Chem. Pharm. Bull. 30(6)2133—2140(1982)

Antitumor Activity of P-MSY, a Protein from Bovine Parotid Glands, and Some Observations relating to the Activity[1)]

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(Received September 24, 1981)

When P-MSY, a component of bovine parotid glands, was injected at a dose of 1 μg/head/d, daily for 10 d starting 24 h after the transplantation of ascites Ehrlich carcinoma cells, the solid tumor formed was rejected in 6 of 10 mice, and the tumor inhibition rate was 86.4%. Subcutaneous injection of 1 μg/head/d, daily for 7 d before transplantation of sarcoma-180 cells resulted in prolongation of the life span of ascites tumor-bearing mice, the mean survival period being 19.4 d against 14.1 d in the control; T/C was 138%. Such a life-prolonging effect was not observed when P-MSY was administered after the transplantation of the tumor.

Electrophoresis of the serum from mice in which the tumor had been rejected after the administration of P-MSY revealed increased quantities of some protein components.

By examinations of delayed-type skin reaction, P-MSY was found to hinder significantly the lowering of cellular immunity in tumor-bearing mice.

Keywords——P-MSY; antitumor activity; solid and ascites Ehrlich carcinoma; life-prolongation; serum proteins; delayed-type skin reaction

A protein with a molecular weight of 66000, which was designated as P-MSY, was isolated from bovine parotid glands.[2,3)] This sample (P-MSY) promoted immune competence[4)] and exhibited a strong antitumor activity against solid sarcoma-180 in ICR–SLC strain or in Swiss–Webster strain mice.[5)]

This paper describes the antitumor activity of P–MSY against solid and ascites tumors of Ehrlich carcinoma and sarcoma-180 in two strains of mice. The effect of P–MSY on the contents of serum proteins and delayed-type skin reaction was also examined.

Materials and Methods

Materials——P-MSY and a partially purified preparation, PAIA, were obtained as described previously.[3)] Male rabbits, fed on a combination diet (bran: wheat: rice-bran=1: 2: 1), were used for hypocalcemic assay.[2)] Hypocalcemic rates in rabbit serum after intravenous injection of these samples were 9.99±2.04% with PAIA at a dose of 0.1 mg/kg and 13.57±1.61% with P-MSY at a dose of 10 μg/kg, and both were found to be effective. Each material was dissolved in saline and used for injection.

Animals——In experiments 1—5 and in experiment 6, 4-week-old littermate mice of ICR-SLC strain weighing 17—20 g and of Swiss-Webster strain weighing 18—20 g, bred in our laboratory, were used, respectively. The littermates were divided into test and control groups in which males and females were distributed as equally as possible. Male 4-week-old ddY mice, weighing about 20 g, were purchased from the Shizuoka Agricultural Co-operative for Experimental Animals, Hamamatsu, Japan.

Tumor——Ascites tumor cells of Ehrlich carcinoma and sarcoma-180 were routinely transplanted into the abdominal cavity of new mice every 7th d. For the formation of the solid tumor, ascites tumor cells (4—8×10^6 cells) were subcutaneously inoculated into the right inguinal region of mice.

Antitumor Experiments——i) Effect on Solid Tumor: Mice in the test group were injected with a sample solution at a dose of 0.1 ml/head in each injection before or after the tumor transplantation, and those in the control group were injected with saline instead of sample solution.

Tumor growth was determined once a week by measuring the size of the tumor (longer diameter × shorter diameter, cm^2) from over the skin, using a pair of calipers. The size and weight of the tumor extirpated at the 5th week after the transplantation were also measured. Tumor inhibition rate was calculated relative to the control. The significance of antitumor effect was examined by means of Student's *t*-test, and when

the result was significant at the 5% level or better, the sample was considered effective.

