Antitumor Activity of Polygalactosamine Isolated from *Paecilomyces* sp. I-1 Strain

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The inhibitory effect of polygalactosamine (PF102), which was isolated from *Paecilomyces* sp. I-1 strain, on a syngeneic murine solid tumor and its antitumor mechanism were studied.

After an intravenous injection of PF102, 1 μ g/kg, an increase in cell mediated and humoral immunities in mice was observed and the growth inhibition of MM46 solid tumor *in vivo* was also evident. Macrophages induced by PF102 into the peritoneal cavity inhibited deoxyribonucleic acid synthesis of target cells. Moreover, PF102 caused a significant increase in the incorporation of 3 H-thymidine into the thymic cells and the culture supernatant of T lymphocytes, stimulated with PF102, exhibited a marked activation of the cytostatic effect of the peritoneal macrophages. Furthermore, this culture supernatant fluid was found to contain interferon (IFN). Therefore, the antitumor activity of PF102 might be due in part to the activation of the macrophage lineage cells by macrophage activating factor and/or IFN produced from T lymphocytes stimulated by PF102.

Keywords — macrophage; polygalactosamine; antitumor activity; *Paecilomyces* sp. I-1; interferon

Introduction

It is well known that various polysaccharides such as lentinan,^{1,2)} yeast mannan,^{3,4)} chitin, and chitosan⁵⁾ isolated from plants or animals exhibited inhibitory effects against allogeneic and/or syngeneic tumors. More recently, it was reported that *N*-acetylchitohexaose and chito-hexaose, the lower water-soluble homologs of chitin and chitosan, respectively, also exhibit antitumor activity against S-180 and MM46 solid tumors.⁶⁾ It has been previously shown that large amounts of polymers of glucosamine and their derivatives exist as chitin and chitosan in nature. However, there are not many polymers composed of galactosamine and only few reports have been published on galactosamine polymers produced by microbes.⁷⁻¹¹⁾

Polygalactosamine (PF102) investigated in the present study is a polysaccharide composed of D-galactosamine, and 80% of the galactosamine residues are *N*-unsubstituted and 7 to 8% are *N*-acetylated.¹²⁾ *N*-unsubstituted galactosamine residues are mainly linked by $\alpha(1\rightarrow4)$ linkages, with small amounts of $\alpha(1\rightarrow2)$ and/or $\alpha(1\rightarrow6)$ linkages in the polymer.¹²⁾ Because PF102 resembles chitosan chemically and func-

tionally, we thought that PF102 might have an antitumor effect similar to that of chitosan. Therefore, we carried out an investigation to study of the antitumor effect of PF102. The present report shows that polygalactosamine which was isolated from the culture supernatant fluid of *Paecilomyces* sp. I-1 strain, PF102, exhibited a marked antitumor activity against syngeneic tumor, MM46, in C3H/HeSlc mice.

Materials and Methods

Mice — Inbred C3H/HeSlc male mice, 4 to 8 weeks old, were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan.

Tumor — MM46 tumor cells were donated by Dr. T. Tachibana, Research Institute for Tuberculosis and Cancer, Tohoku University. The tumor cells were maintained continuously in ascites form in C3H/HeSlc mice in our laboratory.

PF102 — PF102, which was obtained from the culture supernatant fluid of *Paecilomyces* sp. I-1 strain, was supplied by Higeta Soy Sauce Co., Ltd., Choshi, Chiba, Japan.^{12,13)}

Delayed Type Hypersensitivity — The anti-

gen used for sensitization was sheep red blood cells (SRBC). A SRBC suspension of 1×10^8 cells suspended in 0.05 ml of saline was injected into one of the foot pads of each mouse. At the same time, 10 ng/kg to 1 mg/kg of PF102 were injected intravenously (i.v.) in some groups of mice and only saline in the control group. Four days after sensitization, a cell suspension of SRBC (1×10^8 cells/0.05 ml in saline) was injected into another foot pad of each mouse. Thereafter, the foot pads were examined for reaction after 24 h.

