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The Culture Fluid of *Isaria japonica* YASUDA Augments Anti-Sheep Red Blood Cell Antibody Response in Mice

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***Isaria japonica* YASUDA was cultured in a liquid medium, and its culture fluid (IJCE) was tested for stimulatory activity to humoral antibody production. IJCE significantly enhanced the production of anti-sheep red blood cell (SRBC) plaque forming cells (PFC) by oral ingestions at 10 and 30 mg/kg/d for 4 consecutive days, either before or after SRBC challenge. It also recovered the reduction of anti-SRBC PFC response and number of spleen cells caused by treatment with 5-fluorouracil. It is suggested that IJCE is a promising source for an immunomodulating tool or medicine.**

Key words *Isaria japonica*; culture; antibody response; plaque forming cell; immunopotentiation; SRBC

The fungi of *Cordyceps* and *Isaria* (Clavicipitaceae) have a unique lifecycle. These fungi infect live insects in the larval and pupal stages, proliferate inside the insects, form clubs out of the joint tissues of the hosts and finally kill them. The details of the process have not yet been clarified. The clubs together with the host bodies are called "Toh-Chu-Kasoh" in Japanese, and have traditionally been used to assure longevity in China[1] and for treatment of cancer in China and Japan.

Some bioactive compounds have been isolated from *Cordyceps* species. An adenosine derivative cordycepin isolated from the culture fluid of *C. sinensis* and *C. militaris*[2-4] showed antibacterial[2] and Ca^{2+}-antagonistic activities *in vitro*.[5] The galactosaminoglucan, a component of the (1→3)-β-glucan polysaccharides isolated from the insect-body portion of *C. ophioglossoides*, increases the period of survival in carcinoma- or lymphoma-bearing mice.[6-8] Ergosterol peroxide is an antitumor component isolated from the insect-body portion of *C. militaris*.[9]

We established the culture line from the wild type of *I. japonica*.[10] In the present study, we examined the effects of the culture fluid (IJCE) on immune responses and found that the per os (*p.o.*) ingestion of IJCE augments anti-sheep red blood cell (SRBC) plaque-forming cells (PFC) response.

MATERIALS AND METHODS

Reagents Beer dry yeast was purchased from Iwaki Pharmaceutical (Tokyo, Japan). RPMI-1640 medium, phosphate-buffered saline (PBS), fetal bovine serum (FBS) and penicillin/streptomicin were from GIBCO (Grand Island, NY, U.S.A.). *N*-2-Hydroxymethyl piperazine-*N'*-2-ethanesulfonic acid and tris(hydroxymethyl)aminomethane were from Wako Pure Chemicals (Osaka, Japan). SRBC was from Nippon Biological Laboratory (Tokyo, Japan). Guinea pig complement (HEMO-LO) was purchased from Cedar Lane (Ontario, Canada). 5-Fluorouracil (5-FU) was obtained from F. Hoffman-La Roche-Kyowa.

Experimental Animals Male CDF_1 mice, 5 to 6 weeks old, were obtained from SLC-Japan (Shizuoka, Japan) and maintained on water and routine chow *ad libitum*.

Culture of *Isaria japonica* YASUDA *Isaria japonica* YASUDA was cultured as reported previously.[10] Briefly, the ascospores isolated from the perithecium of *I. japonica* YASUDA were inoculated into a 200 ml flask containing autoclaved (121 °C, 20 min) culture medium composed of 0.3% yeast, 0.5% glucose and 0.016% inosine (IJ-medium). The flask was left in the dark for 14 d at 18 °C. Conidiospores were grown and the developing colonies of the new hyphae were inoculated into 300 ml flasks containing 100 ml of IJ-medium. A total of 100 flasks were left for 21 d under the same conditions. The hyphae were gently removed and the conditioned media were centrifuged at $10000 \times g$ for 60 min. A pool of the culture supernatants was lyophilized to give dark-brownish powder (IJCE) in a yield of 0.1 to 0.12% (w/v) of the culture media. As an animal experimental control, IJ-medium alone was processed in the same manner as culture supernatants.