Experiment 1: Seven mice were intraperitoneally injected with the partially purified sample, PAIA, at a dose of 200 μg/head/d, daily for 10 d starting 24 h after the transplantation of Ehrlich carcinoma. Eight mice were assigned to the control group.

Experiment 2: Ten mice were intraperitoneally injected with P-MSY at a dose of 1 μg/head/d, daily for 10 d starting 24 h after the transplantation of Ehrlich carcinoma. Nine mice were assigned to the control group.

Experiment 3: Six mice were subcutaneously injected into the back with P-MSY, 20 μg/head, 7 d before the tumor transplantation of Ehrlich carcinoma. Five mice were assigned to the control group.

Experiment 4: Seven mice were intraperitoneally injected with P-MSY, 60 μg/head, 14 d before the transplantation of Ehrlich carcinoma. Seven mice were assigned to the control group.

Experiment 5: Eight mice were intraperitoneally injected with 1 μg/head/d of P-MSY daily for 10 d starting 24 h after the transplantation of sarcoma-180. Seven mice were assigned to the control group.

Experiment 6: Six mice of Swiss-Webster strain were subcutaneously injected into the back with 20 μg/head of P-MSY, 7 d before the transplantation of sarcoma-180. Five mice were assigned to the control group.

ii) Effect on Ascites Tumor (Life-Prolonging Effect): A group of 8—11 mice of ICR-SLC strain or ddY strain was transplanted with 4—8×10^6 cells/head of Ehrlich carcinoma or sarcoma-180, and the mice were observed for 30 d.

Experiment 7: Eight mice of ICR-SLC strain were subcutaneously injected with P-MSY, at a dose of 1 μg/head/d, daily for 7 d starting 24 h after transplantation of Ehrlich carcinoma. Five mice were assigned to the control group.

Experiment 8: Eleven mice of ddY strain were subcutaneously injected with P-MSY, at a dose of 1μg/head/d (dose A) or 5 μg/head/d (dose B), daily for 7 d starting 24 h after the transplantation of sarcoma-180. Eleven mice were assigned to the control group.

Experiment 9: Eight mice of ICR-SLC strain were subcutaneously injected with P-MSY, at a dose of 1 μg/head/d, daily for 7 d before the transplantation of sarcoma-180. Eight mice were assigned to the control group. Mean survival time (MST) in d was calculated from the following formula, and the ratio of MST of the experimental and control groups (T/C, %) was calculated:

$$\mathrm{MST}=\frac{\sum(t\cdot f)}{n}$$

where f is the number of animals that died on the t-th d and n is the total number of animals observed. The result was judged as effective when T/C was more than 125%.

Variation in Serum Protein of Mice after Administration of P-MSY——Blood was drawn from the heart of mice whose tumor had disappeared after the administration of P-MSY, 5 weeks after tumor transplantation of Ehrlich carcinoma or sarcoma-180. The serum obtained was examined by the following three electrophoretic methods, and the sera from normal and tumor-bearing mice were simultaneously examined.

Disc Electrophoresis: The serum was diluted 20-fold with 20% saccharose and subjected to disc electrophoresis by the method of Davis,[6] using 7.5% polyacrylamide gel (pH 8.9) and Tris-glycine buffer (pH 8.6, $\mu=0.05$), at 3 mA and 380 V for 2 h. The gel was stained with Coomassie Brilliant Blue R250 by the method of Fazekas *et al.*,[7] and destained with a mixture of methanol, acetic acid, and water (40: 14: 160).

Electrofocusing: The procedures were performed according to the method of Bates and Deyoe.[8] Serum diluted 5-fold with 20% saccharose was placed on an Ampholine polyacrylamide gel plate (pH range, 4—6.5) (LKB Producter, Sweden) and electrophoresed at 350—1080 V and 20—75 mA for 2 h. After the electrophoresis, the gel was soaked in the fixative (sulfosalicylic acid and trichloroacetic acid dissolved in 7% aqueous methanol at concentrations of 11.5 and 3.4%, respectively) for 2—3 min, stained with Coomassie Brilliant Blue R250, and destained with a mixture of methanol, acetic acid, and water (50: 16: 134).