Plaque Forming Cells (PFC) — One $\mu\text{g/kg}$ of PF102 or saline was injected i.v., and 0, 5, 10, or 15 d after the administration of PF102 or saline, a cell suspension of SRBC (1×10^8 cells/0.1 ml in saline) was injected i.v. Then, 4 d after sensitization by SRBC, PFC reaction was examined by the method of Cunningham.¹⁴⁾

Growth Inhibitory Effect of PF102 against MM46 Solid Tumor — MM46 tumor cells (5×10^5 cells/0.1 ml in saline/mouse) were transplanted into the left groin of each mouse subcutaneously (s.c.). The administrations of PF102 to these mice were as follows: Group 1 was administered i.v. with saline on days 7, 8, 9, 10, 11, 14, 15, 16, 17 and 18 after the transplantation of tumor. Group 2 was administered i.v. with PF102, 1 $\mu\text{g/kg}$, on days 7, 8, 9, 10, 11, 14, 15, 16, 17; and 18. Group 3 received the same dose on days 7, 8, 9, 10, and 11; group 4 the same dose on days 7 and 14; and group 5 received the dose 14 d after the transplantation of tumor. Group 6 was administered i.v. with lentinan as a positive control, 10 mg/kg, on the 14th day. Then, tumor weight was measured 27 d after transplantation of tumor.

Preparation of Peritoneal Macrophages Induced by PF102 or Casein — PF102, 100 $\mu\text{g/kg}$, or 2% casein, 2 ml/mouse, was injected intraperitoneally (i.p.) and the peritoneal exudate cells (PEC) were obtained by lavage using 6 ml of Hanks' balanced salt solution (HBSS) on the third and fourth days after administration of PF102 and 2% casein, respectively. PEC were washed 2 times with HBSS, and these preparations of PEC which were examined by May-Giemsa stain were found to contain more than 95% macrophages.

Cytostatic Effect of Macrophages Induced by PF102 — Cytostatic effect was examined by the study of inhibition of ^3H -thymidine incorporation into tumor cells. One $\times 10^5$ or 2×10^5 of peritoneal macrophages induced by PF102 or 2% casein were placed into wells of 96 well flat-bottomed microplates (Becton Dickinson & Co., Oxnard, CA, U. S. A.). After incubation at 37°C for 1 h in a 5% CO_2 incubator, nonadherent cells were removed by washing with RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and 200 μl of MM46 tumor cells (1×10^5 cells/ml) suspended in RPMI-1640 medium containing 5% fetal bovine serum (FBS) were added into each well. Then, all cultures were incubated at 37°C for 24 h in a 5% CO_2 incubator. During the last 6 h of culture, ^3H -thymidine, 0.4 $\mu\text{Ci/well}$, was added. After incubation, these cells were harvested using glass fiber filters by means of an automated sampler (Labo Mash, Labo Science Co., Ltd., Tokyo, Japan) and the radioactivity incorporated into target cells collected on filters was counted by means of a Beckmann LS-7800 liquid scintillation counter (Beckmann Instruments Inc., Palo Alto, U. S. A.).

Effect of PF102 on Proliferation of Thymic Cells — This assay was performed by the modified method of Mikami *et al.*¹⁵⁾ Briefly, the thymus obtained from normal mice was homogenized in RPMI-1640 medium and the cell suspension was washed 2 times with RPMI-1640 medium. One hundred μl of the cell suspension (1×10^7 cells/ml in RPMI-1640 medium containing 5% FBS) were placed into wells of 96 well round-bottomed microplates (A/S Nunc, Roskilde, Denmark). An equal volume of PF102 solution or Concanavalin A solution (Con A; Sigma Chemical Co., St. Louis, MO, U. S. A.) in RPMI-1640 containing 5% FBS was added to each well containing the thymic cell suspension. The final concentrations of PF102 and Con A were adjusted to 1.25 ng/ml to 12.5 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$, respectively. Thereafter, all cultures were incubated at 37°C for 48 h in a 5% CO_2 incubator. During the last 6 h of culture, ^3H -thymidine, 0.4 $\mu\text{Ci/well}$, was added. After incubation, the radioactivity incorporated in thymic cells was measured by means

of a liquid scintillation counter.