***In Vivo* Promoting Activity to Antibody Production** CDF_1 mice were intravenously (i.v.) immunized with 2×10^8 sheep blood cells. Four days after immunization, a single cell suspension was prepared by teasing spleens gently with forceps, and filtering this through a wire mesh screen into RPMI 1640 medium supplemented with 10 mM HEPES, 1 mM sodium pyruvate and 5% heat-inactivated fetal bovine serum. Erythrocytes in the cell suspension were lysed with 0.75% ammonium chloride in Tris-buffer (pH 7.6) in the usual manner. After washing with PBS, the spleen cells were resuspended at 4×10^7 cells/ml in the same medium. A mixture of 10% (v/v) SRBC and diluted guinea pig complement was added to the spleen cell suspension, and the mixture was transferred into Cunningham chambers.[11] After incubation for 30 min at 37 °C, the number of PFC was counted under a microscope. In all experiments, IJCE was given to mice by intragastric ingestion. In this experiment, mice were also subcutaneously (s.c.) injected with 5-FU (20 mg/kg/d) for 3 d before SRBC challenge.

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Statistical Analysis All results were expressed as arithmetic mean ± S.E. Statistical significance of differences between groups was determined by Student's *t*-test.

RESULTS

Culture of *Isaria japonica* YASUDA As shown in Fig. 1, visible hyphae were grown from the ascospores of *Isaria japonica* YASUDA in liquid medium (approximately 4 d after inoculation) and new carps which seemed to be same as the wild type (see Fig. 1, upper panel) were generated from the hyphae 14 d later (Fig. 1, lower panel). Ascospores prepared from this cultivated fungus did not differ from wild type *Isaria japonica* YASUDA as judged under a microscope.

Fig. 1. Photograph of Culture of *Isaria japonica* YASUDA

Isaria japonica YASUDA was harvested, and the conidiospore was grown in IJ-medium for 21 d. Upper panel, *Isaria japonica* YASUDA grown in nature; lower panel, *Isaria japonica* YASUDA in culture.

Adjuvant Activity of IJCE to Antibody Response in the Mice We examined whether IJCE was stimulative to immune responses, because this fungus has traditionally been used for cancer patients in China[1] and Japan.

Mice were primed i.v. with SRBC (day 0) and treated with IJCE once a day for 7 d (day −3 to day +3). As shown in Table 1 (exp. 1), IJCE significantly enhanced IgM PFC response toward SRBC at 10 and 30 mg/kg, but did not at 3 mg/kg on the bases of either PFC number per 10^6 spleen cells or PFC number per whole spleen. No significant SRBC PFC response was caused by IJCE ingestion in unprimed mice (data not shown).

To learn the effective timing of IJCE administration, IJCE was ingested by mice before (day −3 to day 0) or after (day 0 to day +3) SRBC immunization (on day 0). An increment of anti-SRBC PFC response occurred either by the pretreatment or by the posttreatment, which showed

Table 1. Augmentation of Anti-SRBC Responses by IJCE Given for Different Periods

Treatment	Dose (mg/kg/d)	PFC per whole spleen ($\times 10^3$)	PFC per 10^6 spleen cells	*n*
Exp. 1: IJCE treatment (day −3 to 3)				
Control	—	32.0 ± 5.9	401 ± 19	5
IJCE	3	38.5 ± 8.2 N.S.	436 ± 42 N.S.	6
IJCE	10	44.1 ± 5.9[a]	555 ± 80 N.S.	6
IJCE	30	59.5 ± 6.5[b]	711 ± 67[a]	6
Exp. 2: IJCE pretreatment (day −3 to 0)				
Control	—	38.8 ± 12.4	477 ± 52	5
IJCE	3	69.4 ± 12.0[a]	688 ± 115 N.S.	5
IJCE	10	58.8 ± 5.8[b]	586 ± 60 N.S.	6
IJCE	30	71.9 ± 4.1[b]	719 ± 41[b]	4
Exp. 3: IJCE posttreatment (day 0 to 3)				
Control	—	69.1 ± 17.0	566 ± 172	3
IJCE	3	62.2 ± 8.3 N.S.	619 ± 88 N.S.	4
IJCE	10	77.7 ± 12.7[b]	778 ± 123[a]	4
IJCE	30	140.8 ± 28.2[b]	1408 ± 273[b]	4
Exp. 4: IJ-medium (day −3 to 3)				
IJCE	10	65.7 ± 6.2[a]	657 ± 77[a]	3
Medium	10	23.1 ± 2.1	223 ± 21	4
	30	30.8 ± 7.7	308 ± 89	4

Mice were immunized i.v. with SRBC (day 0). Four days after SRBC challenge, anti-SRBC PFC was assayed. Data were expressed as mean ± S.E. Significantly different from control, *a*) $p < 0.05$ and *b*) $p < 0.01$. N.S., not significant; *n*, number of animals.