Gradient Acrylamide Gel Electrophoresis: The electrophoresis was run by the method of Maeda *et al.*[9,10] The serum was diluted 2-fold with 20% saccharose and subjected to electrophoresis on a gradient gel, PAA 4/30 (Pharmacia Fine Chemicals, Uppsala), using 5 mM Tris-glycine buffer (pH 8.6), at 125 V for 15 h. The gel was stained with 1% Amido Black 10B for 3 min and destained with 7% acetic acid solution.

Delayed-Type Skin Reaction——In accordance with the methods of Asherson,[11] Ptak,[12] and Natume and Ishida,[13] the delayed-type skin reaction of normal and solid tumor-bearing mice was examined by double sensitization with picryl chloride. Littermates of 4- to 5-week-old mice of ICR-SLC strain were divided into two group (test and control) in the same way as in antitumor experiments.

The hair on the abdomen of the mice was shaved off, and 7% picryl chloride in ethanol was pained on the shaved skin for sensitization. After 7 d, an olive oil solution of 1% picryl chloride was painted on both ears as an inducing antigen, and the thickness of both ears was measured with a dial thickness gauge (Ozaki Manufacturing) 24 h after the painting. In the experiment using normal mice, P-MSY was given at a dose of 1 μg/head/d by intraperitoneal injection, daily for 10 d starting 2 weeks before the sensitization. The mice were sensitized firstly 2 weeks after the start of P-MSY administration, induction was performed on the

3rd week, and the effect was judged first at 24 h after the induction. The second sensitization was done on the 25th day after the start of P-MSY administration, followed by induction after 7 d, and the second judgement was performed 24 h later.

In the case of mice bearing solid Ehrlich carcinoma or sarcoma-180, 1 μg/head/d of P-MSY was intraperitoneally injected, daily for 10 d starting 24 h after the tumor transplantation, and its effect on the skin reaction was examined.

Results and Discussion

Effect on Solid Tumor

The changes in tumor size were followed every week after the transplantation of Ehrlich

