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Antioxidant activity and anti-tumor immunity by *Agaricus*, *Propolis* and *Paffia* in mice

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Key Words: Antioxidant activity, Anti-tumor immunity, *Agaricus*, *Propolis*, *Paffia*

Abstract

Various previously intractable diseases have been overcome by the development of many new medicines. However, cancer is still a major cause of death. In the process of carcinogenesis, a multistep accumulation of gene mutations causes malignant transformation, and the probability of gene mutations is different depending on genetic and environmental factors. Individual differences are found in the susceptibility to cancer, and prevention of carcinogenesis is possible. In South America, natural products with unknown drug effects are used as folk remedies and for preventive medicine. Among South American natural products, we directed our attention to *Agaricus*, *Propolis and Paffia*, which have been known as medicinal plants, and examined the mechanisms by which these substances affect antioxidant activity, anti-tumor activity and immunoresponse.

When the antioxidant activities of *Agaricus*, *Propolis and Paffia* were examined by the DPPH and Rhoudan iron methods, since Propolis contains high levels of fravonoids, it is thought that fravonoids may be responsible for the antioxidant activity in this study.

In the examination of immunoenhancement activity, we measured lymphocyte versus polymorophonuclear leukocyte ratios (L/P activity). The number of lymphocytes was significantly increased in groups treated with Proplolis. Specifically, slightly high levels of IFN-γ were measured in mice bearing the S-180 carcinoma, after administration of *Agaricus*, *Propolis and Paffia*. This strongly suggests that cellular immunity is especially activated by treatment with *Agaricus*, *Propolis and Paffia*, because production of IFN-γ is limited to the T cells and NK cells stimulated by mitogen and sensitized antigen. TNF-α shows a different extent and mechanism of action depending on the target cells. When TNF-α was measured in mice bearing the S-180 carcinoma, mice treated with *Agaricus*, *Propolis and Paffia* showed slightly higher TNF-α levels as compared to the control group. This suggests that activated macrophages produce TNF-α in mice treated with *Agaricus*, *Propolis and Paffia*, since activated macrophages and lymphocytes are the source of most TNF-α. When anti-tumor action was examined

using two kinds of sarcoma (Sarcoma-180 carcinoma), tumor-suppressive ratios after treatment with Propolis was 29.1%. When Sarcoma-180 solid carcinoma was used, tumor-suppressive ratios were 62%. Thus, Propolis showed strong anti-tumor activity against two kinds of solid carcinoma. Taken altogether, this strongly suggests that *Agaricus, Propolis and Paffia* enhances original functions of macrophages and NK cells, and as a result, secondarily enhances the immune reaction and suppresses tumor growth.

1. Introduction

Various previously intractable diseases have been overcome by the development of many new medicines. However, cancer is still a major cause of death. In the process of carcinogenesis, a multistep accumulation of gene mutations causes malignant transformation, and the probability of gene mutations is different depending on genetic and environmental factors. Individual differences are found in the susceptibility to cancer, and prevention of carcinogenesis is possible[1]. In South America, natural products with unknown drug effects are used as folk remedies and for preventive medicine. South American natural products, we directed our attention to Propolis, Agaricus and Paffia, which have been known as medicinal plants, and examined the mechanisms by which these substances affect antioxidant activity[2], anti-tumor activity[3] and immunoresponse[4].

