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Antitumor Effect of PSK at a Distant Site: Tumor-specific Immunity and Combination with Other Chemotherapeutic Agents

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The antitumor effect of PSK, a *Coriolus* preparation, was analyzed with the double grafted tumor system in which BALB/c mice received intradermal inoculations of syngeneic Meth-A fibrosarcoma in the right (primary tumor, 10^6 cells) and left (distant tumor, 2×10^5 cells) flanks. Intratumoral administration of PSK significantly inhibited the growth of not only the right but also the left tumor. PSK also inhibited the growth of a methylcholanthrene-induced fibrosarcoma BAMC-1, and a methylurethane-induced adenocarcinoma Colon 26 in the double grafted tumor system of syngeneic BALB/c mice. However, when the left distant tumor was different from the right Meth-A tumor, the intratumoral administration of PSK in the right tumor was unable to inhibit the growth of the left BAMC-1 or RL♂-1 tumor. The PSK-induced immunity, therefore, is tumor-specific and T lymphocytes may play an important role in antitumor memory function. The enhancement of concomitant immunity by PSK treatment was completely impaired by previous intravenous administration of an alkylating agent, cyclophosphamide (CY). The enhancement of sinecomitant immunity by PSK treatment was also impaired by previous CY intravenous administration. The antitumor effect of PSK was suppressed by previous intravenous administration of another alkylating agent, ACNU. It is possible that alkylating agents suppress the function of effector T cells and granulocytes which are very important for the antitumor immune cascade reaction due to PSK treatment. On the other hand, the antitumor effect of PSK was enhanced by previous intravenous administration of an antimetabolite, 5-fluorouracil.

Key words: BRM — Combination therapy — Cyclophosphamide — Tumor specificity — Antagonistic effect

In our previous papers,[1-7] the antitumor effects at a distant site of PSK, a protein-bound polysaccharide preparation, and recombinant human interleukin-1β (IL-1β)[2] were analyzed by using the double grafted tumor system in which male BALB/c mice received simultaneous intradermal inoculations of Meth-A tumor in the right (10^6 cells) and the left (2×10^5 cells) flanks and were then injected with PSK or IL-1β in the right-flank tumor on days 3, 4 and 5. Among several biological response modifiers (BRM) examined, PSK and IL-1β significantly inhibited the growth of not only the right but also the left (non-treated) tumor. The first purpose of the research described herein was to show that the intratumoral administration of PSK causes growth inhibition of various kinds of tumors (BAMC-1, RL♂-1 and Colon 26) and that the intratumoral administration of PSK into the right Meth-A tumor is unable to inhibit the growth of the left, different tumor, developing a Meth-A specific antitumor immunity. The second purpose of this investigation was to examine whether or not antitumor chemotherapeutics including cyclophosphamide (CY), nimustine (ACNU) and 5-fluorouracil (5-FU) have a synergistic antitumor effect on the intratumoral administration of PSK.

MATERIALS AND METHODS

Mice and tumor Six-week-old male BALB/c mice were obtained from the Institute for Experimental Animals, Tohoku University School of Medicine. Meth-A, a methylcholanthrene-induced fibrosarcoma, was administered to syngeneic BALB/c mice in solid form by intradermal inoculation. BAMC-1, a methylcholanthrene-induced fibrosarcoma in BALB/c mice, was established in the Laboratory of Chugai Pharmaceutical Co. Ltd., Tokyo and was supplied by them.[8] RL♂-1, an irradiation-induced leukemia, was also used.[9] Colon 26 is an N-nitroso-N-methylurethane-induced undifferentiated adenocarcinoma in syngeneic BALB/c mice.[10]

Drug PSK was purified from a hot water extract of cultured mycelia from *Coliolus versicolor*, which belongs to Basidiomycetes. The average molecular weight is about 100,000 and its protein content is about 38%. The main glycoside portion of PSK is β-D-glucan. PSK was

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[2] The abbreviations used are: IL-1β, interleukin-1β; CY, cyclophosphamide; 5-FU, 5-fluorouracil; BRM, biological response modifier; IL-8, interleukin-8; MCF, macrophage chemotactic factor; PBS, phosphate-buffered saline.

supplied by Kureha Chemical Industry Ltd., Tokyo. An alkylating agent, CY was purchased from Sigma Chemical Co., St. Louis, Mo. Another alkylating agent, ACNU was supplied by Sankyo Co., Ltd., Tokyo. An antimetabolite, 5-FU was supplied by Kyowa Hakko Kogyo Co., Ltd., Tokyo.

