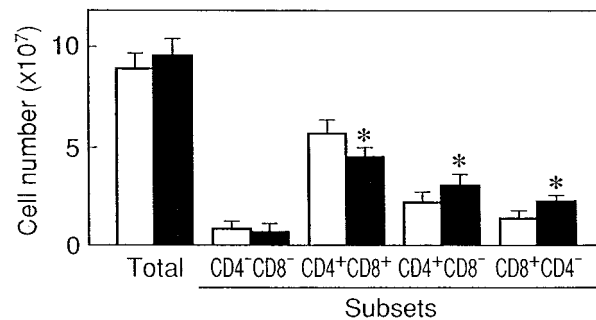


Fig. 7. Effect of neonatal injection of PSK on the thymus of adult male BALB/c mice. Male BALB/c mice received a single i.p. injection of 10 mg/kg PSK or saline within 48 h of birth. C26 tumor cells were s.c. transplanted when the mice reached 8 weeks of age, and the thymus was excised 2 weeks later. The number of thymus cells and the subset ($CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$, $CD8^+CD4^-$) were counted by flow cytometry. Details are described in "Materials and Methods"; A, mice without tumor transplantation; B, mice transplanted with C26. □, saline-injected group; ■, PSK-injected group. Significantly different at $* P < 0.01$ compared with the saline-injected group.

were increased significantly by a combination of PSK treatment during the neonatal period and frequent PSK treatment after maturation compared with those in the groups that received either treatment alone. These findings suggest that the action mechanism of neonatal PSK treatment differs from known mechanisms (5, 6).

Although the detailed mechanisms of action involved are still unclear, the thymus and T cells may somehow be involved in the exertion of the effect, because the findings of PSK injection during the neonatal period were not observed in athymic nude mice or neonatally thymectomized animals (Fig. 6). Furthermore, the antitumor activity of axillary LN cells derived from C26-bearing BALB/c mice administered PSK neonatally was significantly stronger than that of mice treated with saline at the same age, and the PSK effect was mediated possibly through activation of $CD8^+$ T cells. These findings suggest that neonatal PSK treatment strengthens killer T cell function through the thymus in mature tumor-bearing mice.

Neonatal PSK treatment significantly reduced the number of $CD4^+CD8^+$ T cells and significantly increased the number of $CD8^+CD4^-$ T cells and $CD4^+CD8^-$ T cells in the thymus of both tumor-free and tumor-bearing mature mice, although there were almost no differences in the total cell number. The changes in thymic T cell subsets in the neonatally PSK-treated mice may have been attributable to promotion of differentiation to $CD8^+CD4^-$ T cells and $CD4^+CD8^-$ T cells rather than

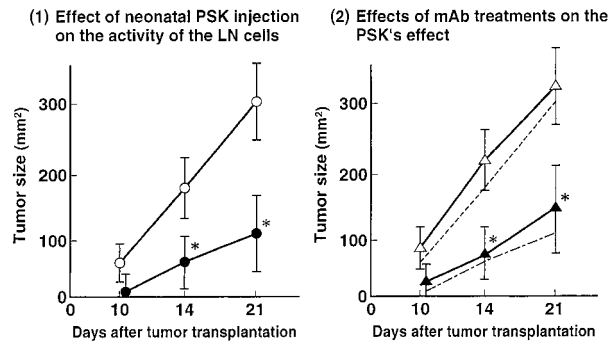


Fig. 8. Effect of neonatal injection of PSK on the antitumor activity of axillary lymph node cells in adult male BALB/c mice bearing C26 tumors. Male BALB/c mice received a single i.p. injection of 10 mg/kg PSK or saline within 48 h of birth, and C26 tumor cells were s.c. transplanted when the mice reached 8 weeks of age. Axillary LN were excised 14 days after the transplantation, and a cell suspension was prepared; A, the LN cells (2×10^6) and C26 tumor cells (2×10^5) were mixed in a total volume of 0.2 ml with HBSS and were s.c. transplanted into male BALB/c mice 8 weeks of age irradiated 2 h previously with 350 rad. ○, saline-injected group; ●, PSK-injected group; B, before the transplantation, the LN cells were incubated with anti- $CD8$ mAb or anti- $CD4$ mAb at $4^\circ C$ for 45 min, followed by incubation with rabbit complement. Treatment of the LN cells with immunoglobulin and complement did not affect the tumor growth (data not shown). △, PSK-injected group treated with anti- $CD8$ mAb and complement; ▲, PSK-injected group treated with anti- $CD4$ mAb and complement; —, saline-injected without mAb and complement treatment; - - -, PSK-injected group without mAb and complement treatment. Significantly different at $* P < 0.01$ compared with the saline-injected group. Bars, SD.

Table 3 The amount of cytoplasmic histone-associated DNA fragments in thymus cells of mature BALB/c mice after neonatal injection of PSK

Treatment during neonatal period ^a	The amount of cytoplasmic histone-associated DNA fragments in the thymus (unit) ^b
Saline	1.8 ± 0.6
PSK	1.9 ± 0.9

^a Male BALB/c mice ($n = 10$) received a single i.p. injection of 10 mg/kg PSK or saline within 48 h of birth. Ten weeks after birth, the thymus was excised, and the amount of cytoplasmic histone-associated DNA fragments in 3×10^7 thymus cells was measured by enzyme immunoassay using cell death detection ELISA (Roche Diagnostic).

