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Effects of the Antitumor Component, F-D-P, Isolated from *Elfvingia applanata* on the Immune Response

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Abstract—An antitumor component, F-D-P, was purified from the hot water extract of the carpophores of *Elfvingia applanata* by precipitation with ethanol, dialysis, and passage through a column of DEAE-cellulose ion exchange. F-D-P inhibited the growth of Sarcoma 180 in mice showing the tumor inhibition ratio of 88.3% in doses of 20 mg/kg for ten days. Chemical analysis of F-D-P showed that it was composed of polysaccharide(65.3%) and protein(6.5%), and that the monosaccharides consisting of the polysaccharide was glucose(89.1%) and mannose(10.9%). The immunomodulatory activities of F-D-P were explored by determining its effect on the proliferation of the whole and subpopulations of lymphocytes, and on the generation of natural killer(NK) cell activity *in vitro*. F-D-P was mitogenic to total lymphocytes and B cells, but not to purified T cells, even in the presence of accessory cells. F-D-P did not increase NK cell activity when added to cultures of resting lymphocytes. From these results, it is clear that F-D-P modulates primarily the humoral immune responses.

Keywords—*Elfvingia applanata* • antitumor activity • lymphoblastogenesis • NK cell activity

Antitumor polysaccharides have been isolated from a number of Basidiomycetes class of fungi, such as *Lentinus edodes*[1~3], *Coriolus versicolor*[4,5], and *Ganoderma lucidum*[6,7]. In general, the fungal antitumor components appeared to be polysaccharides or protein-polysaccharide conjugates with high molecular weight, and exert their antitumor activities *in vivo* through nonspecific stimulation of the immune responses. Some of the fungal antitumor components have already been introduced in the cancer therapy[8].

The carpophores of *Elfvingia applanata*, which is a species of Basidiomycetes and called also as *Ganoderma applanata*[9], have been used in folk medicine for treatment of various ailments including cancers, as have been the carpophores of *Ganoderma lucidum*. However, the therapeutic value of Korean *Elfvingia applanata* has not been well characterized, nor has the biologically active substance. Recently, our laboratory showed that the carpophores of *Elfvingia applanata* contained several kinds of inorganic elements including Germanium[10]. Our laboratory also showed that the water extract of *Elfvingia applanata* contained antimicrobial components which enhanced antimicrobial activity of other antibiotics when used in combi-

nations[11]. Our laboratory also determined the acute toxicity of the water extract of *Elfvingia applanata* in mice, and showed that it did not have acute toxicity upto the concentrations of 5,000 mg/kg when administrated intraperitoneally[12]. In the present study, we isolated an antitumor component, F-D-P, from the carphophores of *Elfvingia applanata*, and examined its immunomodulatory activity *in vitro*.

Materials and Methods

Mice—Specific pathogen-free female BALB/c and ICR strains of mice, 4 weeks of age, were purchased from Korea Research Institute of Chemical Technology, Taejon. The animals were adapted to a normal environment at least for two weeks before use in the experiments.

Purification of antitumor component—Eight hundred grams of *Elfvingia applanata* ($P_{ers.}$) KARST, grown on dead hard wood, were disintegrated, washed with 6 liters of 90% ethanol, and then extracted with 3 liters of boiling water for 8 hours. After filtration, extraction was repeated in the same condition. The filtrates were combined, concentrated to 550 ml and mixed with three volumes of 99% ethanol. The resulting precipitates were collected by centrifugation, dissolved in 100 ml of distilled water, and dialysed against distilled water at 4°C for two days using cellulose membrane (MWCO = 12,000, Spectra). After removing insoluble precipitates formed during dialysis, the dialysate was lyophilized in a freeze-drier to yield 1,554 mg of dark brownish powder.

For further purification, 1,100 mg of the dark brownish powder was dissolved in 100 ml of distilled water, and applied a DEAE-cellulose (Cl^--form) column (bed volume=50 ml). After applying all the sample, the column was developed and washed with distilled water, and then eluted with 0.01 M sodium phosphate buffer, pH 7.4, containing 0.5 M sodium chloride. Macromolecules contained in the effluent (120 ml) and the eluate (140 ml) was precipitated by mixing with three volumes of 99% ethanol, respectively. The precipitates were collected by centrifugation, dissolved in 40 ml of distilled water, and then lyophilized in a freeze-drier, respectively.

