

Note

## In Vitro Augmentation of Natural Killer Activity and Interferon- $\gamma$ Production in Murine Spleen Cells with *Agaricus blazei* Fruiting Body Fractions

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**Aqueous extracts of the *Agaricus blazei* fruiting body prepared at different temperatures were fractionated by ethanol precipitation with various ethanol concentrations. The original aqueous extracts of *A. blazei* failed to stimulate natural killer (NK) cell activity in murine spleen cells *in vitro*, but the strongest effect was observed in a 30% ethanol-soluble-50% ethanol-insoluble fraction prepared from the extract at 40 °C (fraction A-50). Fraction A-50 also showed the strongest augmenting effect on interferon (IFN)- $\gamma$  production. This augmentation of NK activity and IFN- $\gamma$  production by fraction A-50 was completely abrogated by a heat treatment.**

**Key words:** *Agaricus blazei* (*A. blazei*); natural killer (NK); interferon (IFN)- $\gamma$

The basidiomycete, *Agaricus blazei* Murill, a Brazilian native mushroom, has traditionally been used as a health food for preventing cancer, diabetes, hyperlipidemia, arteriosclerosis and chronic hepatitis in Brazil.<sup>1)</sup> The utilization of *A. blazei* as a health food source has been increasing in Japan in recent years. The anti-tumor effect of extracts from the *A. blazei* fruiting body and their fractions has been demonstrated in murine models of such transplantable tumors as Sarcoma 180 and Meth A cells.<sup>1–4)</sup> The mechanism underlying the anti-tumor effect of the mushroom seems to involve the augmentation of immunological responsiveness and the potentiation of the host-defense system through cellular immunity.<sup>3,5–7)</sup> However, details of the activation process are not yet clear. Natural killer (NK) cells play an important role in the innate immunity against infection and tumor development.<sup>8)</sup> To clarify whether *A. blazei* activates NK cells directly or not, we examined the effect of aqueous extracts from the *A. blazei* fruiting body and their ethanol-precipitated fractions on NK activity in murine spleen cells *in vitro*. The effect of the ethanol-precipitated fractions on interferon (IFN)- $\gamma$  production was also investigated.

Dried fruiting bodies (30 g) of *A. blazei* were pulverized and extracted with water (300 ml) at 40, 60, 80

and 100 °C for 4 h. The *A. blazei* powder was also subjected to autoclave extraction (120 °C, 2 h). Each extract was filtered and concentrated under reduced pressure. The aqueous extracts prepared at different temperatures were further fractionated by ethanol precipitation with various ethanol concentrations. The fractions derived from the aqueous extracts at 40, 60, 80, 100 and 120 °C are shown as fraction series A, B, C, D and E, respectively. Fraction series A is composed of fraction A-0 (the original extract at 40 °C), fraction A-10 (10% ethanol-insoluble fraction), fraction A-30 (10% ethanol-soluble-30% ethanol-insoluble fraction), fraction A-50 (30% ethanol-soluble-50% ethanol-insoluble fraction), fraction A-70 (50% ethanol-soluble-70% ethanol-insoluble fraction) and fraction A-70S (70% ethanol-soluble fraction). Fraction series B, C, D and E also have the same fraction composition. The fractionation procedure is briefly described next. To the powder (1.5 g) of each aqueous extract, 10 volumes of water (w/v) were added. After the undissolved matter had been removed by centrifugation, 1/9 (v/v) of cold ethanol was added. The solution was allowed to stand for 24 h at 4 °C and then centrifuged at 1670  $\times$  g for 30 min. Each first fraction of the 10% ethanol precipitate was designated a fraction A-10 to E-10. Cold ethanol was added to the supernatant to a final concentration of 30%. The solution was allowed to stand for 24 h at 4 °C and then centrifuged to give fractions A-30 to E-30. The stepwise addition of ethanol up to 70% and separation of the precipitates gave 5 fractions.