Preparation of Culture Supernatant Fluid of Splenic T Lymphocytes — The culture supernatant fluids of splenic T lymphocytes incubated with PF102, Con A, or lipopolysaccharide (LPS) were examined for macrophage activating factor (MAF). The preparation of the culture supernatant fluid was as follows: The spleen obtained from normal mice was homogenized in RPMI-1640 medium and after hemolysis by a NH_4Cl solution, the cell suspension was washed twice with RPMI-1640 medium. Then, splenic T lymphocytes were prepared by passage over a column of nylon wool, 1.5×4.5 cm (Wako Pure Chemical Industries Ltd., Tokyo, Japan), using the method of Julius *et al.*¹⁶⁾ The purity of T lymphocytes was 95.4% as determined by fluorescein isothiocyanate-conjugated anti-Thy-1 antibody. One hundred μl of the T lymphocyte suspension (1×10^7 cells/ml in RPMI-1640 medium containing 5% FBS) were placed into wells of 96 well round-bottomed microplates, followed by an equal volume of PF102, Con A, or LPS. The cells were incubated at 37°C for 48 h in a 5% CO_2 incubator, and the culture supernatant fluids were collected, centrifuged and stored at -20°C .

Activating Ability of Macrophages by Culture Supernatant Fluid of Splenic T Lymphocytes Incubated with PF102 — Fifty μl of the suspension of the peritoneal macrophages induced by 2% casein (4×10^6 cells/ml in RPMI-1640 medium containing 5% FBS) and 50 μl of MM46 tumor cell suspension (2×10^5 cells/ml in RPMI-1640 medium containing 5% FBS) were placed into wells of 96 well flat-

bottomed microplates, and 100 μl of the culture supernatant fluid of splenic T lymphocyte were added. These cells were incubated at 37°C for 24 h in a 5% CO_2 incubator. During the last 6 h, 0.4 μCi /well of ^3H -thymidine was added. After incubation, the radioactivity incorporated in the target cells was measured by means of liquid scintillation counter.

Interferon (IFN) Assay — IFN was assayed by measuring the reduction of the cytopathic effect (CPE) of vesicular stomatitis virus on L929 cell. The IFN titer is expressed as the reciprocal of the highest serial two-fold dilution of the sample showing 50% reduction of CPE. A standard reference mouse IFN- α/β (NIH standard G-002-904-511) was included in this assay. One unit (U) of IFN in the assay corresponds to 0.48 U of the reference IFN.

Results

Effect of PF102 on Cell Mediated Immunity

In order to examine whether PF102 enhanced cell mediated immunity in mice and to determine the optimal dose of PF102 administration, delayed type hypersensitivity (DTH) response against a heterologous antigen, SRBC, was investigated. In comparison of administration routes of PF102, we have established that i.v. administration of PF102 was the best among i.v., i.p., or s.c. administration (data not shown). As shown in Table I, it is clear that 1 $\mu\text{g}/\text{kg}$ of PF102 was most efficient in enhancing cell mediated immunity. However, it was also observed that 1 mg/kg of PF102 inhibited cell mediated immunity.

TABLE I. Effect of PF102 on DTH Induced by SRBC in Mice

Dose of PF102	Swelling ($\times 0.1$ mm)	Relative %
	8.3 ± 0.9 ^{a)}	0
10 ng/kg	10.0 ± 0.7	20.5
100 ng/kg	9.2 ± 0.7	10.8
1 $\mu\text{g}/\text{kg}$	12.1 ± 0.1	45.8
10 $\mu\text{g}/\text{kg}$	11.1 ± 1.5	33.7
100 $\mu\text{g}/\text{kg}$	10.3 ± 0.2	24.1
1 mg/kg	5.4 ± 0.9	-34.9

^{a)} These values were expressed as means $\pm$ S.E.