Propolis is a resin prepared by combining leaf bud and sap collected by honey bees with secreted salivary juice, bee solder and pollen. Poplar, truffle, fagus, red horsechesnut, and eucalyptus are major trees for collection. The taste and smell of Propolis differs depending on the species of tree. Therefore, ingredients are subtly different. Honeybees combine sap with saliva, and then used it for repairing beehives. This keeps the interior of the beehives warm and sterile. Major constituents of Propolis, a natural antibiotic, are flavonoids, organic acids, phenols, various kinds of enzymes, vitamins and minerals[2] Flavonoids are further classified into about 2000 species such as flavone, flavonol, and flavanone. Flavonoids promote the formation and regeneration of cells and tissues which are resistant to the invasion of viruses and bacteria by promoting cell activation involved in clearing of the blood and strengthening of the cellular membranes. Thus, Propolis has many physiological functions, and there are reports that Propolis has important direct actions on capillary vessels and in reducing inflammation[3]. These activities include antibacterial action[4], analgesic/anti-inflammatory activity[5], antioxidant activity[6)-8)], immunoenhancement activity[9], anti-tumor activity[10)-11)], and anti-allergic activity[12].

A kind of basidiomycetes, the Agaricus mushroom (*Agaricus blazei Murill*), is native to the highlands located in the suburbs of São Paulo in Brazil. This mushroom, which has been used for food in the Piedade Mountains since the Inca era, was named "Cogumelo do Sol" about twenty years ago. Agaricus extracts contain vitamins, minerals, nucleic acids, amino acids, and polysaccharides (α-glucan, β-glucan, xyloglucan, β-galactoglucan). Lipids from this mushroom are characterized by a high content of phosphorus. Major fatty acids include linoleic acid, palmitic acid, stearic acid, and oleic acid and unique fatty acids such as palmitoleic acid have been indentified[13)-14)]. There are also reports that oleic acid and linoleic acid show anti-tumor effects on ascites carcinoma[15)-18)]. In addition, it is reported that the active constituents of Agaricus differ depending where they are produced[19].

Paffia (*Pfaffia paniculata*), which is called Brazil carrot or Amazon carrot, is a perennial plant (*Amaranthaceae)*, and the countries of origin include Brazil, Ecuador, Panama, Peru, and Venezuela. Paffia is called "Paratoda", meaning "useful for all". Paffia has long been used as an old wives' remedy. The nutritional constituents of Paffia consist of 19 kinds of amino acids, electrolytes, minerals (magnesium, cobalt, silica, and zinc), vitamins (A, B_1, B_2, E, K) and saponins[20]. It is thought that Paffia from North America and South America is a substance which enhances rejuvenation, restoration of various body functions and immunostimulation based on it's tonic

action. In addition, it is known to be effective in viral infectious diseases, chronic fatigue syndrome, hypoglycemia, impotence, arthritis, cancer, and mononuclear leukemia[21)-25)].

We investigated the antioxidant activity, lymphocyte versus polymorphonuclear leukocytes enhancement activity (L/P activity) and anti-tumor activity of these three simple substances (Agaricus, Propolis and Paffia) as well as a mixture of these three kinds of substances (ABP), and reported in The Pharmaceutical Society of Japan, The Japan Society of Oriental Medicine and The Japanese Cancer Association[26)-28)]. In the present paper, we summarize findings reported at these meetings.

2. Experimental samples

2.1 Test materials

Original samples of Agaricus, Propolis and Paffia, produced in Brazil, were supplied by Shizenkyosei Corp.

A powdered mixture (ABP) of these three substances was prepared using an agate mortar.

For the extraction, 2,000 ml of water was added to 300g of finely powdered Agaricus, and then stirred for 2 hr in a water bath at 40℃. After centrifugation for 10 min at 5000 rpm, the supernatant was filtered using folded filter paper (TOYO Roshi, Ltd., NO. 131.) Distilled water (2,000 ml) was added to the precipitates, and extraction was repeated in the same way. Dried Agaricus was obtained by combining the supernatant from the first extraction with the supernatant from the second extraction (yield: 48.0g, recovery: 16%). Propolis (100g) was powdered, and 300 ml of 70% ethanol was added. After drying at room temperature, filtration was carried out using folded filter paper. The filtrates were dried using a evaporator. Dried Propolis was obtained by freezing and thawing (yield: 53.5g, recovery: 53.5%). Distilled water (2000 ml) was added to 200g of finely powdered Paffia. Dried Paffia was obtained by the same technique (yield: 83.6g, yield: 41%).

