Potentiation of Cytotoxic Activity in Naïve and Tumor-Bearing Mice by Oral Administration of Hot-Water Extracts from *Agaricus brazei* Fruiting Bodies

Hiroaki TAKIMOTO,[a] Daiko WAKITA,[a] Kiichiro KAWAGUCHI,[b] and Yoshio KUMAZAWA*,[a]

[a] *Department of Biosciences, School of Science, Kitasato University; and* [b] *Medicinal Plant Garden, School of Pharmaceutical Sciences, Kitasato University; 1–15–1 Kitasato, Sagamihara, Kanagawa 228–8555, Japan.*
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When hot-water extracts of mushroom *Agaricus brazei* MURRILL fruiting bodies (Agaricus extracts) were administered orally into Meth A-bearing mice, tumor growth was significantly inhibited compared with controls given water orally. Treatment with Agaricus extracts for five successive days significantly increased natural killer activity of spleen cells in naïve BALB/c mice. In Meth A-bearing BALB/c mice, Agaricus extracts enhanced the induction of antigen-specific cytotoxic T lymphocytes and interferon γ production, indicating that Agaricus extracts potentiate cytotoxic activity in innate and adaptive immunity.

Key words *Agaricus blazei*; Meth A; anti-tumor; natural killer (NK); cytotoxic T lymphocyte (CTL); interferon (IFN)-γ

The mushroom *Agaricus brazei* MURRILL, known as "Cogumelo do Sol", occurs naturally in a special region in the south of Sáo Paulo State, Brazil and has been cultivated under the name Kawarihiratake in Japan. Extracts from fruiting bodies of *A. brazei* MURRILL have been used on cancer patients for the purpose of tumor regression. The anti-tumor activity of these extracts were demonstrated using allogeneic Sarcoma 180 cells by either intraperitoneal injections of water insoluble residue[1] and β-glucan[2] or oral administration of lipid fractions.[3] In syngeneic Meth A tumor cell systems, either intratumoral injections of peptide-glucan preparation or oral administration of β-1, 6-D-glucan caused tumor regression.[4,5] However, the mechanisms by which extracts from *A. brazei* MURRILL and their fractions cause such regression are not yet clear. Recently, we established experimental conditions where oral administration of hot-water extracts from *A. brazei* MURRILL (Agaricus extracts) to Meth A-bearing BALB/c mice caused a significant suppression of tumor growth. To clarify the reason why Agaricus extracts cause such suppression, we investigated the possibility that the extracts stimulate different types of cells involved in innate and adaptive immunity in naïve and tumor-bearing mice.

MATERIALS AND METHODS

Mice Female BALB/c mice, purchased from Japan Clea Inc. (Tokyo), were used at 6 weeks of age and housed under specific pathogen-free conditions in the animal facility of Kitasato University School of Science. Mice were given daily oral administration of Agaricus extracts at a dose of 300 mg/d/mouse in a volume of 0.5 ml/d/mouse from 5 d after intradermal transplantation of 4×10^4 Meth A cells.

Preparation of Agaricus Extracts A commercially available Kohjusen, composed only of hot-water extracts of *A. brazei* MERRILL, was packaged by Asuke Pharmaceutical Co., Suwa, Nagano, and freeze-dried by our laboratory. The freeze-dried material was dissolved in distilled water before use. The content of β-glucan, estimated by a colorimetric analysis using the G-test, Seikagaku Corp., Tokyo, was 422 ng/ml.

Measurement of the Size and Weight of Tumors The tumor size was measured three times a week up to 21 d after transplantation using a micrometer and expressed as a value multiplying the short length (mm) and the long one (mm). The tumor weight was estimated on day 21.

Determination of Natural Killer (NK) Activity Naïve mice were administered Agaricus extracts orally for five consecutive days. The NK activity of spleen cells was determined by a ^{51}Cr-release assay.[6] Briefly, NK-sensitive YAC-1 cells as a target were labeled with 3.7 MBq of $Na_2{}^{51}CrO_4$ (specific activity; 7.4—18.5 GBq/mg Cr, Amersham Biosciences Corp., Piscataway, NJ, U.S.A.) at 37 °C for 1 h and washed three times. The labeled target cells (1×10^4) were mixed with effector spleen cells at the indicated ratios of effector (E) and target (T) cells. Supernatants were collected after incubation for 4 h and their radioactivity was determined by an Automated Gamma Counter (Wizard, Perkin-Elmer).