Antitumor test—Antitumor activity was tested on the basis of the method of Kim *et al.*[3] Briefly, 0.1 ml of Sarcoma 180 ascitic cells (1×10^7/ml) grown in the peritonial cavity of ICR mice were inoculated subcutaneously into the right groin of ICR mice. Injection of sample was carried out intraperitoneally from the 3rd day after tumor implantation for 10 days. Average tumor weight was measured on the 28th day after tumor implantation. Inhibition ratio of tumor growth was calculated by comparing the average tumor weight of treated group with that of the control group.

Chemical analysis—Total polysaccharide content was determined by the method described by Herbert *et al.*[13] Glucose was used as a standard sugar. Total protein content was determined by Bicinchoninic acid protein assay method[14]. Bovine serum albumin was used as a standard protein. Both the monosaccharide and amino acid composition analysis were performed as described by Kim *et al.*[15]

Cell preparation—Spleen cells were prepared from spleens of BALB/c mice by gentle disruption, and red blood cells in the cell suspension were lysed with ACK lysing buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2). Splenic lymphocytes were isolated from spleen cells by the Ficoll-Hypaque density centrifugation method of Boyum[16].

B cells were enriched from spleen cells by removal of plastic surface adherent cells and depletion of Thy-1.2 antigen-bearing cells with

monoclonal anti-Thy 1.2 antibody, J1j. 10 (ATCC), and rabbit complement(ICN).

T cells were prepared from spleen cells by removal of nylon wool adherent cells and depletion of major histocompatibility complex (MHC) class II antigen-bearing cells with monoclonal antibody, M5/114.15.2(ATCC), and rabbit complement(ICN). More than 99% of the cell population was T cells as determined by FACS analysis with monoclonal anti-Thy 1.2 antibody, J1j. 10(ATCC).

Epidermal cell(EC) suspensions were prepared from ears of BALB/c mice by limited trypsinization as described previously by Tang and Udey[17]. The viability of EC was in the range of 85~92% as determined by trypan blue exclusion.

Cell culture—The cells were cultured in RPMI-1640(GIBCO BRL) supplemented with 10% fetal bovine serum (Hyclone), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin(Sigma), 10 mM Hepes(Sigma), 1 mM sodium pyruvate(GIBCO BRL), 0.1 mM non-essential amino acids(GIBCO BRL), and 50 μM 2-mercaptoethanol (Sigma), hereafter referred to as complete media, at 37°C in a humidified atmosphere with 5% CO_2 in air.

Lymphoblast-formation stimulatory activity—Cells were seeded onto a 96 well flat-bottom plate(Falcon) and cultured in complete media in the presence or absence of different concentrations of stimulants. At the end of cultivation, 0.5 μCi of ^{3}H-thymidine(2 Ci/mM, New England Nuclear) were added and incubated for another 6 hrs or 18 hrs. The cells were harvested with a automated cell harvester (Inoteck) onto a glass filter and dried at 85°C. Filter discs for each sample were placed in 2 ml of Lipoluma(LUMAC, LSC), and the radioactivity was counted in a beta counter(Packard).

Cytotoxicity — Effector cells used in the cytotoxicity assay were generated by culturing total or T cell-depleted lymphocytes(0.2 ml/well of 4×10^6/ml or 2×10^6/ml) for 72 hrs at 37°C in complete media in the presence or absence of different concentrations of stimulants. A 96 well U-bottom plate was used in this culture.

Target cells used in the cytotoxicity assay were prepared by culturing Yac-1 cells(5×10^5/ml) or P815 cells(5×10^5/ml) in complete media containing ^{3}H-thymidine(5 μCi/ml, New England Nuclear) for 24 hrs at 37°C. Labelled target cells were washed with HBSS, resuspended in complete media supplemented with 50 μg/ml of DNase I(Boehringer, Mannheim), and incubated for 2 hrs at 37°C. Labelled target cells were washed with HBSS, and finally resuspended in complete media supplemented with 2 mM of thymidine(Sigma) at the density of 2×10^5/ml.