2.2 Animals and feeding conditions

Male ICR Mice, purchased from Japan Clea Inc., were used at 4 weeks of age. After preliminary feeding for one week, these mice were used for experiments. Animals were housed in the animal facility at 23 ± 10℃ humidity, 55 ± 5% with a 12 h light-12h dark cycle. The animals had food (Japan Clea Inc., CE-2) and water ad libitum.

3. Experimental Methods

3.1 Antioxidant activity of the mixture (ABP) and of each simple constituent (Agaricus, Propolis and Paffia)

3.1.1 Measurement of DPPH radical scavenging activity

To examine radical scavenging activity, we measured the reactivity of DPPH, a stable free radical as a model of unsaturated fatty acid radicals.

DPPH (1,1-diphenyl-2-picrylhydrazyl), MES buffer (2-morpholinoethanesulfonic acid, monohydrate) and ethanol were purchased from Wako Pure Chemical Industries, Inc. Trolox, used as positive standard material, was purchased from Aldrich Chemical Co. lnc., and 400 μM DPPH, 0.2 M MES buffer, 80% ethanol, 20% ethanol and 0.2mM Trolox were prepared. All reagents were commercially available specialgrades.

A mixture consisting of 15 ml of 400 μM DPPH, 15 ml of 0.2M MES buffer (pH6.0) and 15 ml of 20% ethanol was prepared. To 0.9 ml of the mixture, (300-a) μl of 80% ethanol was added. As test samples, 1% of ABP, 0.5% Agaricus, 0.20% Propolis and

0.15% Paffia were prepared, and then (0, 30, 60, 90, 120 and 150) μl of samples were serially added at intervals of 30 seconds. The solution was stirred using a vortex mixer and the absorbance at 520 nm was serially measured at intervals of 30 seconds and 20 min after adding the samples. Trolox, which was positive standard material and a water-soluble derivative of tocopherol, was adjusted to concentrations as low as 0.2 mM, and added in the order of 0, 30, 60, 90, 120 and 150 μl[29)-30)].

3.1.2 Antioxidant activity based on Rhodan iron method

The Rhodan iron method was used for evaluating peroxide lipids generated by lipid oxidation. Divalent iron was oxidized to trivalent iron by the reaction of divalent iron with peroxide lipid (LOOH). Red Rhodan iron, $Fe(SCN)_3$ was generated by reacting this trivalent iron with ammonium thiocyanate (SCN). Lipid peroxidation was evaluated by colorimetric analysis.

Linoleic acid was purchased from Nacalai Tesque, Inc. Ammonium thiocyanate, Iron (II) chloride tetrahydrate, HCl solution, ethanol, potassium phosphate dibasic, and potassium phosphate monobasic were purchased from Wako Pure Chemical Industries, Inc. After dissolving 0.08413g of linoleic acid in 3 ml of ethanol, this solution was diluted to 15 ml with 0.1 M phosphate buffer at a final concentration of 0.02 M, and used for experiments. To the mixture prepared by combining 1 ml of 0.2 M of the original linoleic acid solution, 0.8 ml of phosphate buffer, and 0.2 ml of 1% Agaricus, 0.5% Propolis or 0.15% of Paffia were added as a sample. After stirring, auto-oxidation of linoleic acid was carried out by incubation at 37℃ for 24, 72, 120 and 150 hr. Trolox and distilled water were used as a control and blank, respectively. Then, 4.7 ml of 75% ethanol, 0.1 ml of 30% Rhodan ammonium and 0.1 ml of 0.2 mM ferrous chloride in 3.5% HCl were added to the reaction mixture after 1, 3, 5 and 7 days, and absorbance at 500 nm was measured after 3 min[31)-33)].