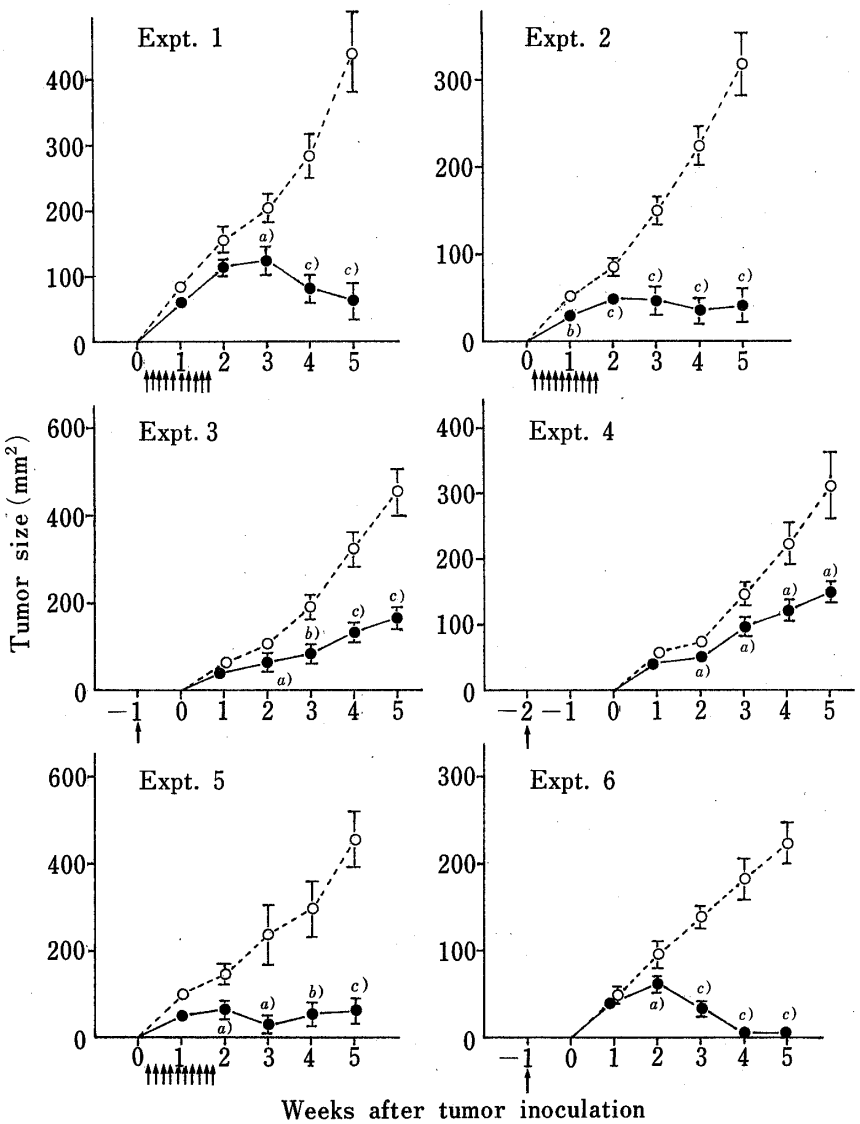

Fig. 1. Effect of Bovine Parotid Products on Solid Ehrlich Carcinoma and Sarcoma-180 in Mice

Expt. 1—4: Ehrlich carcinoma was inoculated subcutaneously in ICR–SLC strain mice.
Expt. 5: Sarcoma-180 was inoculated subcutaneously in ICR–SLC strain mice.
Expt. 6: Sarcoma-180 was inoculated subcutaneously in Swiss–Webster strain mice.
···●···; control group, injected with saline at a dose of 0.1 ml/head/d.
——●——; test group.
○ ●; mean ± S.E.
Significant at *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$.

Table I. Antitumor Effect of Bovine Parotid Extracts on Ehrlich and Sarcoma-180 Solid Tumors

Expt. No.	Sample	Dose (μg/head) ×d)	Days of treatment	Route	No. of mice	Average tumor weight (g ± S.E.)	Tumor inhibition ratio (%)	Complete regression
1	PAIA	200×10	+1～+10	IP	7	0.95±0.38[c)]	82.3	3/7
	Control	0.1×10[a)]	+1～+10	IP	8	5.40±1.24	/	0/8
2	P-MSY	1×10	+1～+10	IP	10	0.28±0.15[c)]	86.4	6/10
	Control	0.1×10[a)]	+1～+10	IP	9	2.10±0.44	/	0/9
3	P-MSY	20×1	(−1 week)	SC	6	1.25±0.26[b)]	67.8	0/6
	Control	0.1×1[a)]	(−1 week)	SC	5	3.88±1.04	/	0/5
4	P-MSY	60×1	(−2 week)	IP	7	0.81±0.23[c)]	72.8	0/7
	Control	0.1×1[a)]	(−2 week)	IP	7	2.98±0.65	/	0/7
5	P-MSY	1×10	+1～+10	IP	8	0.87±0.30[c)]	82.7	3/8
	Control	0.1×10[a)]	+1～+10	IP	7	5.05±1.02	/	0/7
6	P-MSY	20×1	(−1 week)	SC	6	0.10±0.06[b)]	95.4	4/6
	Control	0.1×1[a)]	(−1 week)	SC	5	2.18±0.34	/	0/5