Cytotoxic T Lymphocyte (CTL) Assay Spleen cells (1×10^6/ml) were removed from mice that had been administered the extracts orally for 9 consecutive days from 5 d after the tumor inoculation, mixed with mitomycin C (MMC)-treated Meth A cells (1×10^5/ml) and incubated for 5 d to induce antigen-specific CTL. The CTL activity of antigen-stimulated spleen cells was determined using ^{51}Cr-labeled Meth A cells (1×10^4) as a target at different E : T ratios as described previously.[7]

Determination of IFN-γ by ELISA Spleen cells (1×10^6/ml) were removed from mice administered the extracts orally for 9 consecutive days from 5 d after the tumor inoculation, mixed with MMC-treated Meth A cells (1×10^5/ml) and incubated for 3 d to induce IFN-γ production. IFN-γ levels in culture supernatants were measured by ELISA as described previously.[6]

Statistics The statistical significance of differences between groups was evaluated by Student's *t*-test. A p value of less than 0.05 after comparison with the control group was considered significant.

RESULTS

Anti-tumor Activity of Agaricus Extracts To demon-

* To whom correspondence should be addressed. e-mail: kumazawa@jet.sci.kitasato-u.ac.jp
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strate the anti-tumor activity of Agaricus extracts, Meth A-bearing BALB/c mice received oral administration of either 300 mg of the extracts (containing 211 ng β-glucan) or distilled water in a volume of 0.5 ml once a day from 5 d after Meth A-transplantation until the end of the experiments. Agaricus extracts exhibited significant effects on inhibiting tumor growth (Fig. 1A) and decreasing in tumor weight (Fig. 1B).

Enhancement of NK Activity To estimate whether Agaricus extracts stimulate NK cells, naïve mice received oral administration of the test sample for 5 consecutive days. The NK activity of spleen cells from mice treated with the extracts increased significantly compared with those of controls (Fig. 2).

Activation of CTL To demonstrate whether *in vivo* treatment with Agaricus extracts increases in CTL induction, spleen cells from Meth A-bearing mice were incubated together with MMC-treated Meth A cells to induce antigen-specific CTL *in vitro*. Induction of higher CTL activity than that of controls was observed in spleen cells from Agaricus-treated mice (Fig. 3).

Increase in IFN-γ Production It is known that the magnitude of CTL induction is dependent on cytokines produced by Th1 cells. Since CTL induction was upregulated by treatment with Agaricus extracts, it was tested whether IFN-γ production was upregulated when spleen cells from Agaricus-treated Meth A-bearing BALB/c mice were stimulated *in vitro* with MMC-treated Meth A for 3 d. Treatment with the extracts resulted in significant enhancement of IFN-γ production (Fig. 4), indicating that this increase is due to upregulation of Meth A-specific T cell responses induced by Agar-

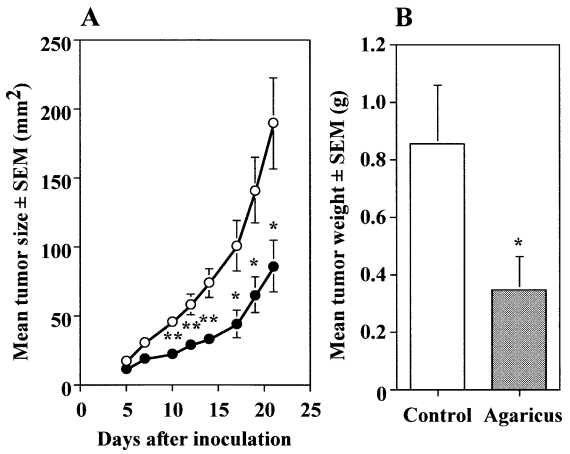

Fig. 1. Effect of Orally Administered Agaricus Extracts on Tumor Regression

BALB/c mice were administered *p.o.* with Agaricus extracts (closed circles) or water (open circles) every day from 5 d after inoculation with 4×10^4 Meth A fibrosarcomas into the right flank. (A), tumor size was estimated three times per week starting on day 5. (B), tumor weight was estimated on day 21. Data represent arithmetic mean±S.E.M. ($n=10$). $*p<0.05$ and $**p<0.01$ (by Student's *t*-test, *vs.* tumor alone control).

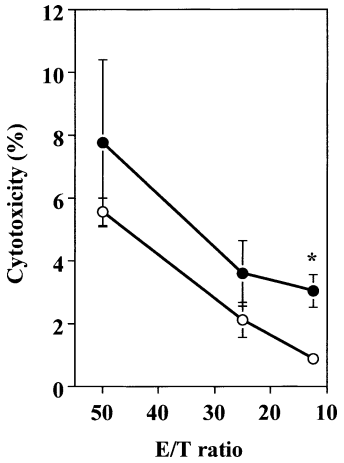

Fig. 2. Enhancement of NK Activity by Treatment with Agaricus Extracts

BALB/c mice were administered *p.o.* with Agaricus extracts once a day for 5 d. Splenocytes from mice treated with these extracts (closed circles) or with water (open circles) were mixed with ^{51}Cr-labeled YAC-1 cells (1×10^4). Chromium release in the supernatant was measured after 4 h of incubation. Data show mean±S.E.M. ($n=3$) and asterisk shows significant difference ($*p<0.05$). The data represent two independent experiments with similar results.