Target cell killing was initiated by adding 0.1 ml of target cell suspension to each well of effector cell culture, and continued for 4 hrs. At the end of incubation, 10 μl of DNase I (1 mg/ml) was added to each well, and incubated for another 30 minutes. A total of 100 μl of the supernatant was collected from each well after centrifugation at 400 g for 10 minutes, and the radioactivity was counted in a beta counter (Packard) after mixing with 3 ml of Lumagel-Safe(LUMAC, LSC). Percent specific lysis was calculated as follows:

$$\% \text{ Specific lysis} = \frac{\text{cpm experimental} - \text{cpm SP}}{\text{cpm MR} - \text{cpm SR}} \times 100$$

Spontaneous release(SR) was defined as the counts per minute(cpm) released from targets incubated with medium alone. Maximum release (MR) was defined as the cpm in the supernatant after lysis of the targets with 1% Triton X-100.

Results and Discussion

Purification of antitumor component—From 800 g of the dried carpophores of *Elfvingia applanata*, a total of 1,554 mg of dark brownish powder (F-T) was obtained by hot water extraction followed by dialysis, precipitation with ethanol, and freeze drying. For further purificaton, 1,100 mg of the dark powder was applied to a DEAE-cellulose ion exchange column, and then eluted with 0.5 M NaCl in a sodium phosphate buffer, pH 7.4. Macromolecules in the effluent and the eluate from the column were precipitated by adding 3 volumes of ethanol, and freeze dried to yield 320 mg of white powder (F-D-P) and 598 mg of dark brownish powder (F-D-B), respectively.

Antitumor activity—Antitumor activity of the preparations from *Elfvingia applanata* was examined against Sarcoma 180 in mice, and the results were shown in Table I. The tumor inhibition ratio of F-T was 78.8% when administrated in doses of 50 mg/kg for ten days. When F-T was further separated into two fractions with DEAE-cellulose ion exchange, the antitumor activity was resided in the fraction that passed DEAE-cellulose column. The tumor inhibition ratios of F-D-P and F-D-B was 88.3% and 60.3%, respectively, when administered in doses of 20 mg/kg for ten days. Thus, F-D-P was subjected to further analysis on the chemical nature and the immunomodulatory activities.

Chemical analysis—Total polysaccharide and protein contents of F-D-P were 65.3% and 6.5%, respectively (Table II). The monosaccharides consisting of the polysaccharide were shown in Table III. Glucose was the major monosaccharide consisting of the polysaccharide. Mannose was also detected from the polysaccharide, but the amount was approximately one-tenth compared to that of glucose. Other monosaccharides such as fucose, galactose, and xylose were not detected. It has been well documented that β-D-glucans isolated from several species of Basidiomycetes such as *Ganoderma applanatum*[18] and *Ganoderma lucidum*[6] posess a strong antitumor activity. Studies on the purification of the antitumor β-D-glucans from *Ganoderma applanatum* showed that they were not bound to DEAE-cellulose(Cl^--form)[18,19], as did not the antitumor component, F-D-P. Taken together, it appears that the antitumor active component of F-D-P would be β-D-glucans.

The amino acids consisting of the protein were shown in Table IV. F-D-P contained sixteen kinds of amino acids. Whether the protein and the polysaccharide moieties were

Table I. Antitumor activity of *Elfvingia applanata* on sarcoma 180 solid tumor

Sample[a]	Dose (mg/kg)	Average tumor weight(g)±S.E.	Inhibition ratio (%)	Complete regression
Saline	—	9.38±0.67	—	0/8
F-T	50	1.99±0.41	78.8[b]	1/8
	20	2.92±0.42	68.1[b]	0.8
F-D-P	20	1.10±0.41	88.3[b]	2/8
F-D-B	20	3.72±0.72	60.3[b]	0/8

[a] *Elfvingia applanata* was extracted with hot H_2O, and the extract was concentrated, and then precipitated with 3 volumes of ethanol. The precipitates (F-T) were then further separated into two fractions by a DEAE-cellulose column chromatography, one that passed DEAE-cellulose (F-D-P) and another that bound to DEAE-cellulose (F-D-B), as described in detail in the text.