Tumor cells ($4—8\times10^6$) were inoculated subcutaneously on day 0.
The tumor inhibition ratio was determined at 5 weeks after tumor grafting.
Tumor inhibition ratio (%)=(1− tumor weight of test group/tumor weight of control group)×100
Expt. 1—4: Ehrlich carcinoma was inoculated subcutaneously in ICR-SLC strain mice.
Expt. 5: Sarcoma-180 was inoculated subcutaneously in ICR-SLC strain mice.
Expt. 6: Sarcoma-180 was inoculated subcutaneously in Swiss-Webster strain mice.
a) Saline, ml/head.
b) Significantly different from control, $p<0.05$.
c) Significantly different from control, $p<0.01$.
d) Significantly different from control, $p<0.001$.

carcinoma (Fig. 1). The tumor weight, inhibition rate, and tumor regression found on the 5th week after the transplantation are summarized in Table I.

Experiment 1——As shown in Fig. 1 (1), the tumor grew until the 2nd week after transplantation and then regressed until the 5th week in the treated group. The excised tumors weighed significantly less ($p<0.01$) than those from the control group (Table I, 1). The tumor disappeared in 3 of 7 mice, and the tumor inhibition rate was 82.3% (Table I, 1).

Experiment 2——As shown in Fig. 1 (2), the tumor grew gradually for 2 weeks after the transplantation and tended to regress thereafter until the 5th week. The excised tumors clearly weighed less ($p<0.01$) than those of the control group (Table I, 2). The tumor disappeared in 6 of 10 animals, and the tumor inhibition rate was 86.4%.

Experiment 3——As shown in Fig. 1 (3), the tumor size in the mice given P-MSY was significantly smaller than in the control group at the 3rd, 4th, and 5th weeks after the transplantation, the growth being very gradual. The excised tumors weighed significantly less ($p<0.05$) than those of the control, and the tumor inhibition rate was 67.8% (Table I, 3).

Experiment 4——The tumor growth in the experimental group was very gradual, as in Fig. 1 (3). The tumor size at the 3rd, 4th, and 5th weeks after the transplantation was significantly suppressed as compared to the control, and the tumor inhibition rate was 72.8% (Table I, 4).

Experiment 5——As shown in Fig. 1 (5), the sarcoma-180 in the experimental mice showed about constant size at the 2nd, 3rd, and 5th weeks after the transplantation, indicating a marked inhibition as compared to the control. The tumor disappeared in 3 of 8 mice at the 5th week after the transplantation, and the tumor inhibition rate was 82.7% (Table I, 5). This experiment was done in order to compare the antitumor activity against Ehrlich carcinoma with that against sarcoma-180 using the same lot of sample as used for experiments 1—4.

Experiment 6——As shown in Fig. 1 (6), remarkable antitumor activity was found. The tumor disappeared in 4 of 6 mice, and the tumors excised from the remaining 2 mice were extremely small. The tumor inhibition rate was 95.4% (Table I, 6). Antitumor activity

against sarcoma-180 was examined again using Swiss–Webster mice.

In ICR–SLC strain mice, tumor growth was relatively rapid and the suppressive effect was not as marked as in Swiss–Webster strain mice.

In the preceding work,[5] P-MSY was found to have a strong suppressive effect on solid sarcoma-180 in Swiss–Webster mice. A marked antitumor effect of P-MSY was again found in the present experiments on solid Ehrlich carcinoma in ICR–SLC strain mice by either subcutaneous or intraperitoneal injection before or after transplantation of the tumor. Further, the effect was again confirmed to be most marked when P-MSY was administered before transplantation. It is not surprising that the present results are similar to those of the previous experiment since Ehrlich cacinoma is an allogeneic tumor, like sarcoma-180 used previously.

Effect on Ascites Tumor (Life-Proloning Effect)

A life-prolonging effect of P-MSY on mice bearing ascites tumor was not expected on the basis of its immunological behavior. However, we examined ascites tumor by using Ehrlich carcinoma and sarcoma-180.

Experiment 7——The mean survival period was 19.3 d against 16.2 d in the control group, and T/C was 119%, indicating that the treatment was ineffective.