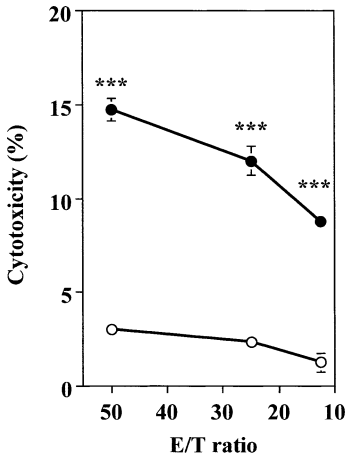

Fig. 3. Enhanced Expansion of CTL Precursors by Treatment with Agaricus Extracts

BALB/c mice were inoculated with 1×10^6 Meth A fibrosarcomas in the right flank, and then administered *p.o.* once a day with Agaricus extracts (closed circles) or water (open circles) from 5 d after tumor inoculation and thereafter. Splenocytes, obtained from mice treated with Agaricus extracts or water on day 14 after tumor inoculation, were cultured with mitomycin C-treated Meth A for 5 d, and then cultured together with ^{51}Cr-labeled Meth A fibrosarcomas (1×10^4). Chromium release into the supernatant was measured after 4 h of incubation. Data represent arithmetic mean±S.E.M. ($n=3$) and asterisks show significant difference ($***p<0.001$). The data represent two independent experiments with similar results.

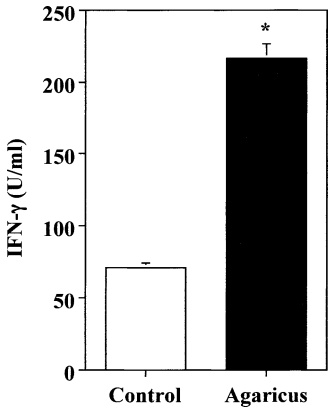

Fig. 4. Enhancement of IFN-γ Producing Activity by Treatment with Agaricus Extracts

Spleen cells, obtained from mice treated with Agaricus extracts (closed) or water (open) on day 15 after 1×10^6 Meth A inoculation, were stimulated *in vitro* with MMC-treated Meth A for 3 d. The amounts of IFN-γ in culture supernatants were determined by ELISA. Data show mean±S.E.M. ($n=3$) and asterisk shows significant difference ($*p<0.05$). The data represent two independent experiments with similar results.

icus extracts.

DISCUSSION

Natural products stimulate us to investigate their immunomodulating activities, since hot-water extracts of *Angelica acutiloba* exhibited significant anti-tumor activity.[8] Chinese medicines and plant extracts such as glucans, alkaloids, saponins and flavonoids exhibit immunomodulating properties in terms of anti-tumor activity,[9] activation of macrophages,[10—12] inhibition of γ-irradiation-induced leukopenia,[13] enhanced protection against microbial infections,[14] and suppression of lipopolysaccharide-induced TNFα production.[15,16] In the present study, we demonstrated that commercially available Agaricus extracts, such as Kohjusen in Japan, exhibited significant anti-tumor activity when the extracts were administered orally to Meth A-bearing BALB/c mice at a dose of 300 mg/d/mouse. The oral administration of Agaricus extracts to these mice caused activation of NK cells, and induced CTL to increase innate and adaptive immunity, respectively. Upregulation of NK and CTL activity are triggered by IL-12-dependent activation.[17] It is not yet clear whether oral administration of Agaricus extracts enhances IL-12 production *in vivo*. Generally, tumor-bearing mice tend to shift to Th2 rather than Th1 in the Th1/Th2 balance. Thus the mechanisms by which oral administration of Agaricus extracts resulted in significant induction of CTL might be expressed by the shift to Th1 due to IL-12-dependent activation. In our preliminary experiments, oral administration of Agaricus extracts decreased antigen-specific IgE production and infiltration of eosinophils into the lungs, indicating that the extracts inhibit these Th2-shifted responses. In addition, the IFN-γ producing ability of spleen cells from Agaricus-treated Meth A mice significantly increased (Fig. 4). Thus, it is possible that Agaricus extracts express anti-tumor activity *via* IL-12-dependent activation, following the induction of a Th1 response. In conclusion, daily uptake of Agaricus extracts may have potential for the treatment of human cancers.

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