Chem. Pharm. Bull. 29(11)3298—3304(1981)

Effects on Antibody-Producing Cells and Peripheral Bloody Lymphocyte Subpopulations (T- and B-cells) of P-MSY, a Substance with Antitumor Activity from Bovine Parotid Gland[1)]

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(Received April 30, 1981)

The effects of an antitumor protein P-MSY on the numbers of IgM producing cells and IgG producing cells were investigated. Groups of ICR-SLC newborn mice were injected intraperitoneally with P-MSY in doses of 1 to 10 μg/animal and their immune response was assessed at 21 days of age by measuring the antibody production in response to ovine erythrocytes according to the method of Jerne *et al.* The group of mice administered P-MSY at a dose of 1 μg/animal showed significant increases of the numbers of both direct PFC (IgM-producing cells) and indirect PFC (IgG-producing cells). The number of direct PFC of the test group on day 4 was $962.0 \pm 153.8/10^6$ cells, compared with $453.0 \pm 77.16/10^6$ cells of the saline-treated control group. The number of indirect PFC of the test group on day 6 was $479.6 \pm 70.52/10^6$ cells compared with $192.8 \pm 32.14/10^6$ cells of the control group. Experiments were conducted to assess the effect of P-MSY on the peripheral blood lymphocyte subpopulations (T- and B-cells) in groups of newborn guinea pigs by identification and counting of T- and B-cells according to the microplate assay technique of Wilson *et al.* Guinea pigs dosed *i.p.* at the neonatal stage with 10 or 20 μg of P-MSY displayed a significant elevation of the T-cell percentage in peripheral blood with a significant relative decrease in the B-cell subpopulation ($p<0.05$).

Keywords——direct PFC; indirect PFC; antitumor protein; P-MSY; parotid; IgG producing cells; IgM producing cells; peripheral blood lymphocyte subpopulations (T- and B-cells); hypocalcemic protein

We previously reported the properties of some physiologically active proteins purified from bovine parotid[3)] and thymus.[4)] In the meantime, a hypocalcemic protein, P-MSY, was isolated from the mother liquor after separation of a fraction precipitating at pH 5.4 from bovine parotid extract.[5)] This substance has proven to be markedly active in suppressing the growth of solid form tumor of sarcome-180 and Ehrlich carcinoma cells.[6)] This antitumor effect was considered to be hostmediated, involving the enhancement of immunologic responsiveness.[7)] Suzuki has recently reported on the structures of the N- and C-terminals of this compound.[8)]

The purpose of this study was to ascertain what types of antibody-producing cells might be involved in the mechanisms of antitumor activity of P-MSY by assessment of the influence of the substance on the numbers of mouse spleen direct (IgM-producing) and indirect (IgG-producing) plaque-forming cells (PFC). Experiments were performed to assess the effect of P-MSY on the peripheral blood lymphocyte subpopulations (T- and B-cells) in guinea pigs and to explore its relation to the antitumor activitity of the substance.

Materials and Methods

Materials——The sample P-MSY (purified preparation) and its intermediate (PAIA) obtained in the course of purification were prepared according to the method described previously.[5)] Namely, the supernatant of an aqueous extract from the bovine parotid gland at pH 5.4 was mixed with acetone to give 50% (w/w) concentration and the resulting precipitate was dried with acetone. The acetone-dried powder was extracted with glacial acetic acid and the extract (PAIA) was then purified by DEAE-cellulose chromatography followed by gel filtration on a column of Sephadex G-100 and the purified P-MSY was obtained. The hypocalcemic activity in rabbits was determined by the previously reported procedure[9)] and the lowering

rates were 9.99±2.04% with PAIA (0.1 mg/kg *i.v.*) and 13.57±1.61% with P-MSY (10 μg/kg *i.v.*), both being statistically significant at the 1% level as compared to a saline-treated group.

Animals——Litters of neonates from inbred colonies of ICR-SLC mice and from those of Hartley strain guinea pigs maintained at this laboratory were used for PFC assays and determination of lymphocyte subpopulations, respectively.

