AUGMENTATION OF ANTITUMOR ACTIVITY BY COMBINED CRYO-DESTRUCTION OF SARCOMA 180 AND PROTEIN-BOUND POLYSACCHARIDE, EA₆, ISOLATED FROM *FLAMMULINA VELUTIPES* (CURT. EX FR.) SING. IN ICR MICE

TETSUO OHKUMA, KENJI OTAGIRI,* TETSURO IKEKAWA,** AND SHIGEO TANAKA***

Tumor Research Division, Division of Special Surgery,*** Hokushin General Hospital, Nishi 1-5-63, Nakanoshi, Nagano, 383, Japan and Chemotherapy Division, National Cancer Center Research Institute,** Tsukigi 5-1-1, Chuoku, Tokyo, 104, Japan*

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Augmentation of antitumor activity by combined *in situ* freeze-destruction of tumor (cryosurgery) and administration of antitumor active substances isolated from a hot water extract of an edible mushroom, *Flammulina velutipes* (Curt. ex Fr.) SING., was investigated in sarcoma 180 bearing ICR mice. Antitumor active substances of the mushroom included β -(1,3)-glucan (EA₃) and protein-bound polysaccharide (EA₆). Autitumor activity was evaluated by the growth rate of the solid tumor rechallenged subcutaneously (*s.c.*) or by cumulative mortalities of the mice rechallenged intraperitoneally (*i.p.*) with the ascites tumor, on day 7 after cryosurgery. Weak antitumor effect induced by cryosurgery against challenged solid tumor of sarcoma 180 was markedly augmented by *i.p.* administration of EA₆ (10 mg/kg). Cryosurgery of the solid sarcoma 180 apparently did not induce any antitumor effect against challenged ascites sarcoma 180. However, when cryosurgery was combined with oral administration of the polysaccharides (50 mg/kg), prolonged survival of the mice challenged with ascites sarcoma 180 *i.p.* was recognized by EA₆, but not by EA₃. Timing of the administration of EA₆ (*i.p.*) with cryosurgery was best in pre- and post-cryosurgery. Immunological activity of EA₆ to the host was discussed.

Keywords—oncology; cryosurgery; *Flammulina velutipes*; mushroom; polysaccharide; protein-bound polysaccharide; sarcoma 180; tumor immunology

T. Ikekawa *et al.*¹⁻³⁾ isolated 4 antitumor active substances from a hot water extract of an edible mushroom, *Flammulina velutipes* (Curt. ex Fr.) SING., and studied the antitumor effect of these substances by “host-mediated” antitumor bioassay and by the total packed cell volume method.

We previously reported⁴⁾ that tumor specific as well as non-specific immunity estimated by delayed hypersensitivity reactions was augmented by a combined treatment with cryo-destruction (cryosurgery) of subcutaneous solid VX2 carcinoma and oral administration (*p.o.*) of the hot water extract of the mushroom (FEH-1) in rabbits. Among these antitumor active substances of *F. velutipes*, a protein-bound polysaccharide (EA₆) augmented an antitumor effect highly

against intraperitoneal (*i.p.*) challenging of ascites sarcoma 180 when combined with cryosurgery in ICR mice.

This paper related further studies of the combined treatment with cryosurgery and the polysaccharides of the mushroom in mice allogeneic tumor systems.

MATERIALS AND METHODS

Animals—Five-week-old female ICR mice weighing 20 ± 2 g (Japan Clea Inc. Tokyo) were used.

Tumors—Sarcoma 180 and Ehrlich carcinoma were used and maintained by *i.p.* inoculation of ascites form into ICR mice at weekly intervals.

Materials — Antitumor active substances²⁾ were isolated from the hot water extract of fruit bodies of *Flammulina velutipes* (Curt. ex Fr.) SING. Of these substances, β -(1,3)-glucan (EA₃) and protein-bound polysaccharide (EA₆) were used. Normal saline solutions of these samples were sterilized by autoclave prior to administration.

Cryosurgical Destruction of the Tumor — Ascites tumor of sarcoma 180 (5×10^6 cells) was subcutaneously (s.c.) transplanted to the right back of female ICR mice. Cryosurgical destruction of the established solid tumors was performed on day 7 after the tumor transplantation. Frigitrionics Linde CE-2B cryosurgical unit (Frigitrionics of Connecticut Inc., Shelton, CONN, U.S.A.), using liquid nitrogen as the cryogen, was used with a probe PR-6 attached with a copper cylindrical probe-tip adaptor of 1.0 cm in diameter. *In situ* freezing of the solid tumor was performed by the contact method through shaved skin at -80°C , for 20 seconds plus 15 seconds (2-cycle-freezing). In consequence, the solid tumors were completely eradicated by the cryosurgery.