Table 2. Effects of IJCE on 5-FU-Induced Reduction of Spleen Weight, Cell Number and Anti-SRBC PFC Response in Mice

Treatment			Spleen weight (mg)	Number of cells ($\times 10^6$)	PFC per whole spleen ($\times 10^3$)	PFC per 10^6 spleen cells	*n*
SRBC	5-FU	IJCE dose (mg/kg/d)					
−	−	—	70 ± 6	68 ± 5	—	—	3
+	−	—	127 ± 6[b]	152 ± 15[b]	60.3 ± 4.9	448 ± 24	5
+	+	—	98 ± 7[a]	121 ± 7[a]	36.9 ± 1.6[a]	302 ± 25[a]	9
+	+	3	108 ± 3 N.S.	119 ± 5 N.S.	62.6 ± 7.7[a]	558 ± 102[a]	9
+	+	10	107 ± 3 N.S.	120 ± 5 N.S.	71.3 ± 6.3[b]	652 ± 62[b]	9
+	+	30	118 ± 4[a]	142 ± 7[a]	93.3 ± 13.0[b]	715 ± 94[b]	9

Mice were immunized with SRBC (day 0) and treated s.c. with 5-FU (20 mg/kg) once daily for 3 consecutive days before SRBC immunization. IJCE was ingested *p.o.* for 7 d (day −3 to day +3). Spleens were removed from mice 4 d after immunization and their weight, cell numbers and anti-SRBC PFC were assayed. Data are expressed as mean ± S.E. Significantly different from SRBC-immunized, 5-FU-treated control, *a*) $p < 0.05$ and *b*) $p < 0.01$. N.S., not significant; *n*, number of animals.

a dose-dependent manner (Table 1, exps. 2 and 3). The extract of IJ-medium used for culturing *Isaria japonica* was confirmed not to cause any stimulation of PFC response (Table 1, exp: 4).

Recovery of 5-FU-Induced Decrease of Lymphocytes and Anti-SRBC PFC Response by IJCE In the next series of experiments, the effect of IJCE on the 5-FU-induced immunotoxicity[12] was investigated. When the mice were s.c. treated with 20 mg/kg of 5-FU for three consecutive days before SRBC immunization, a significant reduction of spleen weight and cell number was observed (Table 2). However, with the same treatment after SRBC immunization, the reduction of spleen weight and cell numbers was significantly restored at 30 mg/kg.

The anti-SRBC PFC response was reduced with 5-FU to about 50% of that in normal mice (PFC/whole spleen cells). When IJCE was ingested at 10 and 30 mg/kg, anti-SRBC PFC production was increased over that in the normal mice (Table 2).

DISCUSSION

Under the present culture conditions, the hyphae of *Isaria japonica* YASUDA began to grow in 3 d and the proliferation reached a plateau 21 d after inoculation, thus obtaining a large quantity of homogeneous products by *Isaria japonica*. The *p.o.* administration of IJCE, which was prepared when the hyphae and carps were well grown in culture, exerted a significant adjuvant activity to anti-SRBC PFC production in mice. Moreover, IJCE restored the reduction of anti-SRBC PFC response and spleen cell number in 5-FU-treated mice.

It was not known what mechanisms were employed by IJCE to stimulate anti-SRBC production, which was one of the T cell-dependent immune responses requiring the cooperative involvement of B cells, T cells and macrophages. It is possible that IJCE is involved in the rather broad process of immune response, because it enhanced anti-SRBC response when given to mice either prior to or after immunization.

The active component(s) of IJCE remains to be determined. Thus far, many kinds of polysaccharides with adjuvant and/or host-mediated antitumor activities have been isolated from various natural sources. Recently, a polysaccharide derived from the genus *Cordyceps* was also found to give long-term survival to tumor-bearing mice.[6-8] All these polysaccharides are indeed effective when they are given animals *via* the i.p. and i.v. routes, however, they are not as effective when given *via* the *p.o.* route even at a high dose.[13-18] IJCE, a crude preparation, on the other hand, exhibited significant adjuvant activities upon *p.o.* ingestion in rather small doses (10 and 30 mg/kg), therefore, its active component(s) is suggested to be something other than polysaccharides.

IJCE did not show any significant toxicity to mice, judged by the lack of change in the weight of body and organs and the activity of hepatic marker enzymes (transaminases) in serum, even when used at a higher dose (>300 mg/kg/d for 7 consecutive days)(data not shown). All the results in the present study suggest that IJCE might have potential as a useful tool for immunological study or for relief from myelotoxicity caused by cancer chemotherapeutics.

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