3.2 Lymphocyte versus polymorphonulcear leukocyte ratio enhancement activity (L/P activity)

The Hand et al.[35)] modification of the method of Metcalf et al.[34)], was used. Immunologically immature neonatal litters of Swiss-Webster mice (Japan SLC, Inc.), within 6-12 hr after birth were divided into two groups. In the control group, 0.02 ml of saline was injected intraperitoneally, and in the sample groups, ABP (200 μg/mouse), Agaricus (200 μg/mouse), Propolis (200 μg/mouse) or Paffia (200 μg/mouse) was injected in 0.02 ml intraperitoneally. Blood was collected from the tail vein before injection, and 6, 10 and 14 days after injection. Thin-layer blood smears were prepared and stained by Wright's staining method. The number of lymphocytes and polymorphonuclear leukocytes were counted, so that the total cell number was 100, using a microscope with a mechanical stage, and the ratio of the number of lymphocytes to the number of polymorphonuclear leukocytes (L/P ratio) was calculated Efficacy was evaluated according to the method of Mizutani et al.[36)]. A p value < 0.05 by t-test was considered statistically significant.

3.3 Anti-tumor activity

3.3.1 Anti-tumor activity in Ehrlich solid carcinoma

Ten male ICR mice (Japan SLC, Inc.) were used at 4 weeks of age per group. Twenty-four hours after transplantation of Ehrlich solid carcinoma (1×10^6 cells, 0.05 ml), distilled water (10 ml/kg), ABP (400 mg/kg B. W.), Agaricus (200 mg/kg B. W.), Propolis (80 mg/kg body weightB. W.) or Paffia (60 mg/kg B. W.) were administered orally for 34 days. The size of

the tumor (long length × short length × thickness, in mm^3) was measured using a micrometer five times every other week for 35 days. Tumors were removed 35 days after transplantation, and the weight of the tumor (g) was measured. Inhibitory ratio in tumor growth was calculated according to the following formula.

3.3.2 Anti-tumor activity in Sarcoma-180 solid carcinoma

Ten male ICR mice (Japan SLC, Inc.) at 4 weeks of age were used per group. Sarcoma-180 solid carcinoma (1×10^6 cells, 0.05 ml) was transplanted into the right inguinal region subcutaneously. Twenty-four hours after tranplantation, distilled water (10 ml/kg B. W.) was administered orally to the control group, and ABP (400 mg/kg B. W.), Agarics (200 mg/kg B. W.), Propolis (80 mg/kg B. W.) or Paffia (60 mg/kg B. W.) was administered orally to the other groups for 34 consecutive days. The size of tumor was measured by measuring the length of the largest diameter using a micrometer from the surface of the skin five times every other week from 7 days to 35 days after transplantation. At 35 days after transplantation, tumors were removed, and the weight (g) of tumors were measured. The inhibitory ratio in tumor growth compared with the control group treated with distilled water was calculated according to the formula described above.

3.4 Mechanism affecting immunoresponse of tumor-bearing mice

3.4.1 Measurement of interferon gamma (IFN-γ)

An ELISA (enzyme-linked immunosorbent assay) method was used for the measurement of IFN-γ using a mouse IFN-γ ELISA kit (Amersham Biosciences, Inc.).