Evaluation of Antitumor Activity — 1) Effect of Combined Cryosurgery and Polysaccharides on s.c. Solid Tumor: Cryosurgery of solid tumor of sarcoma 180, inoculated s.c. on day -14 , was carried out on day -7 . The test materials were administered *i.p.* 10 mg/kg daily for 10 d, starting on day -11 and ending on day -1 . Effect of the

combination treatment was evaluated by growth rate of the solid tumor of sarcoma 180 challenged s.c. (5×10^5 cells/mouse) on day 0. Animals were sacrificed on day 32 after transplantation for autopsy. Numerical values in the evaluation were indicated by inhibition ratio (%).

2) Effect of Combined Cryosurgery and Polysaccharides on the Ascites Tumor: Cryosurgery of solid tumor of sarcoma 180, inoculated s.c. on day -14 , was carried out on day -7 . The test materials were administered *p.o.* 50 mg/kg daily for 10 d, starting on day -11 and ending on day -1 . Effect of the combination treatment was evaluated by cumulative mortalities of the mice challenged ascites sarcoma 180 *i.p.* (5×10^4 cells/mouse) on day 0.

3) Timing of the Administration of EA₆ with Cryosurgery: Cryosurgery of solid tumor of sarcoma 180, inoculated s.c. on day -14 , was carried out on day -7 . EA₆ was administered *i.p.* 10 mg/kg 4 times daily prior to cryosurgery, or pre- and post-cryosurgery, or post cryosurgery only. Seven days after cryosurgery, mice were challenged with ascites tumor of sarcoma 180 *i.p.* (5×10^4 cells/mouse) on day 0.

4) Effect of Combined Cryosurgery of Sarcoma 180 and Polysaccharides on Ascites Ehrlich Carcinoma: Cryosurgery of solid tumor of sarcoma 180, inoculated s.c. on day -14 , was done on day -7 . The test materials were administered *i.p.* 10

TABLE I. *Effect of Combined Cryosurgery and EA₃ or EA₆ Treatment on Sarcoma 180 Solid Tumor*

Treatment	Tumor weight (g) (mean $\pm$ SD)	Inhibition ratio, (%)	Disappeared
			total
EA ₃ + Cryo	ND ^{a)}	100	8/8
EA ₃	ND	100	8/8
EA ₆ + Cryo	0.3 ± 0.3 ^{b)}	73	4/7
EA ₆	1.2 ± 0.7	0	0/8
Cryo	0.8 ± 0.9	27	2/7
Control	1.1 ± 0.5	0	0/8

Mice were sacrificed on day 32 after sarcoma 180 (5×10^5 cells) s.c. transplantation

a) Not detected.

b) Significantly different ($p < 0.01$) from control.

mg/kg daily for 10 d, starting on day -11 and ending on day -1. Animals were challenged with ascites Ehrlich carcinoma *i.p.* (5×10^4 cells/mouse) on day 0.

5) Induction of Transplantation Immunity by Polysaccharides: Challenge of sarcoma 180 (5×10^4 cells/mouse) was made *i.p.* on day 0 to the mice bearing solid tumor of sarcoma 180, inoculated on day -14, or to the tumor-free control mice. The test materials were administered *i.p.* to the sarcoma 180-bearing mice or to the tumor-free mice 10 mg/kg for 10 consecutive days, starting 24 h after *s.c.* challenge of the tumor.

Statistical Analysis — Statistical analysis was made by student's *t*-test.

RESULTS

1. Effect of the Combined Treatment on solid Tumor of Sarcoma 180 (Table I)

Administration (*i.p.*) of EA₆ alone did not show any antitumor effect against the solid tumor of sarcoma 180. Cryosurgery exhibited a weak antitumor effect. However, the mice received combination treatment with cryosurgery and EA₆ exhibited high inhibition ratio against the tumor challenged *s.c.* Incidentally, EA₃ showed marked antitumor effect on the solid tumor of sarcoma 180.

2. Effect of the Combined Treatment on Ascites Tumor of Sarcoma 180 (Fig. 1)

Cryosurgery alone or the polysaccharides (EA₃ or EA₆) *p.o.* only did not exhibit any antitumor effect on ascites sarcoma 180. However, when the materials were administered in conjunction with cryosurgery, only EA₆ induced a remarkable antitumor effect against the challenged ascites tumor (ILS of 85 %).

3. Timing of EA₆ Administration with Cryosurgery (Table II)

All of the groups administered EA₆, *i.p.* exhibited more than 35.9 % of ILS. Among the 3 schedules, EA₆ given pre- and post-combining with cryosurgery, showed the largest ILS, and 3 of 8 mice survived for more than 60 d.