Double grafted tumor system As described in previous papers,[1-7] we devised the double grafted tumor system as a new experimental tumor model. BALB/c mice receive simultaneous intradermal inoculations of Meth-A tumor cells in both the right (1×10^6 cells, primary region) and the left (2×10^5 cells, distant region) flanks. Drugs are injected into the right-flank tumor on days 3, 4 and 5, and the left (non-treated) tumor is observed for 21 days.

Evaluation of antitumor activity Tumor diameter was measured 3 times a week with calipers and the tumor size was calculated as the square root of the long diameter$\times$ short diameter (mm). After 3 weeks, the animals were killed and each tumor was weighed to obtain the mean value (g) $\pm$ standard deviation. Each experimental and control group consisted of 7 to 10 mice. The difference in tumor growth (tumor size or tumor weight) between the control and experimental groups was tested statistically by using Student's t test. The differences in cure rates were statistically evaluated by means of the chi-square test. Survival rates were statistically evaluated by the generalized Wilcoxon test.

Adoptive transfer test *In vivo* assay of effector cells was conducted in recipient mice inoculated intradermally with 1×10^6 cells. CY (2 mg/0.2 ml/mouse) was given intravenously one hour prior to administration of effector cells on day 3. Effector cells (2×10^7) in 0.1 ml of PBS (pH 7.2) were injected intratumorally into the recipient mice.

Concomitant immunity Primary Meth-A cells (10^6 cells) were inoculated intradermally at day -11 or -7 into the right flanks and 2×10^5 Meth-A cells were inoculated on day 0 into the left flank. The growth of the second challenge tumor was observed for 21 days to evaluate the generation of concomitant immunity. PSK was injected intratumorally into 3-, 4- and 5-day primary tumors.

Sinecomitant immunity Initially, primary tumor cells (10^6 cells) were inoculated intradermally in the right flank. On day 6 after tumor inoculation, the primary tumor was resected, and on day 21, 10^6 secondary tumor cells were rechallenged into the left flank. The growth of the secondary tumor was followed for 21 days to evaluate the generation of sinecomitant immunity. PSK was administered intratumorally on days 3 and 5 into the primary tumor.

RESULTS

Double grafted tumor system with various tumors In our previous reports,[1-3, 5, 6] we showed that PSK inhibited the growth of not only the right but also the left syngeneic Meth-A fibrosarcoma in the double grafted tumor system in BALB/c mice and this result was confirmed in the present work, as shown in the left part of Fig. 1 and the upper column of Table I. A similar antitumor effect was observed in another methylcholanthrene-induced fibrosarcoma BAMC-1, as shown in the right part of Fig. 1. Intratumoral PSK administration into a syngeneic Colon 26 adenocarcinoma also showed effective prolongation of survival time in BALB/c mice. It was, therefore, shown that PSK induced a significant antitumor effect on both sarcoma and carcinoma.

Tumor-specific immunity after PSK treatment Next, in order to detect tumor-specific immunity after PSK treatment, we investigated the antitumor activity of PSK on a left different tumor in the double grafted tumor system. As shown in Fig. 2, when another fibrosarcoma BAMC-1 was used as the left tumor, an intratumoral administration of PSK into the right Meth-A tumor did not inhibit the growth of BAMC-1. When the right tumor and the left tumor were reversed, the same result was obtained

Table I. Tumor Specificity of Antitumor Effect of PSK in the Double Grafted Tumor System

Group			Right tumor (1×10^6)		Left tumor (2×10^5)	
Right tumor	Left tumor		Tumor-free /tested	Tumor weight (g$\pm$SD)	Tumor-free /tested	Tumor weight (g$\pm$SD)
Meth A	Meth A	Control	0/8	5.0$\pm$0.78	0/8	2.8$\pm$0.31
		PSK	2/8	1.6$\pm$1.10**	5/8*	1.3$\pm$1.55
Meth-A	RL♂-1	Control	0/6	5.3$\pm$0.58	0/6	1.3$\pm$0.65
		PSK	2/6	1.3$\pm$0.29**	0/6	1.3$\pm$0.88

Mice received simultaneous intradermal inoculations of Meth A fibrosarcoma or RL♂-1 leukemia cells in both the right (1×10^6 cells) and left (2×10^5 cells) flanks on day 0. PSK (5 mg/0.1 ml/mouse/day$\times$3, days 3, 4 and 5) was injected into the right tumor, and a 21-day observation period followed. Significant difference from the each control group: * $P<0.05$, ** $P<0.01$. SD: Standard deviation.