Experimental Procedure

I. Effect on Antibody-producing Cells——The litter was divided into two groups; one group was injected intraperitoneally with 20 μg/animal of bovine serum albumin, 80 μg/animal of PAIA, or 1, 2.5, 5, or 10 μg/animal of P-MSY, and the other group was given saline as a control. The mice of both groups received at 21 days of age an injection of sheep erythrocytes (SRBC) as an antigen by the method of Jerne *et al.*[10] On day 0 and from days 4 to 12 after immunization the numbers of splenic direct and indirect PFC were determined (expressed as numbers per 10^6 splenic cells) and the results for the test group and control groups were compared. The assay procedures are summarized in Fig. 1.

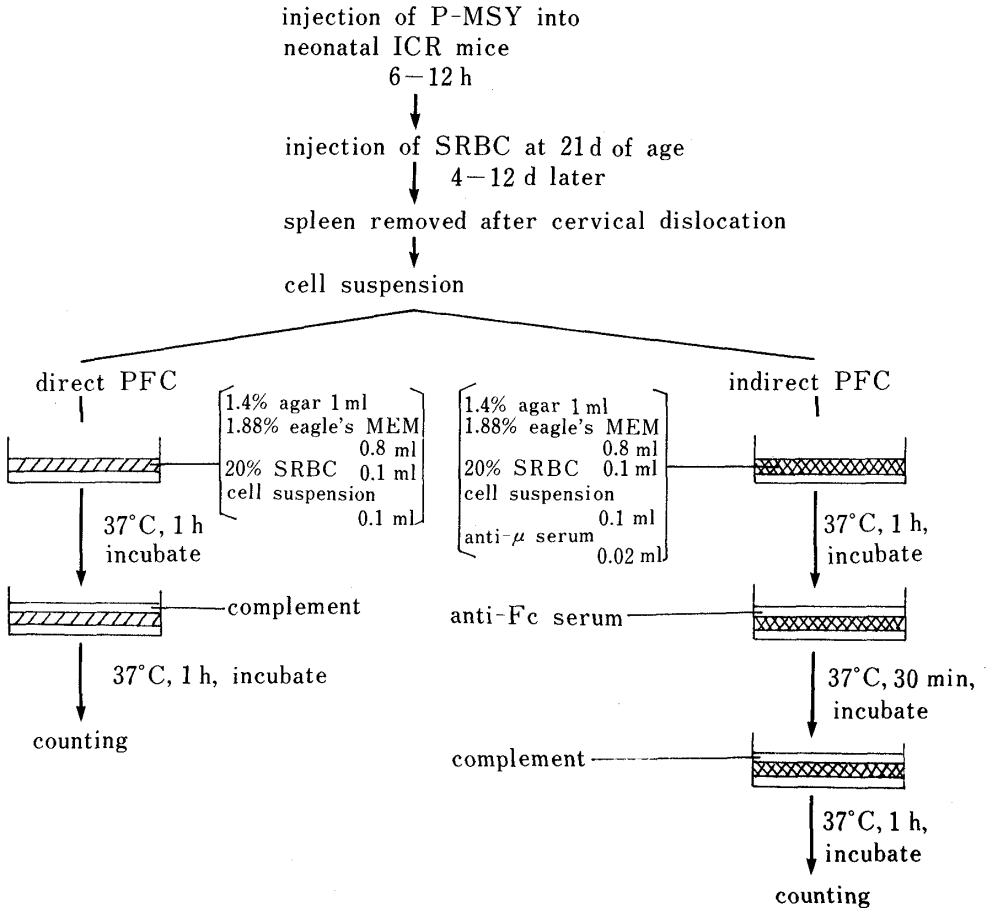

Fig. 1. Flow Chart of the PFC Assays

i) Preparation and Sensitization of Spleen Cells: Mice in the test and control groups were intraperitoneally injected for immunization with 20% SRBC (for complement fixation; Kyokuto Pharmaceuticals, Tokyo) in physiological saline at a dose of 0.4 ml/10 g B.W. Animals were sacrificed by cervical vertebral dislocation at days 4—12 of sensitization, and the spleen was removed, disrupted into cells aseptically in Eagle's medium (Nissui Pharmaceutical Co., Ltd., Tokyo) and passed through a metal sieve (200 to 400 mesh). The cell suspension was centrifuged at 1500 rpm for 3 min, and the supernatant was discarded. After several cycles of washing by centrifugation, the cell deposit was resuspended in fresh Eagle's medium.