Experiment 8——At doses A and B, the values of T/C were 91 and 106%, respectively, and thus the treatments were ineffective.

Experiment 9——The mean survival period was 19.4 d against 14.1 d in the control, and T/C was 138% (Fig. 2). This treatment was effective.

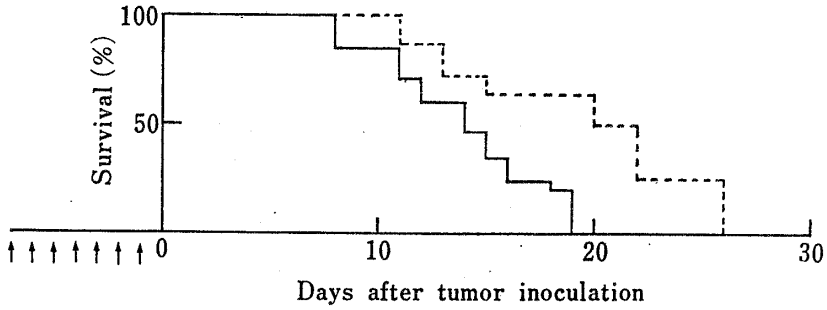

Fig. 2. Effect of P–MSY on the Survival of Mice bearing Ascites Sarcoma-180 (Expt. 9)

ICR–SLC strain mice were inoculated intraperitoneally with tumor cells ($4–8 \times 10^6$ cells/head) on day 0.
·····P–MSY 1 μg/head/d×7 SC ($n=8$).
——Control ($n=8$).

In the case of ascites tumor, P-MSY was effective in prolonging the life of mice when it was administered before the tumor transplantation, but not after. This suggests that P–MSY does not exert a direct cytolytic action on cancer cells but that its effect is mediated through the host. Some days may be required, therefore, to promote the host's immunity to a level sufficient to affect the tumor cells. Consequently, it was considered that mice die of rapid multiplication of the ascites tumor cells before the suppressive effect of P-MSY appears when P-MSY is administered after tumor transplantation. Life-prolongation was observed, in contrast, when P-MSY was admisistered before the transplantation. Similar results were obtained in the experiment on solid tumors with pre-administration of P-MSY.

Electrophoretic patterns of mouse serum obtained by disc electrophoresis, electrofocusing, and gradient polyacrylamide gel electrophoresis after the administration of P-MSY are shown in Fig. 3a—c.

In disc electrophoresis of the serum from mice whose tumor had disappeared after the administration of P-MSY, there was an increase in the band at the position of globulin, as

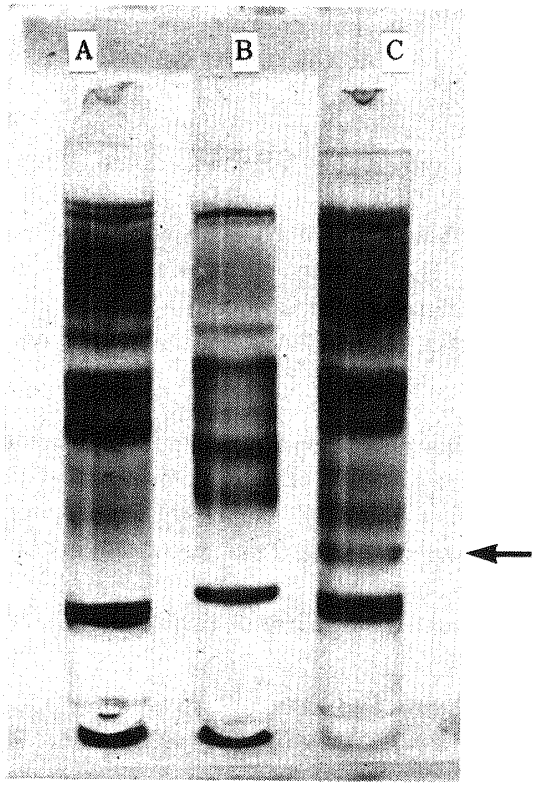

Fig. 3a. Disc Electrophorograms of Serum Samples from P-MSY-Treated Mice

A: tumor-bearing mice, 5th week after inoculation.
B: normal mice (tumor free)
C: cured mice.