[b] $p<0.01$

Table II. Polysaccharide and protein contents of F-D-P

Component	Content (%)
Polysaccharide	65.3±3.4[a]
Protein	6.5±3.5

[a]The values are means±S.D. from three experiments.

Table III. Monosaccharide composition of F-D-P

Monosaccharide	Content (%)
Glucose	89.1[a]
Galactose	N.D.[b]
Mannose	10.9
Fucose	N.D.
Xylose	N.D.

[a]The values are expressed as the area percentage of the monosaccharide peaks obtained from GLC chromatogram.
[b]Not detected.

Table IV. Amino acid composition of F-D-P

Amino acid	Content (%)
L-Aspartic acid	8.90[a]
L-Threonine	12.67
L-Serine	24.46
L-Glutamic acid	5.56
Glycine	11.85
L-Alanine	15.64
L-Valine	4.38
L-Methionine	2.05
L-Isoleucine	1.00
L-Leucine	3.23
L-Tyrosine	0.56
L-Phenylalanine	2.00
L-Lysine	0.86
L-Histidine	1.33
L-Arginine	0.79
L-Proline	4.72

[a]The values are expressed as the mole percentage.

bound each other is not clear at present.

Immunomodulatory activity—Blast-formation stimulatory activity of F-D-P was examined on the whole and subpopulations of lymphocytes.

Table V. Lymphoblast-formation stimulatory activity of F-D-P

Addition to culture[a]	Final concentration (μg/ml)	^{3}H-thymidine incorporation (CPM×10^{-3})[b]
None	—	6.3±0.7
Con-A	1.0	72.2±3.7
F-D-P	1,000.0	64.0±6.4
	500.0	39.5±4.0
	100.0	20.9±1.4
	50.0	18.0±2.4

[a]Mouse splenic mononuclear cells (5×10^5/well) were cultured with the indicated amounts of stimulus for 48 hrs, and then pulsed with ^{3}H-thymidine for additional 6 hrs. The cells were harvested onto a glass filter, dried, and then counted in a scintillation counter after mixing with scintillation fluid.
[b]Values are means±S.D. of triplicate experiments.

Table VI. Blast-formation stimulatory activity of F-D-P on B-lymphocytes

Addition to culture[a]	Final concentration (μg/ml)	^{3}H-thymidine incorporation (CPM×10^{-3})[b]
None	—	8.9±0.1
LPS	50.0	55.2±2.1
F-D-P	1,000.0	78.9±5.4
	100.0	28.5±0.8
	10.0	16.7±1.1
	1.0	13.8±1.9

[a]B-lymphocytes (5×10^5/well) were cultured with the indicated amounts of stimulus for 72hrs, and then pulsed with ^{3}H-thymidine for additional 18 hrs. The cells were harvested onto a glass filter, dried, and then counted in a scintillation counter after mixing with scintillation fluid.
[b]Values are means±S.D. of triplicate experiments.

F—D—P showed mitogenic activity to total splenic lymphocytes (Table V) in a dose-dependent manner. The mitogenic activity of F-D-P was the highest at concentration of 1,000 μg/ml, showing that F-D-P is not directly cytotoxic to cells at this high concentration. Toexamine the lymphocytes primarily responsible for the proliferation, mitogenic activity of F-

Table VII. Blast-formation stimulatory activity of F-D-P on T-lymphocytes

Addition of accessory cells[a]	Addition of stimulus	^{3}H-thymidine incorporation (CPM)[b]
None	None	79±10
	Con A(1 μg/ml)	487±123
	F-D-P	
	1,000 μg/ml	147±69
	100 μg/ml	78±2
	10 μg/ml	81±8
	1 μg/ml	85±16
EC	None	2,619±845
	Con A(1 μg/ml)	63,925±4,502
	F-D-P	
	1,000 μg/ml	1,691±177
	100 μg/ml	1,832±543
	10 μg/ml	2,283±449
	1 μg/ml	1,592±302

[a]T lymphocytes(2×10^5/well) were cultured in the absence or presence of epidermal cells(1×10^5/well) isolated from syngenic mouse ear skin.