Ten male ICR mice at 4 weeks of age were used per group. Sarcoma-180 solid carcinoma (1×10^6 cells) was transplanted into the right inguinal region subcutaneously. Distilled water (10 ml/kg B. W.) was administered orally to the control group, and ABP (400 mg/kg B. W.), Agaricus (200 mg/kg B. W.), Propolis (80 mg/kg B. W.) or Paffia (60 mg/kg B. W.) was administered orally to the sample groups Four weeks after administration, 5 ml of PBS was injected into the mice intraperitoneally. After rubbing the abdominal region, peritoneal fluid was collected. The resulting peritoneal fluid was frozen at − 20℃ for 3 hr. After defrosting the frozen fluid, the supernatant obtained by centrifugation (3000 rpm, 10 min) was transferred to a dialysis membrane. The solution was concentrated using ~~of~~ polyethylene glycol (#4000) and used as a sample. Fifty micro liters of the sample and the standard were distributed to each well and incubated at room temperature (20 − 25℃) for 120 min after covering with a plate cover. Then, 50 μl of biotinylated antibody reagent was added to each well, and incubation was carried out at room temperature (20 − 25℃) for 60 min. After washing three times with wash buffer, 100 μl of streptavidin-HRP incubation medium was added and wells were covered with a plate cover. After incubation at room temperature for 30 min, washing was carried out three times with wash buffer. One hundred micro liters of TMB solution was added and incubation was carried out for more than 30 min. Incubation times were determined according to the extent of development of blue color. After incubation, 100 μl of stop solution was added. Absorbance at 450 nm was measured using a Labsystems Multiskan MS-UV (Dainippon Pharmaceutical Co., Ltd) within 30 min and the amount of IFN-γ was estimated based on absorbance using a standard calibration curve.

3.4.2 Measurement of tumor necrosis factor-alpha (TNF-α)

Measurement of TNF-α was carried out by an ELISA method using a mouse TNF-α ELISA kit (Pierce Biotechnology, Inc.).

Ten male ICR mice at 4 weeks age were used per group. Sarcoma-180 solid carcinoma was transplanted into right the inguinal region subcutaneously. Distilled water (10 ml/kg B. W.) was administered orally to the control group, and ABP (400 mg/kg B. W.), Agaricus (200 mg/kg B. W.), Propolis (80 mg/kg B. W.) or Paffia (60 mg/kg B. W.) was administered orally to the experimentsl groups Four weeks after administration, 5 ml of PBS was injected intraperitoneally. After rubbing the abdominal region, peritoneal fluid was collected. The resulting peritoneal fluid was frozen at -20℃ for 3 hr. After defrosting the frozen fluid, the supernatant obtained by centrifugation (3000 rpm, 10 min) was transferred to a dialysis membrane. The solution was concentrated using polyethylene glycol (#4000) and used as a sample. Fifty micro liters of the sample and the standard were distributed to each well and incubated at room temperature (20 − 25℃) for 120 min after covering with a plate cover. Then, 50 μl of biotinylated antibody reagent was added to each well, and samples were incubated at room temperature (20 − 25℃) for 2 hr. After washing five times with wash buffer, 100 μl of HRP solution was added and wells were covered with a plate cover. After incubation at room temperature (20 − 25℃) for 30 min, washing was carried out five times with wash buffer. One hundred micro liters of TMB solution was added and incubation was carried out for more than 30 min. Incubation time was determined according to the extent of development of blue color. After incubation, 100 μl of stop buffer was added. Absorbance at 450 nm was measured using a Labsystems Multiskan MS-UV (Dainippon Pharmaceutical Co., Ltd) within 30 min after addition of stop buffer and the amount of TNF-α was estimated based on absorbance using a standard calibration curve.

3.4.3 Measurement of IgM

IgM was measured by an ELISA method using a mouse IgM ELISA quantitation kit (Bethyl Laboratories, Inc., catalog No.: E90-101) and an ELISA starter accessory package (Bethyl Laboratories, Inc., catalog No.: E101).

Ten male ICR mice at 4 weeks of age were used per group. Sarcoma-180 solid carcinoma (1×10^6 cells) was transplanted into the right inguinal region subcutaneously. Distilled water (10 mg/kg B. W.) was administered orally to the control group, and ABP (400 mg/kg B. W.), Agaricus (200 mg/kg B. W.), Propolis (80 mg/kg B. W.), or Paffia (60 mg/kg B. W.) was administered orally into the sample groups Four weeks after administration, blood was collected from the eyeground. Resulting serum was used as sample.