^b Unit = [absorbance ($A_{406 \text{ nm}} - A_{490 \text{ nm}}$) of experimental group (3×10^7 thymus cells)/absorbance ($A_{406 \text{ nm}} - A_{490 \text{ nm}}$) of positive control group (1×10^4 U937 cells after incubation with $2 \mu\text{g/ml}$ camptothecin at $37^\circ C$ for 4 h)] $\times 100$.

acceleration of apoptosis of $CD4^+CD8^+$ T cells (Fig. 8 and Table 3). It is unlikely, however, that PSK acts simply and only on the function of specific cells or cell precursors in the thymus of neonate animals. During the neonatal period, not only the thymocytes but also nonlymphoid cells, such as the stroma cells in the thymus, are functioning actively. It is possible that PSK acts on the microenvironment of the thymus in neonate animals by affecting the generation of immunocompetent T cells responsible for immunological defenses and by enhancing the defense mechanism of tumor-bearing mice against tumors as evidenced after maturation, which results in an increased number of tumor-rejecting mice and extension of survival duration. Furthermore, the findings of the present study suggest that the period showing a high sensitivity to PSK is within 48 h of birth. The detailed mechanisms, such as an identification of the effector molecules responsible for the PSK effect, remain to be elucidated.

PSK injection during the neonatal period reduced the incidence of all precancerous lesions induced by AOM in F344

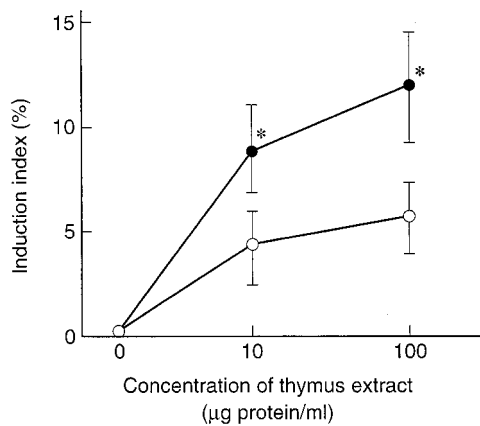


Fig. 9. Effect of neonatal injection of PSK on thymic hormone-like activity of thymus in adult male BALB/c mice. Male BALB/c mice received a single i.p. injection of 10 mg/kg PSK or saline within 48 h of birth, and the thymus was excised when the mice reached 8 weeks of age. Thymus extract was prepared following the method of Goldstein *et al.* (15) after modification. The extract was incubated *in vitro* with spleen cells from healthy BALB/c *nu/nu* mice at 37°C for 24 h, followed by analysis of CD90.2 positive cells using FACScan. Bars, SD. Induction index was calculated by [(% of CD90.2 negative cells cultured without thymus extracts) - (% of CD90.2 negative cells cultured in the presence of thymus extracts)] ÷ (% of CD90.2 negative cells cultured without thymus extracts) × 100. ●, mean induction index of thymus extracts derived from PSK-injected group; ○, mean induction index of thymus extracts derived from saline-injected group. Significantly different at * $P < 0.01$ compared with the saline-injected group.

rats. AOM-induced colon ACF in rats are regarded as precancerous lesions that predict the possible transition from initiation to progression in the multistep carcinogenesis process and are used as an intermediate marker for screening of chemopreventive agents (24–26). The effects of most ACF-inhibiting agents are classified into: a) blocking activity against carcinogens; b) antioxidative activity; and c) antiproliferation or antiproliferation activity (27). As in the experiments using neonatally thymectomized rats, the effect of PSK disappeared, and the involvement of T cell-mediated immunity in inhibition of AC or ACF by neonatal PSK treatment is considered, but it may be completely different from the actions of vitamin E, cyclooxygenase inhibitor 2, and nonsteroid agents, which are categorized in classification c. It is unlikely that PSK has the same antigenicity as those of mutant cells or cancer cells, which suggests that T-cell activity that recognizes antigens expressed on mutant cells in AC or ACF is strengthened. Alternatively, other mechanisms, *e.g.*, modulation of carcinogen metabolism and colon cell sensitivity to the active metabolite, methylazoxymethanol, are possible. However, during later stages of progression, rather than early stages, in multistep carcinogenesis, killer T cells are anticipated to act on cancer cells expressing a variety of tumor-related or tumor-specific antigens on the surface, which results in the inhibition or progression of cancer growth. To confirm whether T cells augmented by neonatal injection with PSK may contribute to the delay of the onset or reduced mortality in cancer bearing hosts, experiments that investigate the effects of neonatal inoculation with PSK on the formation of AOM-induced colon cancer in rats are now in progress.

Finally, the findings of the present study suggest that modulation of the response by BRM during the neonatal period is effective in augmenting the host defense mechanism against tumors after maturation. Although the effect of PSK injection during the neonatal period was relatively modest in the present

study, it may offer a clue toward intervention by a procedure that is relatively easy to carry out. However, additional studies of the detailed mechanisms by which neonatal injection of PSK causes an antitumor effect are needed before the effects of this type of BRM treatment can be exploited fully for the application of prevention studies. It was suggested also that the effect of neonatal treatment is not characteristic of PSK alone, and the investigation of BRM with a superior activity is beneficial.

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