TABLE II. Effect of PF102 on Plaque Forming Cells against SRBC in Mice

No. of exp.	Time after PF102 administration	PFC/spleen	Relative %
1	Control	37600 ± 4115	
	4 d	49917 ± 22268	32.8
2	Control	36899 ± 4511	
	9 d	74711 ± 18632	102.5
3	Control	14245 ± 3228	
	14 d	26904 ± 4332	88.9
4	Control	14701 ± 6389	
	19 d	29654 ± 1757	101.7

TABLE III. Growth Inhibitory Effect of PF102 against MM46 Solid Tumor in Mice

No. of group	Schedules of PF102 administration after tumor implantation (d)	Tumor weight (g)	Inhibition %
1		5.07 ± 0.45 ^{a)}	
2	7, 8, 9, 10, 11, 14, 15, 16, 17, 18	3.82 ± 0.31	24.7
3	7, 8, 9, 10, 11	3.57 ± 0.61	29.6
4	7, 14	2.37 ± 0.60	53.2
5	14	1.91 ± 0.53	62.3
6	14 (Lentianan 10 mg/kg)	2.29 ± 0.65	54.8

^{a)} These values were expressed as means ± S.E. of seven mice.

TABLE IV. Cytostatic Effect of Peritoneal Macrophages Induced by PF102 against MM46 Tumor Cells

Agents ^{a)}	Dose	Effector: target	dpm ± S.E.	Cytostasis %
2% casein	2 ml/mouse	10 : 1	149673 ± 6101	
PF102	100 µg/kg		64117 ± 4258	57.2
2% casein	2 ml/mouse	5 : 1	202820 ± 2296	
PF102	100 µg/kg		108674 ± 902	46.4

^{a)} Casein and PF102 were administered i.p., and peritoneal macrophages were obtained on 4 and 3 d after administration of casein and PF102, respectively.

Effect of PF102 on Humoral Immunity

An investigation of the acceleration of antibody production to heterologous antigen, SRBC, by PF102 using the method of Cunningham was performed. The administration conditions were 1 µg/kg and an i.v. route. As shown in Table II, the enhancing effect of humoral immunity was 32.8% and approximately 100% on 4 and 9 to 19 d, respectively, after the administration of PF102. The result clearly demonstrated that the effect of PF102 on the humoral immunity was durable.

Inhibition of Growth of MM46 Solid Tumor by PF102

Table III shows the effect of PF102 on MM46 solid tumor in C3H/HeSlc mice using various schedules of administration. Seven mice/group were used and lentinan was used as a positive control. As shown in Table III, the administration of PF102 exhibited a significant growth inhibition of MM46 solid tumor in most of the administered groups. Additionally, it was obvious that a single administration at 14 d, after the transplantation of MM46 tumor cells, had the

TABLE V. Effect of PF102 on Proliferation of Thymic Cells *in Vitro*

Agents	Concentration	dpm $\pm$ S. E.	S.I. ^{a)}
Control		404 $\pm$ 63	
Con A	10 μ g/ml	8864 $\pm$ 1901	21.9
PF102	1.25 ng/ml	924 $\pm$ 207	2.3
	12.5 ng/ml	1494 $\pm$ 207	3.7
	125 ng/ml	1494 $\pm$ 275	3.7
	1.25 μ g/ml	5719 $\pm$ 788	14.2
	12.5 μ g/ml	838 $\pm$ 141	2.1

a) Stimulation index.

TABLE VI. Effect of Culture Supernatant Fluids of Splenic T Lymphocytes on Cytostatic Effect of Mouse Peritoneal Macrophages against MM46 Tumor Cells ^{a)}

Treatment of T lymphocytes	Concentration	dpm $\pm$ S. E.	Inhibition %
None		898 $\pm$ 53 ^{b)}	0
LPS	5 μ g/ml	829 $\pm$ 53	7.7
PF102	1.25 μ g/ml	355 $\pm$ 68	60.5
Con A	10 μ g/ml	99 $\pm$ 22	89.0

a) Effector macrophages induced by 2% casein were used in this assay. b) The ratio of effector to target cells was 20 : 1.

strongest effect.