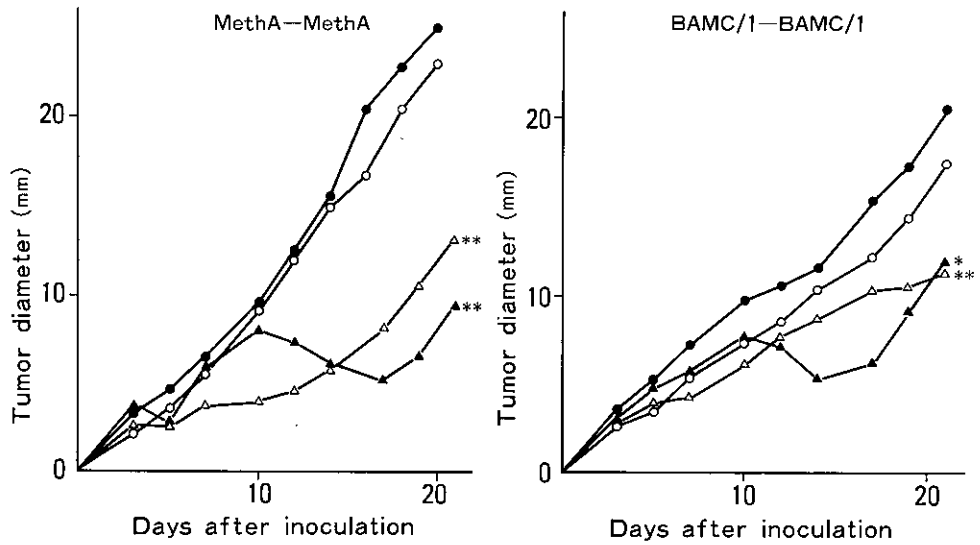

Fig. 1. Antitumor effect of intratumoral injection of PSK on the growth of Meth-A or BAMC-1 tumor in the double grafted tumor system. ● control, right tumor (1×10^6 cells); ○ control, left tumor (2×10^5 cells); ▲ PSK, right tumor; △ PSK, left tumor. Values are means of eight mice per group. Significant difference from the control group: ** $P<0.01$, * $P<0.05$.