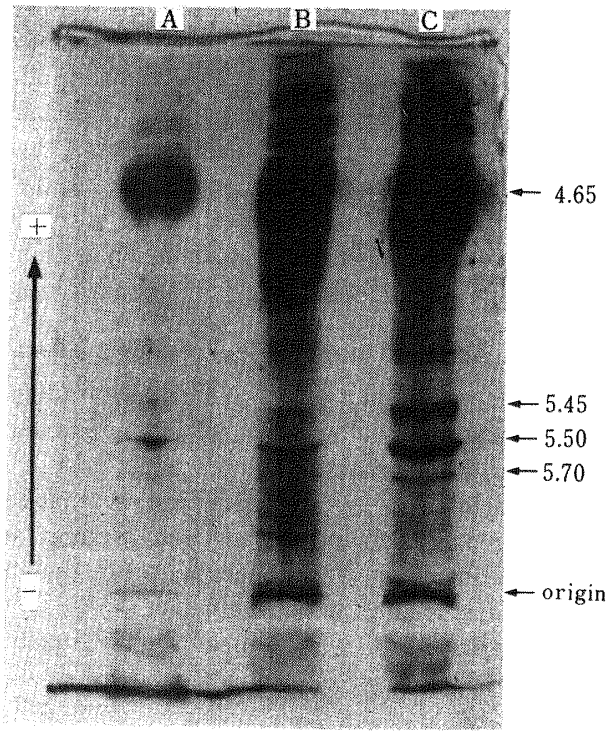

Fig. 3b. Electrofocusing on Polyacrylamide Gel Plate

pH range: pH 4.0—6.5.
A: tumor-bearing mice, 5th week after inoculation.
B: normal mice (tumor free).
C: cured mice.

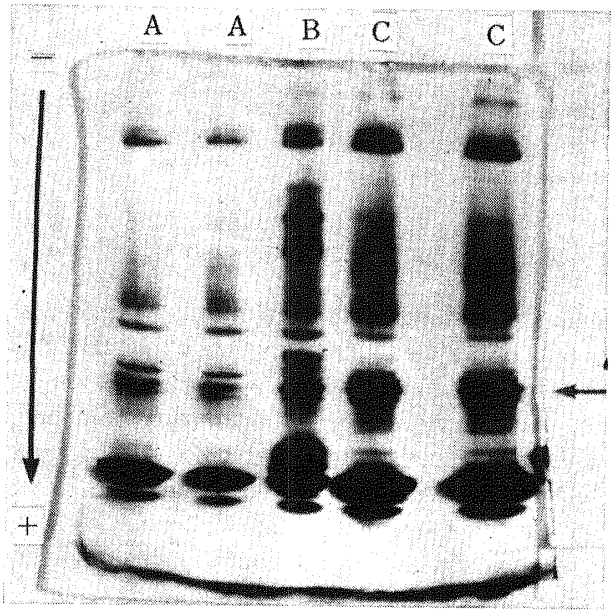

Fig. 3c. Polyacrylamide Gradient Gel Pattern of Serum from P-MSY-Treated Mice

A: tumor-bearing mice, 5th week after inoculation.
B: normal mice (tumor free).
C: cured mice.