4. Effect of Combined Cryosurgery of Sarcoma 180 and Poly-saccharides on Ascites Ehrlich Carcinoma

(Table III)

Antitumor effect induced by combining cryosurgery of sarcoma 180 and the test materials was examined on a heterogeneous tumor, ascites Ehrlich carcinoma. Cryosurgical treatment for the solid tumor of sarcoma 180 induced strong antitumor effect against Ehrlich carcinoma

FIG. 1. Combined Treatment with Cryosurgery and EA₃ or EA₆ in Oral Administration

1) EA₃ or EA₆ was orally administered to ICR mice 50 mg/kg daily for 10 d. Cryo-destruction of solid sarcoma 180 was done on day -7. Mice were challenged with ascites tumor of sarcoma 180 *i.p.* (5×10^4 cells) on day 0.

2) a) $p < 0.05$ as compared with the control groups.

3) —; cryosurgery, ---; *s.c.* solid tumor bearing.

challenged. EA₆ exhibited a marked antitumor effect on Ehrlich carcinoma challenged, in *i.p.* administration for ICR mice bearing sarcoma 180 (solid type), but this was not shown by EA₃ with any combination. Combination with cryosurgery and EA₆ indicated the largest ILS in this experiment, and all of the mice in the group survived for more than 60 d.

5. Effect of EA₆ on Induction of Transplantation

Immunity (Table IV)

When EA₃ or EA₆ was administered to *s.c.* tumor-free mice, the polysaccharides exhibited a weak antitumor activity. Obvious prolongation of life span was found when EA₆ was given to the mice bearing solid sarcoma 180, but not by EA₃.

DISCUSSION

Cryoimmunologic reaction elicited by cryosur-

TABLE II. *Timing of EA₆ Administration with Cryosurgery*

EA ₆ administration	Cryosurgery	MST ^{a)} (mean ± SD)	ILS ^{b)} (%)	Deaths total
Pre-admn.	+	31.4 ± 18.1	35.9	5/7
Pre-admn.	—	34.1 ± 16.3	47.6	6/8
Pre & post admn.	+	37.8 ± 20.2 ^{c)}	63.6	5/8
Pre & post admn.	—	32.6 ± 18.7	41.1	6/8
Post-admn.	+	32.0 ± 17.7	38.5	6/8
Post-admn.	—	35.2 ± 15.9 ^{c)}	52.4	6/8
None	+	24.8 ± 13.5	7.4	7/8
Tumor-bearing Control	—	23.1 ± 7.7	0	8/8

Mice were challenged with ascites tumor of sarcoma 180 *i.p.* (5×10^4 cells) on day 0.

a) Mean survival time, days.

b) Increase in mean survival time over control.

c) Significantly different ($0.05 < p < 0.1$) from control.

TABLE III. *Effect of Combined Cryosurgery of Sarcoma 180 and EA₃ or EA₆ Administration on challenged Ehrlich Ascites Carcinoma*

Samples	Cryosurgery	MST ^{a)} (mean ± SD)	ILS ^{b)} (%)	Deaths total
EA ₃	+	43.5 ± 15.8	15.0	3/4
EA ₃	—	25.5 ± 15.5	—32.5	5/6
EA ₆	+	60.0 ± 0.0 ^{c)}	58.7	0/5
EA ₆	—	57.0 ± 5.1	50.8	2/6
None	+	59.5 ± 1.1 ^{c)}	57.4	1/6
None (Control)	—	37.8 ± 18.2	0	4/5

Mice were challenged with ascites Ehrlich carcinoma *i.p.* (5×10^4 cells) on day 0.

a) Mean survival time, days.

b) Increase in mean survival time over control.

c) Significantly different ($p < 0.05$) from control.

gery of malignant tissues is expected to be tumor-specific.⁵⁾ In animal tumor systems,⁵⁻⁸⁾ antitumor immunity following cryothermic treatment of tumors has been studied in comparison with surgical excision. The mechanism of host's immune response and tumor-specific immune reaction have been discussed by many investigators.^{5,8)} It is reported that tumor-specific transplantation immunity was higher in cryosurgically destroyed group than in surgically amputated group.^{5,6)} In humoral or lymphocyte-mediated cytotoxicity test, cryosurgery group showed significantly higher immunity than untreated tumor-bearing animals or the mice undergone tumor excision.^{7,8)} The suppressed tumor growth in the rabbits received cryosurgery of VX2 carcinoma was considered to be the result of interstitial reaction by tumor circumscribing reactive granulation tissue rich in immunoreactive cells.⁹⁾ In the clinical observations of human cancer^{10,12)} the verifications of the manifestations of cryoimmunologic antitumor reaction to metastatic lesions were reported. And cryosurgery of pulmonary metastases induced an increase in tumor-specific cell-mediated cytotoxicity without producing serum blocking factors.¹³⁾ But remission of the metastatic lesions following

cryothermic treatment was seen in not all of the patients. Therefore, augmentation of the tumor-specific cryoimmunologic reaction by some means is required for the treatment of the cancer patients.