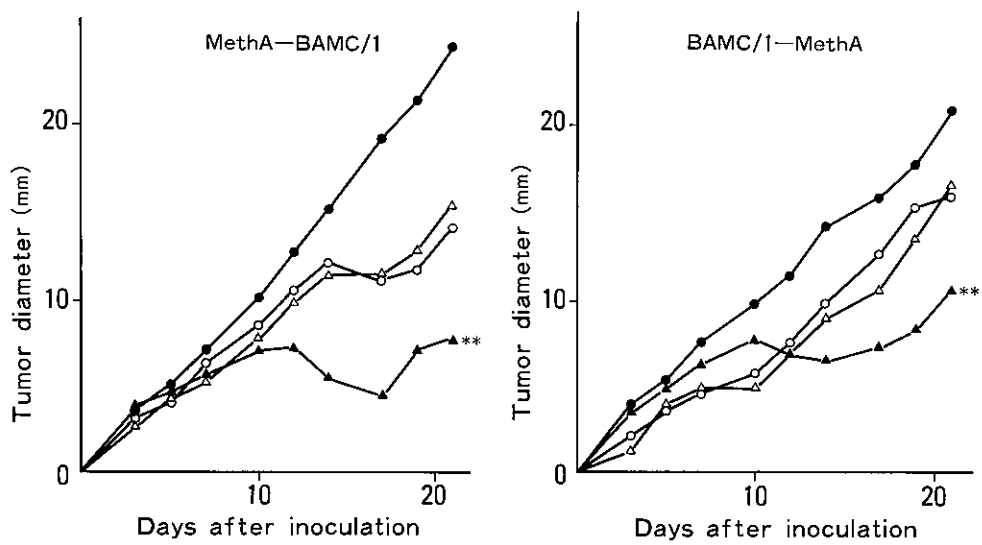

Fig. 2. Tumor specificity of antitumor effect of PSK in the double grafted tumor system. Mice received simultaneous intradermal inoculations of Meth-A in the right flank (1×10^6 cells) and BAMC-1 in the left flank (2×10^5 cells) on day 0 (left part), and *vice versa* (right part). PSK (5 mg/mouse/day) was injected in the right tumor on days 3, 4 and 5. ● control, right tumor; ○ control, left tumor; ▲ PSK, right tumor; △ PSK, left tumor. Significant difference from the control group: ** $P<0.01$.

(the right part of Fig. 2). PSK also was unable to inhibit the growth of syngeneic leukemia RL♂-1 tumor as the left tumor of the double grafted tumor system, as shown in the lower column of Table I. As described in a previous paper,[1] immunized spleen cells were taken from mice which had been cured by the intratumoral administration

Table II. Effect of Spleen Cells Obtained from PSK-treated Meth A-bearing Mice on the RL♂-1 Recipient

Recipient	Group	Treatment	Tumor-free /tested	Tumor weight (g±SD)	Relative weight (%)
RL♂-1	CY control	None	0/8	3.8±0.88	100
	Control	None	0/8	4.0±0.56	105
	Meth A	None	0/8	3.5±0.72	92
	bearer cells	PSK	0/8	4.1±0.87	108
Meth-A	CY control	None	0/8	2.6±0.95	100
	Control	None	0/8	4.3±0.90	165
	Meth A	None	0/8	1.6±0.46	61
	bearer cells	PSK	6/8**	0.3±0.17**	11

Donor spleen cells were obtained from Meth A-bearing mice treated with PSK (5 mg/mouse/day×3 on days 3, 4 and 5 after Meth A implantation). Recipients were inoculated intradermally with 1×10^6 RL♂-1 leukemia cells or Meth-A sarcoma cells and were injected intravenously with 2 mg of cyclophosphamide (CY) one hour prior to intratumoral adoptive transfer of donor spleen cells (2×10^7) on day 3, and a 3-week observation period followed. Significant difference from the CY and control groups: ** $P<0.01$. SD: Standard deviation.

Table III. Impairment of Concomitant Immunity by Cyclophosphamide (CY)

Inoculation day of primary tumor	Drug treatment	Tumor-free /tested	Tumor diameter (mm±SD)	Tumor weight (g±SD)
−11 day	None	0/10	16.40±2.12	1.90±0.14
−11 day	PSK[a)]+CY[b)]	0/9	16.40±2.12	1.90±0.14
−11 day	PSK[a)]	9/10**	0.70±2.20**	0.03±0.09**
−7 day	PSK[a)]	10/10**	0	0

Mice were inoculated intradermally with 1×10^6 Meth A cells in the right flank on day −11 or day −7 and further inoculated intradermally with 2×10^5 Meth-A cells in the left flank on day 0, and the growth of the left tumor was monitored for 21 days.
a) PSK (5 mg/day) was injected into the 3-, 4- and 5-day primary tumor.
b) CY (2 mg/mouse) was injected intravenously one hour prior to PSK treatment.
Significant difference from the none-treated group: ** $P<0.01$. SD: Standard deviation.

of 5 mg of PSK and were injected into the Meth-A tumor on day 3. Adoptive transfer of PSK-immunized spleen cells caused the complete regression of Meth-A tumors. The effector was Lyt-1-positive and L3T4-positive T cells.[2,5] Then PSK-immunized spleen cells (2×10^7) on day 21 were obtained from BALB/c mice which had been cured by an intratumoral PSK administration into Meth-A tumor and were injected into a different RL♂-1 tumor on day 3. PSK-immunized spleen cells did not inhibit the growth of RL♂-1 by day 21, as shown in Table II. Therefore, it was shown that the intratumoral administration of PSK induced a Meth-A tumor-specific antitumor effect on a distant tumor in the double grafted tumor system.