Female C57BL/6 mice, 6 or 7 weeks old, were purchased from Charles River Japan, Inc. (Yokohama, Japan). The care and use of the animals in this study followed the guidelines of Okayama University. All mice were used at 7 to 12 weeks of age. They were housed in a room with controlled temperature (23–25 °C) and humidity (50–60%) and a preset light-dark cycle (12 h:12 h), provided with sterile wood-chip bedding and given food and water *ad libitum* under specific pathogen-free conditions. These mice were sacrificed, and their spleens were removed. The spleens were

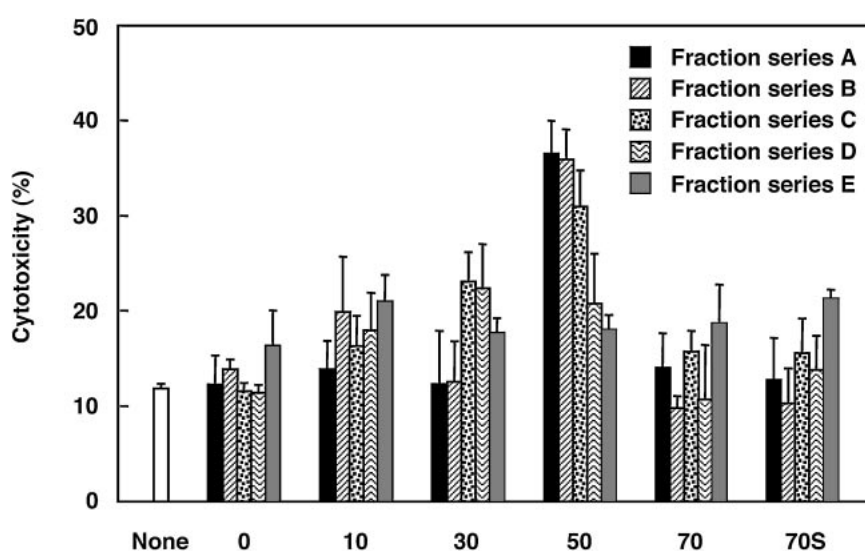
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minced, and a single-cell suspension was obtained in MEM. Spleen cells ( $1 \times 10^6$  cells/200  $\mu$ l/well), in which the red blood cells had been lysed by adding an ammonium chloride solution ( $\text{NH}_4\text{Cl}$ , 144 mM; Tris-HCl, 16.5 mM; pH 7.2), were cultured with various *A. blazei* fractions in 96-well flat-bottom microplates (167008; Nunc, Roskilde, Denmark) for 24 h. A phenol red-free RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml of penicillin G, and 100  $\mu$ g/ml of streptomycin was used as the culture medium. NK cell activity was determined by the target cell retention of the fluorescent dye, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, OR, U.S.A.).<sup>9,10</sup> YAC-1 cells were suspended in Hanks' balanced salt solution containing 5% FBS at  $1-3 \times 10^6$  cells/ml. BCECF-AM was added by 100-fold dilution of a 2.5 mmol/l stock solution in DMSO, and the cells were incubated at 37  $^\circ\text{C}$  for 30 min. After three washes in the cold medium, the target cells ( $10^4$ /well) were added to the pre-cultured spleen cells (effector cells) at an E:T ratio of 100:1 and immediately centrifuged at  $120 \times g$  for 3 min, before being cultured at 37  $^\circ\text{C}$  in an atmosphere of 5%  $\text{CO}_2$  and 95% air. After 4 h of incubation, the culture plate containing all the samples was centrifuged at  $420 \times g$  for 10 min. The resulting supernatant was removed from the cellular fraction by rapidly inverting the plate and flicking the supernatant out. The plate was blotted dry, and 200  $\mu$ l of 0.1% Triton X-100 in a 0.05 mmol/l borate buffer (pH 9.0) was added to each well. After allowing to dissolve for 10 min at room temperature, individual wells were assayed for fluorescence with a microplate fluorometer. The wavelengths of the filters used were excitation at 485 nm and emission at 527 nm. The

percentage of cytotoxic activity was calculated as: Cytotoxic activity (%) = (largest fluorescence-experiment group fluorescence)/(largest fluorescence-smallest fluorescence)  $\times$  100.