[b]Cultures were incubated with the indicated amounts of stimulus for 72 hrs, and then pulsed with ^{3}H-thymidine for additional 6 hrs. The cells were harvested onto a glass filter, dried, and then counted in a scintillation counter after mixing with scintillation fluid. Values are means±S.D. of triplicate experiments.

Table VIII. Effect of F-D-P on the generation of NK cell activity *in vitro*

Stimulus[a]	% Specific lysis of target cell[b]	
	Effector to target ratio	
	40 : 1	20 : 1
Medium	6.1±0.5	3.2±0.3
F-D-P		
1,000 μg/ml	7.4±1.2	3.6±0.6
100 μg/ml	5.2±0.9	2.1±0.7
10 μg/ml	6.2±0.4	3.2±0.3
1 μg/ml	5.6±0.6	3.6±0.6

[a]Mouse splenic lymphocytes were cultured with the indicated amounts of stimulus for 72 hr, and then mixed with 2×10^4 labelled target cells in a short term cytotoxicity assay.

[b]The assay was performed in triplicate and expressed as mean percent±S.D.

D-P was tested on the subpopulations of lymphocytes. F-D-P was strongly mitogenic to B lymphocytes, as shown in Table VI. The mitogenic activity of F-D-P was shown even in the concentration of 1 μg/ml, and the mitogenic activity was increased in a dose-dependent manner upto the concentration of 1,000 μg/ml. However, F-D-P was not mitogenic to T lymphocytes (Table VII). Because many of the T-cell mitogens such as concanavalin-A require the presence of accessory cells to show mitogenicity[20], epidermal cells isolated from mouse ear skin were supplemented with purified T lymphocytes in examining the mitogenic properties of F-D-P on T lymphocytes. F-D-P was not mitogenic to T lymphocytes regardless of the presence of accessory cells. Results showing that F-D-P induces B lymphocyte proliferation are in agreement with previous studies that have shown that the antitumor polysaccharides or antitumor protein-polysaccharide conjugates isolated from Basidiomycetes activate humoral immune responses in normal and cancer-bearing mice[21,22]. In our experiments, F-D-P did not induce proliferation of T lymphocytes, even though administration of the crude extract of *Ganoderma applanatum* had been reported by others to induce amplification of nonspecific T cells resulting in enhancement of delayed type hypersensitivity reaction[23]. This may be due to the differences in the purity of antitumor preparations because the possibility of amplification of nonspecific amplifier T cells without involving proliferation is very low.

Several investigators have shown that administration of the antitumor polysccharides or antitumor protein-polysaccharide conjugates isolated from mushrooms can restore the suppressed NK cell activity in tumor-bearing mice[5,24]. Thus, we examined the NK cell stimulatory activity of F-D-P *in vitro*. F-D-P, however, did not increase NK cell activity when added

to cultures of resting lymphocytes(Table VIII). We examined the NK cell stimulatory activity of F-D-P in several different conditions varying the total numbers of lymphocytes and the duration of stimulation, but we were unable to find any significant differences in NK cell activity between the stimulated cultures and the normal cultures, as shown a representative result in Table VIII.

The mechanisms by which B lymphocytes affect anticancer immunity are not clarified yet, but recent studies have shown that activated B lymphocytes or B lymphoblastoid cell lines produce a variety of lymphokines such as interleukin (IL)-1, IL-6, IL-12, interferon (IFN)-α, IFN-β, and lymphotoxin[25~29]. These findings suggest that the outcome of the stimulation of B lymphocytes can result in activation of antitumor effector cells such as macrophages and NK cells, which lyse tumor cells in a contact-dependent, nonphagocytic process[30,31]. Antitumor effector cells activated by F-D-P are under investigation.

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