Affinity-purified antibody was diluted 100-fold with coating buffer and capture antibody solution was prepared. One hundred micro liters of solution was transferred to each well, and incubation was carried out for 60 min. Capture antibody solution was then aspirated, and each well was washed twice with wash solution. Next, 200 μl of postcoat solution was added to each well, and incubation was carried out for 30 min. Postcoat solution was then aspirated, and washing was carried out twice with wash solution. One hundred micro liters of sample diluted 500-fold and mouse reference serum prepared by dilution with standard diluent were transferred into each well, and incubation was carried out for 60 min. After washing the well four times with wash solution, 100 μl of antibody/HRP conjugate in conjugate diluent,

which was prepared by diluting goat anti-mouse IgM-Fc-HRP 120000-fold with sample/conjugate diluent, was transferred into each well. After incubation for 60 min, wells were washed 4 times with wash solution. Finally, 100 μl of substrate solution (TMB) was added to each well. After incubating for 10 min, the reaction was stopped by adding 100 μl of 2 M H_2SO_4 to each well, and absorbance at 450 nm was measured using a Labsystems Multiskan WS-UV (Dainippon Pharmacetutical Co., Inc.).

3.4.4 Measurement of IgG

IgG was measured by an ELISA method using a mouse IgG ELISA quantitation kit (Bethyl Laboratories, Inc., catalog No.: No. E90-131) and an ELISA starter accessory package (Bethyl Laboratories, Inc., catalog No.: E101).

Ten male ICR mice at 4 weeks of age were used per group. Sarcoma-180 solid carcinoma (1×10^6 cells) was transplanted into the right inguinal region subcutaneously. Distilled water (10 ml/kg B. W.) was administered orally to the control group, and ABP (400 mg/ml B. W.), Agarics (200 mg/kg B. W.), Propolis (80 mg/kg B. W.) or Paffia (60 mg/kg B. W.) was administered orally to experimental groups. Four weeks after administration, blood was collected from the eyeground and serum was used as samples.

Capture antibody solution was prepared by diluting affinity-purified antibody 100-fold with coating buffer. One hundred micro liters of the resulting solution was transferred to each well. After incubation for 60 min, capture antibody solution was aspirated, and each well was washed twice with wash solution. Next, 200 μl of postcoat solution was added to each well. After incubation for 30 min, postcoat solution was aspirated, and the well was washed twice with wash solution. In addition, 100 μl of the sample diluted 1000-fold and the standard prepared by diluting mouse reference serum with standard diluent were transferred to each well. After incubation for 60 min, the well was washed four times with wash solution. Then, 100 μl of antibody/HRP conjugate in conjugate diluent, which was prepared by diluting goat anti-mouse IgG-Fc-HRP conjugate ~~to~~ 90000-fold with sample/conjugate diluent, was transferred to each well. After incubation for 60 min, the well was washed four times with wash solution. Finally, 100 μl of substrate solution (TMB) was added to each well, and incubation was carried out for 7 min. The reaction was stopped by adding 100 μl of 2 M H_2SO_4, and absorbance at 450 nm was measured using a Labsystem Multiskan MS-UV (Dainippon Pharmacetutical Co., Inc.).

4. Results

4.1 Antioxidant activity

4.1.1 Results of DPPH radical scavenging activity

With respect to radical scavenging activity, water and Paffia, both of which had no radical scavenging activity, showed no marked difference, whereas Agaricus showed slight radical scavenging activity. Both ABP and Propolis showed greater radical scavenging activity than 0.2 mM Trolox, which was used as a positive standard (Fig. 1).