ii) Plaque-forming Cell (PFC) Counting: For underlay, Difco Noble agar (1.4%) in Eagle's medium was added to a 5 cm plastic Petri dish, and allowed to solidify. One ml of soft agar solution (1.4% Difco Noble agar), maintained at 45°C, was mixed with 0.8 ml of 2×concentrated Eagle's medium, 0.1 ml of 20% SRBC suspension and 0.1 ml of cell suspension. The mixture (0.5 ml) was immediately pipetted on the 1.4% agar in a Petri dish, which was kept at 45°C. After incubation at 37°C for 1 h, guinea pig serum complement was added to the overlay, and the number of plaques formed was counted (direct PFC assay for IgM-producing cells).

The procedure for indirect PFC assay (for IgG-producing cells)[11] was essentially the same as in the case

of the direct PFC assay except in the following respects. A soft agar overlay containing spleen cell suspension was mixed with 20 μl of rabbit anti-mouse μ chain serum (Medical–Biological Research Laboratories, Inc.) and poured over the underlay. After incubation at 37°C for 1 h, rabbit anti-mouse IgG(Fc) (Medical–Biological Research Laboratories, Inc.) was added to the overlay and further incubated at 37°C for 30 min. One ml of 1: 10 dilution of guinea pig serum in Eagle's medium was added to each plate then the plates were incubated at 37°C for 1 h, and the numbers of plaques of hemolysis (PFC) were counted.

II. Effect on Peripheral Blood Lymphocyte Subpopulations (T- and B-Cells)——Identification and counting of T- and B-cells were carried out by the microplate technique[12] of Wilson *et al.*,[13] using the JIMCO microassay kit (JIMCO Kotai–Kaseikogyo, Ltd.). Groups of newborn guinea pigs at 6—12 h after birth were injected intraperitoneally with 80 μg/mouse of PAIA or 10 or 20 μg/mouse of P-MSY. The animals were bled by cardiac puncture on the 14th day after the injection, and lymphocytes separated from the blood were assayed to determine the percentages of their two immunocompetent subpopulations by way of rosette formations with indicator cells in plastic plates with viable lymphocytes adhering to their surface *via* poly-L-lysine (PLL), making use of their properties of binding sheep erythrocytes (E cells) and having surface receptors for complement.

i) Separation and Adhesion of Lymphocytes: A 2 ml sample of heparinized blood was drawn from a guinea pig by cardiac puncture. Lymphocytes were purified from the blood by density gradient centrifugation[14] on Ficoll-Conray, and suspended in an appropriate medium at a concentration of 1×10^6 cells per ml. The lymphocyte suspension was then pipetted in 1 μl aliquots into wells of three PLL-treated microtest plates, for T-cell, B-cell and peroxidase stain assays, respectively. After settlement of cells, fetal calf serum (FCS) was added (10 μl to each well of the plates for T- and B-cell assays, and 0.5 μl to the plate for peroxidase stain assay), and the plates were all incubated at room temperature for 30 min.

ii) Sheep Erythrocyte (E)-Binding Cells: After removal of FCS from the plate for T-cell assay, an indicator erythrocyte E suspension[15] (2×10^8 rabbit erythrocytes per ml of FCS) was added (10 μl to each well) and the plate was incubated at room temperature for 90 minutes then at 4°C overnight. The cells were washed gently with phosphate-buffered saline (PBS), fixed and stained with a 0.005% Brilliant Cresyl Blue–0.25% glutaraldehyde solution in PBS, and the number of E-binding cells was counted with a microscope to calculate the percentage of the E-binding cells in a population of 300 to 400 nucleated cells.

iii) EAC (Erythrocyte–amboceptor–complement)-binding Cells: After removal of the FCS from the plate for B-cell assay, an indicator EAC suspension (complement–coated sheep red cell suspension) was added (10 μl to each well) and the plate was placed in a sufficiently humidified incubator at 37°C for 60 min and then at 4°C overnight. Subsequently, the cells were washed, fixed and stained in the same manner and with the same reagents described above, followed by counting of the number of EAC rosettes to calculate the percentage of EAC-binding cells.

iv) Detection of Peroxidase-positive Cells: To each well of the peroxidase stain assay plate with adhering lymphocytes, 10 μl of 0.4% benzidine solution (PSS) was added, and, after incubation for 1 min, PSS was removed and Giemsa stain solution added. The plate was examined microscopically to determine the percentage of peroxidase-positive cells, *i.e.* cells with brown granules, in a population of 300 to 400 nucleated cells.