Impairment of concomitant immunity by previous cyclophosphamide treatment In our previous paper,[3] we showed that a primary growth of Meth-A sarcoma inoculated into the right flank resulted in the generation of concomitant immunity to the growth of a second graft

Table IV. Suppressive Effect of Cyclophosphamide (CY) on Enhancement of Concomitant Immunity by PSK Treatment

Treatment	Tumor diameter (mm±SD)	Tumor weight (g±SD)
None	15.9±2.9	2.2±1.1
PSK[a)]	1.3±3.7	0.1±0.2
CY[b)] (−1 h)+PSK[a)]	22.8±2.3**	5.9±1.3**
CY[b)] (−12 h)+PSK[a)]	20.3±3.2*	3.9±2.2*
CY[b)] (−24 h)+PSK[a)]	22.9±1.6**	5.7±1.1**

Mice were inoculated intradermally with 1×10^6 Meth-A cells in the right flank on day −7 and further inoculated intradermally with 2×10^5 Meth-A cells in the left flank on day 0, and the growth of the left tumor was monitored for 21 days.
a) PSK (5 mg/day) was injected into the 3-, 4- and 5-day primary tumor.
b) CY (2 mg/mouse) was injected intravenously at the indicated hour prior to PSK treatment.
Significant difference from the PSK group: ** $P<0.01$, * $P<0.05$. SD: Standard deviation.

of the same tumor cells in the left flank. A significant inhibitory effect on the proliferation of the tumor cells inoculated secondarily was shown in mice bearing a primary right tumor that had previously been inoculated 3 times with PSK. In this system, 2 mg of CY was injected intravenously one hour prior to the injection of PSK in order to eliminate the function of suppressor T cells.[11-13] Interestingly, an alkylating agent, CY completely abrogated the enhancement of concomitant immunity by PSK treatment, as shown in Table III. That is, none of the 9 PSK-treated mice became tumor-free. This antagonistic effect of CY was also observed when CY was injected 24 h before PSK treatment (Table IV).

Impairment of sinecomitant immunity by previous cyclophosphamide treatment In a previous report,[6] regarding sinecomitant immunity, tumor inoculation into the right flank followed by intratumoral administration of PSK on days 3 and 5 and surgical excision of the primary tumor on day 6 resulted in complete rejection of a tumor challenge in the left flank on day 21. In this

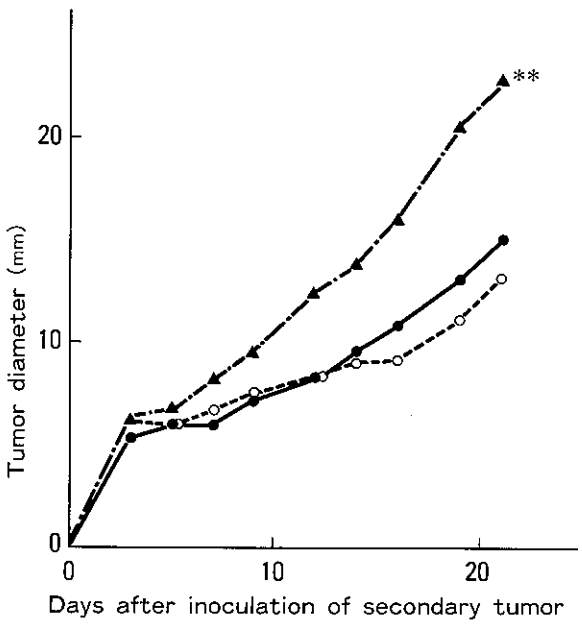

Fig. 3. Impairment of sinecomitant immunity by cyclophosphamide (CY). Mice were inoculated intradermally with 1×10^6 Meth-A cells in the right flank and primary tumor was resected on day 6. The secondary (rechallenge) tumor (1×10^6 cells) was inoculated in the left flank on day 21 and the growth of the secondary tumor was monitored for a further 21 days. PSK (5 mg/day) was injected into the 3- and 5-day primary tumor. CY (2 mg/mouse) was injected intravenously one hour prior to PSK treatment. ● Meth-A control; ○ PSK treatment; ▲ CY and PSK treatment. Significant difference from the control group: ** $P<0.01$.