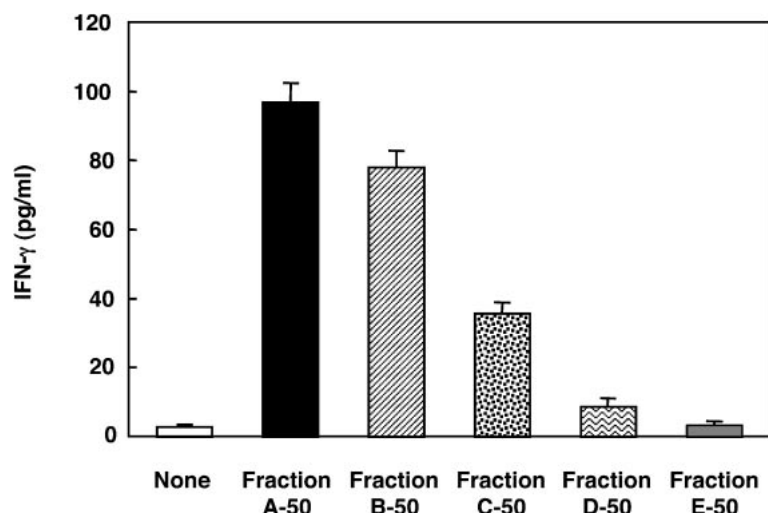
Mice spleen cells ( $1 \times 10^6$  cells/200  $\mu$ l/well) were cultured for 24 h in the presence of various *A. blazei* fractions. The IFN- $\gamma$  level in each cultured supernatant was measured by ELISA. An ELISA plate (Maxisorp 442404, Nunc) was coated overnight with 50  $\mu$ l of anti-mouse INF- $\gamma$  Ab (1.5  $\mu$ g/ml; Endogen, Woburn, MA, U.S.A.) at 4  $^\circ\text{C}$ . The coated plates with 50  $\mu$ l of the culture medium or standard INF- $\gamma$  added were incubated at room temperature for 1 h. Recombinant mouse INF- $\gamma$  (Endogen) was used as a standard. The plates with 50  $\mu$ l of biotin-conjugated anti-mouse INF- $\gamma$  Ab added (0.5  $\mu$ g/ml, Endogen) were incubated at room temperature for 1 h. The plates with 50  $\mu$ l of HRP-conjugated streptavidin added (diluted 1:5000; Zymed Lab, San Francisco, CA, U.S.A.) were further incubated at room temperature for 1 h. The wells were washed, and 100  $\mu$ l of a 3,3',5,5'-tetramethylbenzidine substrate solution was added to each well. The reaction was stopped by the addition of 50  $\mu$ l 0.18 M  $\text{H}_2\text{SO}_4$ /well, and the reaction products were measured with an ELISA reader at 450 nm.

It has been reported that the NK activity of spleen cells was increased by the oral administration of a hot-water extract of *A. blazei*.<sup>11</sup> We examined whether the fractionated extracts of *A. blazei* could directly enhance the NK cell activity in spleen cells *in vitro*. As shown in Fig. 1, fractions A-50, B-50 and C-50 significantly increased NK cell activity, although the original extracts, fractions A-0, B-0 and C-0 did not show such augmentation of NK activity. Fractions A-50, B-50 and C-50 also had an augmenting effect on NK cell activity



**Fig. 1.** Effect of *A. blazei* Aqueous Extracts and Fractions on NK Activity in Mice Spleen Cells.

Various ethanol fractions of *A. blazei* aqueous extracts were used as samples for NK activity. Spleen cells were cultured with 300  $\mu$ g/ml of a sample for 24 h. The NK activity against YAC-1 tumor cells was measured after the incubation. Each value is the mean  $\pm$  S.D. for triplicate cultures.



**Fig. 2.** Effect of Fractions A-50, B-50, C-50, D-50 and E-50 on IFN- $\gamma$  Production in Mice Spleen Cells.

Spleen cells were cultured with 300  $\mu\text{g/ml}$  of each 30% ethanol-soluble-50% ethanol-insoluble fraction for 24 h. The IFN- $\gamma$  level in each culture supernatant was measured by ELISA. Each value is the mean  $\pm$  S.D. for triplicate cultures.

in a concentration-dependent manner between 10  $\mu\text{g/ml}$  and 300  $\mu\text{g/ml}$  (data not shown). This NK cell activity decreased with increasing extraction temperature. The other fractions slightly increased or did not show any increased NK cell activity (Fig. 1). Although the original aqueous extracts of the *A. blazei* fruiting body did not augment the NK activity, ethanol fractionation of the extracts led to the discovery that the components of the *A. blazei* fruiting body directly activated NK cells. These results suggest that these extracts included both active and inactive compounds.

It is known that IL-2 and IFN- $\gamma$  are cytokines which are able to stimulate NK cell activity. The addition of the anti-IFN- $\gamma$  antibody abrogated the IL-2-induced NK activity, suggesting that IFN- $\gamma$  production may be required for the IL-2 induction of NK activity.<sup>12)</sup> Since the fractionated extracts of *A. blazei* increased the NK activity, it was investigated whether IFN- $\gamma$  production would be up-regulated in spleen cells cultured with the fractionated extracts. Fractions A-50, B-50 and C-50 markedly enhanced IFN- $\gamma$  production, the IFN- $\gamma$ -enhancing effect being in the order of fraction A-50 > fraction B-50 > fraction C-50 (Fig. 2). Fraction D-50 showed a slight effect, and fraction E-50 showed none. These results for the enhancement of IFN- $\gamma$  production are nearly in accordance with those observed for the augmentation of NK activity. IFN- $\gamma$  produced by NK cells also induces Th1- and CTL-mediated acquired immunity, suggesting that *A. blazei* expressed its anti-tumor activity *via* innate and adaptive immunity.