Results and Discussion

The number of direct PFC of the test groups injected *i.p.*, with P-MSY in doses of 1 or 5 μg/mouse are compared with those of the control groups in Fig. 2. As can be seen in Fig. 2, the test groups with treated P-MSY displayed the highest direct PFC counts on day 4; the number fell slightly on day 5 and declined from day 6 onwards. The numbers of direct PFC of the test groups were approximately twice those of the control group. On days 10 to 12 plaques were still demonstrable in the direct assays, but the difference between the numbers of the test and control groups was not significant.

The results of indirect PFC assays with the spleen cells after treatment with P-MSY or saline are shown in Fig. 3. The numbers of indirect PFC showed a peak on day 5 (dose of 5 μg/mouse) or 6 (dose of 1 μg/mouse) and the peaks were slightly delayed as compared to those of direct PFC. The numbers in the test groups at doses of 1 μg and 5 μg/mouse were approximately twice those in the control group. The differences between the test and control groups were statistically significant as shown in Fig. 3.

Analyses of the data for dose–response relationships of maximal direct PFC on days 4 to 5 and indirect PFC on days 5 to are shown in Table I and II, respectively. At all four points at doses from 1 to 10 μg/animal, the test groups treated with P-MSY showed significant

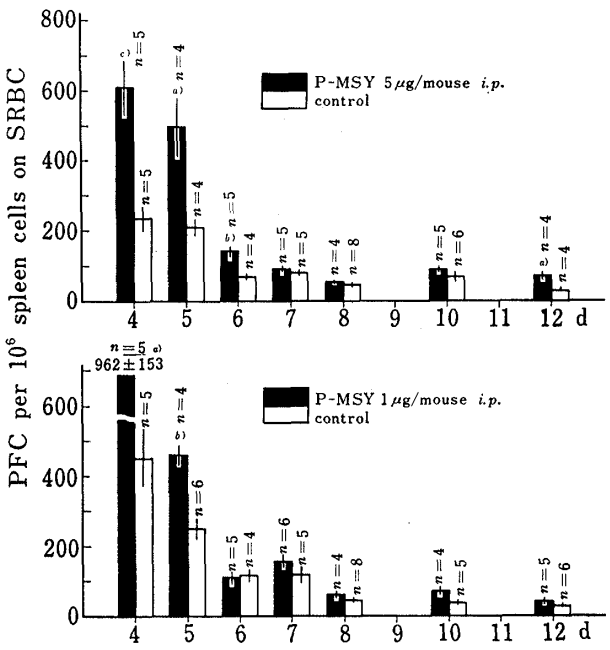

Fig. 2. Direct Plaque-forming Cells of ICR Mice given P-MSY and SRBC Antigen Injection

Each bar of the graph represents a mean value. *a*) significantly different from control $p<0.05$; *b*) significantly different from control $p<0.01$; *c*) significantly different from control $p<0.001$

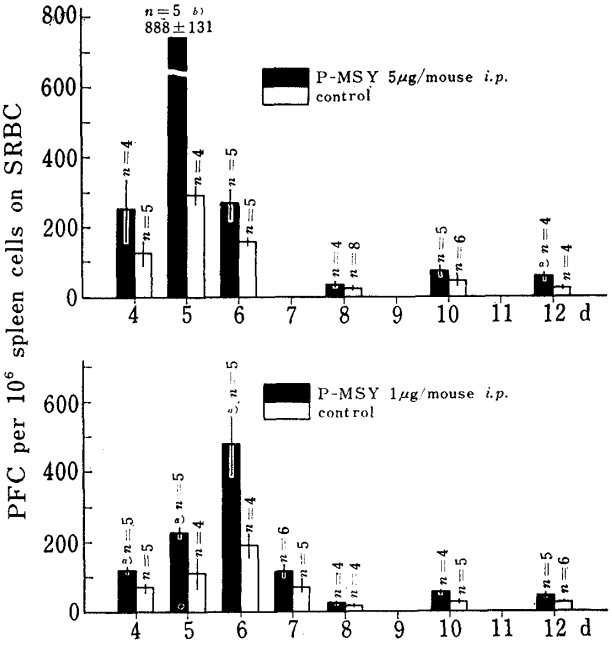

Fig. 3. Indirect Plaque-forming Cells of ICR Mice given P-MSY and SRBC Antigen Injection