Cytostatic Effect of Macrophages Induced by PF102 against MM46 Tumor Cells

In order to determine whether the macrophages induced by PF102 inhibited deoxyribonucleic acid (DNA) synthesis of MM46 tumor cells *in vitro*, the incorporation of ³H-thymidine into the MM46 tumor cells incubated with the macrophages induced by PF102 was examined. As shown in Table IV, percent cytostasis of the macrophages induced by PF102 in a dose of 100 μ g/kg were 57.2 and 46.4% for ratios of effector cells to target cells of 10 : 1 and 5 : 1, respectively.

Effect of PF102 on the Proliferation of Thymic Cells

The results of dose response assay of PF102 on ³H-thymidine incorporation into thymic cells are given in Table V. Maximum DNA synthesis occurred at 1.25 μ g/ml of PF102. In order to compare the degree of mitogenic response, Con A was used as a positive control. Although the mitogenic activity of PF102 was lower than that of Con A, it was obvious that PF102 enhanced

the incorporation of ³H-thymidine into thymic cells.

Effect of the Culture Supernatant Fluid of Splenic T Lymphocytes on Cytostatic Effect of Macrophages

In order to examine whether the culture supernatant fluids of the splenic T lymphocytes incubated with PF102 contained MAF, we investigated the influence of culture supernatant fluids on the cytostatic effect of macrophages, induced by 2% casein, against MM46 tumor cells. As shown in Table VI, the supernatant fluids of splenic T lymphocytes incubated with PF102 caused macrophages to inhibit DNA synthesis of MM46 tumor cells. In a preliminary study, an equal quantity of PF102 contained in the culture supernatant fluid of splenic T lymphocytes (0.625 μ g/ml) could not enhance the inhibitory effect of DNA synthesis of target cells by macrophages. On the other hand, the culture supernatant fluid of T lymphocytes incubated with Con A also displayed a strong inhibition of DNA synthesis of target cells, but the equal quantity of Con A contained in the culture su-

TABLE VII. Production of IFN by T Lymphocytes Stimulated with PF102

Treatment	Concentration	IFN titer (U)
None		0.5
Con A	10 $\mu\text{g/ml}$	12.9
LPS	5 $\mu\text{g/ml}$	3.9
PF102	1.25 $\mu\text{g/ml}$	23.8

pernatant fluid of T lymphocytes (5 $\mu\text{g/ml}$) also exhibited the inhibitory effect of the DNA synthesis of target cells by macrophages. The culture supernatant fluid of T lymphocytes incubated with LPS did not enhance the cytostatic effect. Each value of the direct cytostatic effect by medium, LPS, PF102 and Con A in the preliminary study was 1925 ± 49 , 2038 ± 40 , 2003 ± 62 , and 346 ± 29 , respectively.

Effect of PF102 on the Production of IFN by Splenic T Lymphocytes

Table VII shows the antiviral effect of the culture supernatant fluids of T lymphocytes incubated with PF102, Con A, or LPS. The culture supernatant fluid of T lymphocytes incubated with PF102 contained a larger amount of IFN than that incubated with Con A. Additionally, there was almost no IFN in the culture supernatant fluid of T lymphocytes incubated with LPS. PF102, Con A and LPS did not show direct effect for L929 cells in this assay.

Discussion

Zymosan, a water-insoluble yeast polysaccharide, and chitin and chitosan, two major constituents of the shells of crustaceans, enhanced the host defense function in some experimental animals and showed a non-specific resistance against various experimental infections and/or tumors. Since PF102 used in this study has structure and function similar to chitosan, we examined whether PF102 enhanced the host defense system like chitosan and exhibited an antitumor effect in mice. As shown in Tables I, II and III, PF102 significantly enhanced cell mediated immunity and humoral immunity in mice and displayed an antitumor effect against MM46 solid tumor transplanted in mice. More-

over, the efficient dose of PF102 was very low, 1 $\mu\text{g/kg}$.