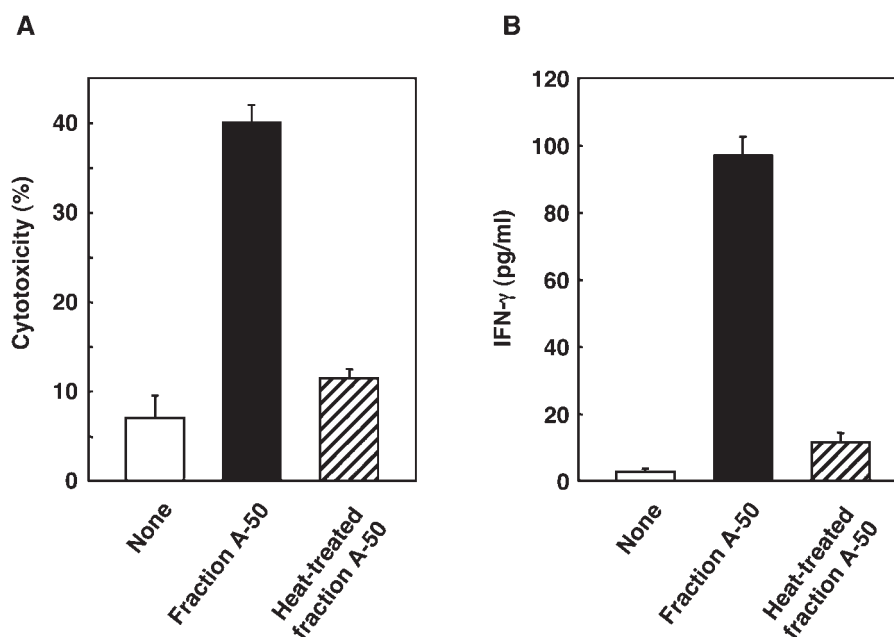
Fraction A-50 showed the strongest effect in augmenting the NK activity and IFN- $\gamma$  production. Since both the NK cell activity and IFN- $\gamma$  production decreased with increasing extraction temperature as already mentioned, we examined whether the effect of fraction A-50 on the NK activity and IFN- $\gamma$  production would be

influenced by heat treatment. A solution of fraction A-50 was heated at 120  $^{\circ}\text{C}$  for 2 h, and spleen cells were cultured with the heated fraction A-50. The augmentation of NK activity and IFN- $\gamma$  production was completely abrogated by the heat treatment (Fig. 3). These results indicate that the active components of fraction A-50 were susceptible to heat.

We found in this study the augmenting effects on NK activity and IFN- $\gamma$  production in fractions A-50, B-50 and C-50, these being the 30% ethanol-soluble-50% ethanol-insoluble fractions prepared from the aqueous extracts of the *A. blazei* fruiting body at 80  $^{\circ}\text{C}$  and below, although the original aqueous extracts did not show such effects. Fraction A-50 had the strongest effect in augmenting NK activity and IFN- $\gamma$  production. The augmentation of NK activity and IFN- $\gamma$  production was completely abrogated by a heat treatment at 120  $^{\circ}\text{C}$ . It is suggested that an *A. blazei* extract for enhancing NK activity *in vitro* requires extraction at below 80  $^{\circ}\text{C}$ , and then fractionation. We propose in the near future to investigate the mechanism for the fraction A-50-induced NK activity and IFN- $\gamma$  production, and to clarify the active compounds in fraction A-50.

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**Fig. 3.** Effect of Heat-Treated Fraction A-50 on NK Activity and INF- $\gamma$  Production in Mice Spleen Cells.

The fraction A-50 solution was heated at 120 °C for 2 h. Spleen cells were cultured with 300  $\mu$ g/ml of heat-treated fraction A-50 for 24 h. A, The NK activity against YAC-1 tumor cells was measured after incubation. B, The IFN- $\gamma$  level in each culture supernatant was measured by ELISA. Each value is the mean  $\pm$  S.D. for triplicate cultures.

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