Each bar of the graph represents a mean value. *a*) significantly different from control $p<0.05$; *b*) significantly different from control $p<0.01$

TABLE I. Formation of Direct Plaque-forming Cells (DPFC) against Sheep Erythrocytes in the Spleen of Mice treated with P-MSY

Days after the immunization	Treatment	Dose (μg/mouse) *i.p.*	No. of mice	DPFC/10^6 sp. cells (mean±SE)	Changes (to control)
4	P-MSY	10	5	508.0±88.87	+1.41
	Control[a)]		5	358.1±34.89	
	P-MSY	5	5	610.6±58.85[b)]	+2.52
	Control[a)]		5	242.1±39.97	
	P-MSY	2.5	6	682.0±99.61[c)]	+1.90
	Control[a)]		5	358.1±34.89	
	P-MSY	1	5	962.0±153.8[c)]	+2.12
	Control[a)]		5	453.0±77.16	
5	P-MSY	10	5	556.7±43.86[b)]	+2.20
	Control[a)]		6	252.4±33.75	
	P-MSY	5	4	504.1±82.18[c)]	+2.36
	Control[a)]		4	213.2±20.82	
	P-MSY	2.5	4	388.1±76.49	+1.60
	Control[a)]		5	242.1±39.97	
	P-MSY	1	4	462.6±35.11[d)]	+1.83
	Control[a)]		6	252.4±33.75	

a) Control: saline, 0.1 ml/mouse *i.p.*
b) Significantly different from control, $p<0.001$.
c) Significantly different from control, $p<0.05$.
d) Significantly different from control, $p<0.01$.

Table II. Formation of Indirect Plaque-forming Cells (IPFC) against Sheep Erythrocytes in the Spleen of Mice treated with P-MSY

Days after the immunization	Treatment	Dose (μg/mouse) *i.p.*	No. of mice	IPFC/10^6 sp. cells (mean±SE)	Changes (to control)
5	P-MSY	10	5	538.0±45.11[a]	+2.66
	Control[b]		5	201.8±33.10	
	P-MSY	5	5	888.1±131.58[c]	+2.97
	Control[b]		4	298.7±55.91	
	P-MSY	2.5	6	602.7±85.40[c]	+2.98
	Control[b]		5	201.8±33.10	
	P-MSY	1	5	234.2±18.18[d]	+2.06
	Control[b]		4	113.2±36.81	
6	PAIA	80	6	695.0±135.01[d]	+2.05
	Control[b]		5	337.9±69.81	
	P-MSY	5	5	267.6±43.38	+1.64
	Control[b]		5	162.4±14.57	
	P-MSY	2.5	5	500.0±46.40[c]	+2.01
	Control[b]		5	261.0±33.72	
	P-MSY	1	5	479.6±70.52[d]	+2.48
	Control[b]		4	192.8±32.14	
	BSA[e]	20	5	225.6±51.33	+1.43
	Control[b]		6	157.0±35.03	

a) Significantly different from control, $p<0.001$.
b) Control: saline, 0.1 ml/mouse *i.p.*
c) Significantly different from control, $p<0.01$.
d) Significantly different from control, $p<0.05$.
e) BSA: Bovine serum albumin.

changes. Increases of 1.9- to 2.9-fold in the number of both direct and indirect PFC compared to the control level were found. However, the increase was not significantly dose-related. The group injected with PAIA *i.p.* in a dose of 80 μg/mouse showed a significant two-fold increase in the number of indirect PFC as compared to the control group. PAIA (80 μg) contained about 0.3 μg of P-MSY.[5] The dose levels of P-MSY employed in these experiments had been determined on the basis of the data obtained in the previous study of the effect of P-MSY on Ehrlich solid tumor in adult mice receiving a single dose of 20 μg/animal[6] and by reference to the body weight relation to neonatal mice. Although further experiments with lower doses seem to be necessary, the finding that the group of neonatal mice injected *i.p.* with 20 μg/animal of bovine serum albumin exhibited a 1.4-fold increase of indirect PFC, as shown in Table II, seems to suggest that there is no distinct dose-response relationship even at further reduced dosage levels.