compared to normal and tumor-bearing mice (Fig. 3a). The electrofocusing pattern of the serum from cured mice showed increases in the serum proteins at pH 5.45, 5.50, and 5.70 (Fig. 3b). Maeda *et al.*[9] reported that three dark bands of globulin appeared when serum from mice administered an antitumor polysaccharide, *e.g.* lentinan,[9,10,14,15] or a streptococcal preparation, OK-432 (Picibanil),[16] was subjected to gradient polyacrylamide gel electrophoresis In our experiment, under similar conditions, a dark band appeared in the position of α-globulin (Fig. 3c, indicated by an arrow) when the serum from mice administered P-MSY was electrophoresed. An increase in the proteins was suggested by Mizuno[17] to be correlated closely with the host's resistance to cancer. The increase in serum proteins after the administration of P-MSY may be related to the appearance of antitumor action as a result of promotion of the host's immu-

nity.

The maximal increases in the three bands of serum protein components were observed at 4 d after the administration of lentinan, 4—10 d after pachymaran, and 7 d after zymosan, and were followed by gradual decreases.[9] In our preliminary experiments using P-MSY, a distinct increase in protein components in the serum from mice cured by administration of P-MSY was found at the 5th week after tumor transplantation as compared to the sera from normal or tumor-bearing mice. The increase in the protein components seems to begin about one week after the discontinuation of P-MSY administration. These observations may be characteristic of P-MSY.

In the experiment on delayed-type skin reaction, the administration of P-MSY to normal mice failed to give a significantly different skin reaction from that in control mice (Table II, group 1). In the case of tumor-bearing mice, the reactions in the mice transplanted with Ehrlich carcinoma (group 2) or sarcoma-180 (group 3) were significantly lower ($p<0.01$) than those of the control normal mice (group 1), indicating the impairment of cellular immunity in tumor-bearing mice.[18] With both kinds of tumor, the lowering of the reaction was significantly inhibited by the administration of P-MSY. Therefore, the tumor suppression by P-MSY was probably due to the restoration of the host's immunity.

Table II. Effect of P-MSY on Delayed Hypersensitivity in Normal and Solid-Tumor-Bearing Mice

	Group No.		No. of mice	Ear thickness (10^{-3} cm) 1st delayed hypersensitivity reaction	Ear thickness (10^{-3} cm) 2nd delayed hypersensitivity reaction
Normal mice	1	P-MSY	8	32.1±2.14	35.3±2.12
		Control	7	34.0±1.61	35.8±1.87
Tumor-bearing mice	2	P-MSY	6	34.6±3.64[a]	39.8±3.67[b]
		Control	5	23.5±0.42	24.6±1.11
	3	P-MSY	7	32.7±1.53[b]	34.3±2.02[b]
		Control	7	26.4±0.85	25.8±1.32

Group 2: Ehrlich carcinoma.
Group 3: Sarcoma-180.
a) Significantly different ffom control, $p<0.05$.
b) Significantly different from control, $p<0.01$.

Thus, the antitumor activity of P-MSY was confirmed in the present experiments, and its effect is considered to be due to the potentiation of the host's immunity as evidenced by the electrophoretic patterns of serum protein and the results of the delayed-type skin reaction. These properties of P-MSY are not identical with those of other antitumor substances such as BCG, OK-432, and lentinan. For example, OK-432 is thought to contain intracellular components, and an increase in plaque-forming cells has been reported when it was administered to 2-month-old mature mice.[16] However, according to Luckey,[19] endotoxin does not increase plaque-forming cells in newborn mice. In contrast, P-MSY does elevate plaque-forming cells[4,19] in newborn mice. While OK-342, BCG, and lentinan are reported to activate the immunity in immunologically mature animals as adjuvants,[20-22] P-MSY may mature the immunity of immunologically immature mice (newborn), promote the immune competence and finally cause tumor suppression.

Acknowledgements The authors wish to express their thanks to prof. Harumi Okuyama for his helpful suggestions and linguistic advice. They are also grateful to Miss M. Nakashima for her technical assistance in the experimental work.

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1) Presented at the 98th Annual Meeting of the Pharmaceutical Society of Japan, Okayama, April 1978.

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