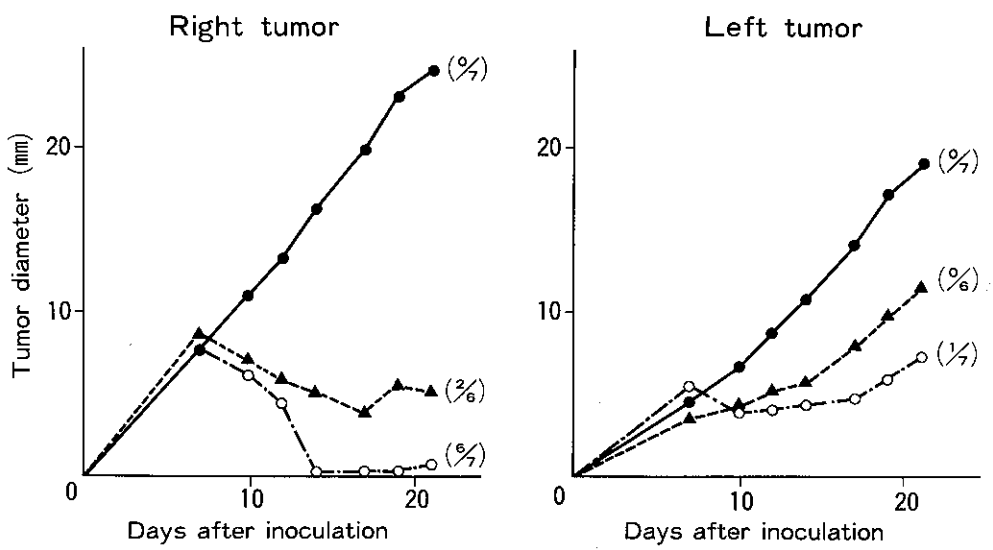

Fig. 4. Suppressive effect of ACNU on antitumor activity of PSK in the double grafted tumor system. Mice received simultaneous intradermal inoculations of Meth-A cells in both the right and the left flanks on day 0. PSK (5 mg/mouse/day) was injected into the right tumor on days 3, 4 and 5. ACNU (1 mg/mouse) was injected intravenously on day 2. ● Meth-A control; ○ PSK treatment; ▲ PSK+ACNU treatment (No. of tumor-free mice on day 21/No. of treated mice).

Table V. Synergistic Antitumor Effect by Combining PSK with 5-FU in the Double Grafted Tumor System

Group	Right tumor			Left tumor		
	Tumor-free /tested	Tumor diameter (mm±SD)	Tumor weight (g±SD)	Tumor-free /tested	Tumor diameter (mm±SD)	Tumor weight (g±SD)
Control	0/4	22.8±0.4	4.1±0.6	0/4	17.5±1.9	2.4±0.6
PSK[a)]	2/5	5.3±5.1**	0.3±0.4**	0/5	11.5±2.3*	1.2±0.8*
5-FU[b)]	0/5	20.5±1.1	3.1±0.5	0/5	15.6±1.3	1.7±0.4
PSK[a)]+5-FU[b)]	6/6**	0.0±0	0.0±0	1/6	8.6±4.6**	0.5±0.3**

Mice received simultaneous intradermal inoculations of Meth-A cells in both the right (1×10^6 cells) and the left (2×10^5 cells) flanks on day 0.
a) PSK (5 mg/0.1 ml/mouse/day) was injected into the right tumor on days 3, 4 and 5.
b) 5-FU (1 mg/mouse) was injected intravenously on day 2.
Significant difference from the control group: * $P<0.05$, ** $P<0.01$. SD: Standard deviation.

system, a previous intravenous injection of CY (−1 h, 2 mg) also suppressed the enhancement of sinecomitant immunity by an intratumoral injection of PSK, as shown in Fig. 3.

Suppressive effect of ACNU on antitumor activity of PSK Another alkylating agent, ACNU, was examined for synergistic antitumor activity with PSK in the double grafted tumor system. One mg of ACNU was intravenously administered in BALB/c mice bearing Meth-A tumor 1 day before PSK treatment. As shown in Fig. 4, a previous intravenous injection of ACNU suppressed the antitumor effect of PSK in the same manner as CY.