It is generally agreed that the ontogenic maturation of the immunolymphoid system takes a course of development with a sequence of IgD→IgM→IgG and IgA in mammals.[16] It is also universally recognized that, in response to injection of SRBC as an antigen, IgM-producing cells proliferate first on the fourth day of immunization and subsequently IgG-producing cells proliferate.[17] Thus, it seemed interesting to explore the possibility of acceleration by administration of P-MSY in the ontogenic maturation of the lymphoid system, by way of indirect PFC assay for IgG producing cells, besides assay for IgM-producing cells. A significant, 2.98-fold increase of the number of indirect PFC occurred in the group treated with P-MSY at a dose of 2.5 μg/animal, as compared with the saline-treated control group. Therefore, P-MSY may potentially accelerate the ontogenic maturation of the immunolymphoid system.

In the experiment to explore the influence of P-MSY on the lymphocyte subpopulation (T- and B-cells) of newborn guinea pig peripheral blood, the control group dosed *i.p.* with

0.5 ml of physiological saline ($n=5$) showed T- and B-cell percentages of 64.3±1.3 and 35.6±1.4%, respectively, at 14 days of age, *i.e.*, a greater subpopulation of T-cells than of B-cells. In a group injected *i.p.* with 80 μg of PAIA ($n=5$), on the other hand, the percentage of T-cells was 75.8±0.3% and that of B-cells was 24.2±0.4%. A significant increase of T-cells and decrease of B-cells were seen in this group, as compared with the control group ($p<0.001$). Similarly, a significant elevation of the percentage of T-cells with a significant relative fall of the B-cell percentage occurred in groups treated *i.p.* with 10 μg of P-MSY ($n=2$; T-cell, 74.8±2.3%; B-cell, 26.0±1.5%) ($p<0.01$) or with 20 μg of P-MSY ($n=4$,; T-cell, 79.3±3.9%; B-cell, 21.1±3.8%) ($p<0.05$). An untreated group of neonates ($n=3$) displayed values of 59.2±4.3 and 40.6±4.6%, respectively, for the corresponding parameters.

The data demonstrate a noticeable increase of the T-cell subpopulation in the peripheral blood of guinea pigs at 14 days of age following administration of PAIA or P-MSY at the neonatal stage. This period of T-cell increase generally coincides with that at which an increase of lymphocytes in terms of L/P ratio and an increase of PFC value, *i.e.* the number of antibody-producing cells in the B-cell population, were noted in mice in a previous study. Although inter-species differences do exist, it is very likely that PAIA and P-MSY have the ability to increase the number of T-cells in the peripheral blood and thus enhance cell-mediated immune function. According to Friedman *et al.*,[18] thymic precursor cells are derived from stem cells in the bone marrow, and are liberated into the blood stream and gain entrance to the thymus where they differentiate into immunologically competent T-cells, which then migrate to peripheral lymphoid organs. Moreover, they function as helpers to stimulate differentiation and proliferation of antibody-producing precursor cells (B-cells) with a consequent increase of antibody-producing cells (a rise in PFC value). What facilitates this chain of events is generally presumed to be the thymic humoral factor (thymic hormone).

The increase in T-cell subpopulation in peripheral blood lymphocytes observed in this study following administration of P-MSY seems to be consequent upon stimulation of stem cell differentiation into immunocompetent T-cells. The data also suggest that the test substance enhanced the helper function of T-cells, which, in turn, stimulated the activity of B-cells.

Acknowledgement The author is deeply indebted to Dr. Takaharu Mizutani for valuable suggestions and encouragement throughout this investigation. The generous support of Dr. Ikukatsu Suzuki during this study is also gratefully acknowledged.

References and Notes

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