Various immunopotentiators were shown to directly activate macrophages of several animal species^{17,18)} so that the activated macrophages were able to produce interleukin 1 (IL-1) and displayed the cytostatic effect against various tumor cells. In the present study, casein-induced macrophages stimulated with PF102 were examined *in vitro* for their production of IL-1 and cytostatic effect on MM46 tumor cells as an index of macrophage activation. The production of IL-1 produced by these macrophages was not significant in comparison with that by control macrophages, but these macrophages demonstrated a cytostatic effect in a dose dependent manner (data not shown). Because Sakai *et al.* reported that peritoneal macrophages obtained from mannan-administered mice had a cytolytic effect against MM46 tumor cells *in vitro*,¹⁹⁾ we examined whether the peritoneal macrophages obtained from PF102-administered mice exhibited the cytostatic effect also. As shown in Table IV, PF102 significantly activated macrophages to cause the cytostatic effect at both ratios of effector to target, 10 : 1 and 5 : 1. The above results demonstrate that PF102 activated the macrophages in both *in vivo* and *in vitro* systems. However, macrophages induced by PF102 *in vivo* did not produce IL-1 in comparison with control macrophages (data not shown). These results suggested that PF102 selectively activated the function of macrophages.

In order to investigate the response of lymphocytes for PF102, we assayed the influence of PF102 on thymic cells proliferation. As shown in Table V, thymic cells incorporated fourteen-fold ^3H -thymidine in comparison with the control group at the optimal dose of 1.25 $\mu\text{g/ml}$ for

PF102. Although PF102 showed inhibitory effect on the antitumor effect *in vivo* at a lower concentration, approximately 0.0125 $\mu\text{g}/\text{ml}$ in blood, as compared with the optimal concentration *in vitro* on the activation of T lymphocytes by PF102, this concentration of PF102 *in vivo* was sufficient to activate T lymphocytes as shown in Table V. It is known that T lymphocyte can produce various soluble factors such as gamma IFN, interleukin 2 (IL-2) and MAF.²⁰⁻²²⁾ Since PF102 can activate T lymphocytes in mice, we examined culture supernatant fluids of T lymphocytes stimulated by PF102 for MAF activity on cytostatic effect. As shown in Table VI, it appears that the culture supernatant fluid exhibited MAF activity. Of course, the equal quantity of PF102 which was contained in these culture supernatant fluids could not activate macrophages in this system. Since there are many reports concerning whether MAF and IFN are identical,^{23,24)} we investigated the IFN titer in the culture supernatant fluid stimulated with PF102. The results showed that PF102 cause T lymphocytes to produce more IFN than Con A, however, one could not conclude that MAF and IFN were identical (Table VII).

There have been several reports concerned with the susceptibility of transformed cells, such as tumor cells, to lectin, indicating that tumor cells are more sensitive to cytotoxicity of lectin than normal cells.^{25,26)} In the case of PF102, the incorporation of ^3H -thymidine by MM46 tumor cells was inhibited by a dose of PF102 which prevented the influence of the thymic cells on the incorporation of ^3H -thymidine (data not shown). It is of interest that PF102 has higher cytotoxicity for transformed cells than normal cells, similar to lectins. Moreover, Shoham *et al.* have reported that Con A which was shown to possess both T lymphocyte mitogenic and carbohydrate-binding properties exhibited an antitumor effect in transplanted tumor systems.²⁵⁾ Moreover, Robinson *et al.* have also reported an antitumor effect of phytohemagglutinin (PHA) which also possesses both mitogenic and lectin-like properties similar to Con A.²⁷⁾ The above and the present findings suggest a possibility that a soluble factor(s) produced by T lymphocytes, stimulated with PF102, activated the host macrophages so that the antitumor activity of PF102 was demonstrable.

phocytes, stimulated with PF102, activated the host macrophages so that the antitumor activity of PF102 was demonstrable.

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