Synergistic antitumor effect of PSK and 5-FU Next, another chemotherapeutic antimetabolite, 5-FU was examined for synergistic antitumor activity with PSK in the double grafted tumor system. One mg of 5-FU was intravenously administered to BALB/c mice bearing Meth-A tumor, 1 day before PSK treatment. As shown in Table V, a previous intravenous 5-FU administration showed a synergistic antitumor effect with PSK treatment.

DISCUSSION

The present data show that the intratumoral administration of PSK inhibited the growth of various types of tumor, i.e., methylcholanthrene-induced fibrosarcomas Meth-A (Fig. 1 and Table I) and BAMC-1 (Fig. 1), and a methylurethane-induced adenocarcinoma, Colon 26. This report also shows that intratumoral immunotherapy with PSK in the primary Meth-A tumor was unable to induce rejection of a distant different tumor, e.g. BAMC-1 (Fig. 2) or RL♂-1 (Table I), in the double grafted tumor system. Adoptive transfer experiments showed that effector T cells from the spleen of cured mice, which had been injected with PSK in the Meth-A tumor, were not able to inhibit the growth of RL♂-1 tumor (Table II). These data suggested that the PSK-induced immunity was tumor-specific and that T lymphocytes play an important role in antitumor memory function. Our data also confirm the previous results that the antitumor activity of PSK in the double grafted tumor system is associated with a sequential immune mechanism in which T cells play an important role[3] and are consistent with findings for OK-432 in the athymic nude mice.[8] Therefore, it has become apparent that the tumor immunity developed in mice treated with PSK is tumor-specific; mice cured of Meth-A with PSK rejected rechallenge with Meth-A but not challenge with syngeneic RL♂-1 leukemia.[1] It is possible that when it is given intratumorally, PSK may come into close contact with tumor cells, whereupon local inflammatory responses occur and result in the non-specific killing of tumor cells, followed by the development of immunity to the tumor-specific transplantation antigen.

The results reported here demonstrate an antagonistic activity of alkylating agents, CY (Table III, IV; Fig. 3) and ACNU (Fig. 4), with respect to PSK antitumor activity in an *in vivo* BALB/c mice-syngeneic Meth-A tumor model. It was shown that a single dose of CY (100 mg/kg) eliminates tumor-induced suppressor T cells.[11–13] However, our present results showed that the same dose of CY (2 mg/mouse) eliminated tumor-induced effector T cells, including the Lyt-1-positive and L3T4-positive T cells.[2,5] Therefore, it is possible that CY suppresses most T cell functions. Many reports have also demonstrated that a dose of 200 mg/kg of CY induces myelosuppression or leukocytopenia.[14–17] Our previous paper[6] showed that the intratumoral administration of PSK first induced neutrophils in the right tumor via an IL-8-like factor,[18] which might be the first vital participant in the cascade of interacting cytokines and effector cells in the double grafted tumor system. Therefore, a previous CY treatment might cause leukocytopenia, so that neutrophils

could not be induced in the right tumor by an intratumoral administration of PSK, as well as showing an antagonistic action on the antitumor effect on PSK treatment. On the other hand, another chemotherapeutic agent, the antimetabolite 5-FU, has been reported to be a synergistic antiproliferative agent interacting with a kind of BRM, interferon.[19,20] Our data confirm those reports (Table V). Therefore, the time of addition of anticancer drugs may be a very important factor for combination therapy with BRM.

Finally, our present and previous studies on the antitumor effect of PSK in the double grafted tumor system show that the intratumoral administration of PSK first induces neutrophils in the right tumor via an IL-8-like factor and then macrophages are induced by a macrophage chemotactic factor (MCF),[6] which might be produced by tumor cells. Then Lyt-1 (L3T4)-positive cells[3,5] are induced in the right regional lymph nodes and in the spleen, probably via IL-1, which might be produced from macrophages in contact with tumor cells. Subsequently, Lyt-1-positive cells reach the left tumor through the blood stream, come into contact with Meth-A tumors and then produce MCF[6] and interleukin-2. Intratumoral administration of PSK in the right tumor thus induces activated macrophages and Lyt-2-positive killer T cells in the left, non-treated tumor, thereby bringing about the regression of the distant tumor. Also Lyt-1-positive memory cells are left in the spleen,[2] possibly causing the rejection of reinoculated tumor cells.[1]

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