In our previous paper,⁴⁾ we reported experimental studies of intensification of cryoimmunologic reaction by *p.o.* administration of a hot water extract of fruit bodies of a Japanese edible mushroom of *F. velutipes*. T. Ikekawa *et al.*^{2,3)} isolated 4 antitumor active substances from the mushroom, β -(1,3)-glucan (EA₃), a heteropolysaccharide (EA₅), a polysaccharide (EA₇), and a protein-bound polysaccharide (EA₆). We tested which polysaccharide, in these 4 antitumor active substances, could augment cryoimmunologic reaction and could intensify antitumor effect elicited by cryosurgery.⁴⁾ Administration of EA₆ (10 mg/kg) *i.p.* showed the highest antitumor effect against *i.p.* challenging of ascites sarcoma 180 in ICR mice when combined with cryosurgery. In this study, combined treatment with cryosurgery and *p.o.* administration of EA₆ resulted in a significant prolongation of survival time of the mice, but EA₃ did not prolong the life of the mice as shown in Fig. 1. Effect of the combined treatments on the growth of *s.c.* solid sar-

TABLE IV. Effect of EA₆ or EA₃ on Host Immunity against Sarcoma 180

Sarcoma 180 solid tumor-bearing	Sample	MST ^{a)} (mean $\pm$ SD)	ILS ^{b)} (%)	Deaths total
+	EA ₃	24.8 $\pm$ 1.9	31.9	6/6
-	EA ₃	26.8 $\pm$ 4.5 ^{c)}	42.6	6/6
+	EA ₆	41.7 $\pm$ 14.4 ^{d)}	121.8	4/6
-	EA ₆	28.5 $\pm$ 6.4 ^{e)}	51.6	6/6
+	None	22.2 $\pm$ 4.7	18.0	6/6
-	None	18.8 $\pm$ 2.2	0	6/6
(Control)				

Mice were challenged with ascites sarcoma 180 *i.p.* (5×10^4 cells) on day 0.

a) Mean survival time, days.

b) Increase in mean survival time over control.

c) Significantly different ($p < 0.02$) from control.

d) Significantly different from control ($p < 0.02$) and from EA₃ administered groups ($p < 0.05$).

e) Significantly different ($p < 0.05$) from control.

sarcoma 180 was tested by *i.p.* administration of EA₃, or EA₆ (Table I). The β -(1,3)-glucan (EA₃) showed excellent antitumor effect both in its single and in combination use. Therefore, EA₃ gave identical immune mechanism to β -(1,3)-glucan purified from another plants.¹⁴⁻¹⁶ EA₆ did not exhibit significant antitumor effect on sarcoma 180 solid tumor by *i.p.* administration. But the cryosurgery-induced weak antitumor effect on solid tumor was markedly intensified by combined use of EA₆ (Table I). In order to intensify these cryoimmunologic reactions, the best administration schedule of EA₆ was prior to- and post-cryosurgery in sarcoma 180 bearing mice.

Cross reactivity of the combined cryosurgery of sarcoma 180 and *i.p.* administration of EA₆ or EA₃ (10 mg/kg) was tested by *i.p.* challenge of Ehrlich carcinoma. Cryosurgery of sarcoma 180 resulted in marked antitumor effect against Ehrlich carcinoma, and EA₆ did augment the effect, but EA₃ failed. However, mechanism of this cross reactive antitumor effect is not known.

When these specimens were administered to the mice bearing solid sarcoma 180, only EA₆ exhibited excellent prolongation of ILS as shown in Table IV. These two antitumor active substances, EA₃ and EA₆ must have a different mode of action. Actually EA₃, β -(1,3)-glucan was effective against the solid tumor of sarcoma 180 in *i.p.* administration, but the protein-bound polysaccharide, EA₆, was effective against the challenged ascites tumor in the tumor-bearing mice of sarcoma 180 by *i.p.* and *p.o.* use as illustrated in the present study. Thus, augmentation of cryoimmunologic reaction and transplantation immunity using protein-bound polysaccharide, EA₆ from *F. velutipes* were demonstrated.

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