4.1.2 Results of antioxidant activity by Rhodan iron

Antioxidant activity of ABP, Agaricus, Propolis and Paffia was measured by observing the auto-oxidation activity of linoleic acid. controls and Paffia showed maximal peroxide values at day 1, and thereafter decreased with time. Agaricus showed maximal values at day 3, and thereafter tended to decrease. Trolox, which was used as a positive standard, showed little or no increase, and high antioxidant activity. On the other hand, ABP and Propolis

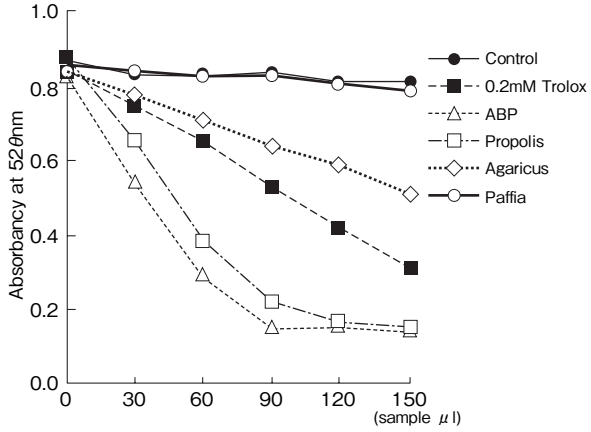

Fig. 1. DPPH radical scavenging effects. Whereas Agaricus showed slight radical scavenging activity. Both ABP and *Propolis* showed greater radical scavenging activity than 0.2 mM Trolox, which was used as a positive standard.

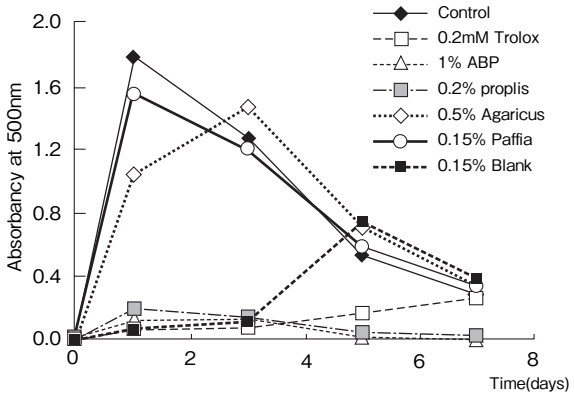

Fig. 2. Rodin iron method. Antioxidant activity of *ABP*, *Agaricus*, *Propolis* and *Paffia* was measured by observing the auto-oxidation activity of linoleic acid. controls and *Paffia* showed maximal peroxide values at day 1, and thereafter decreased with time.

showed little or no peroxide activity, and high antioxidant activity (Fig. 2).

4.2 Lymphocyte versus polymorphonuclear leukocyte enhancement activity (L/P activity)

Ten neonatal Swiss-Webster mice were divided into two groups. Saline was injected intraperitoneally into one group, and Agaricus was injected intraperitoneally into the other group at a dose of 200 μg/mouse, as shown in Fig. 3. The arrow (↑) shows the day of injection. Significant differences from the control group ($p < 0.01$) were shown 6, 10 and 14 days after injection.

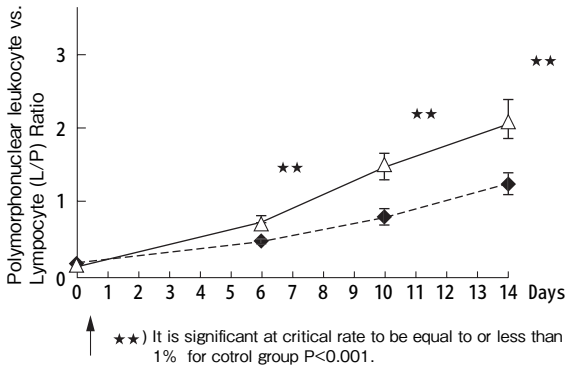

Fig. 3. Days change by *agaricus*. The arrow (↑) shows the day of injection. Significant differences from the control group ($p < 0.01$) were shown 6, 10 and 14 days after injection.

Ten neonatal Swiss-Webster mice were divided into two groups. Saline was injected intraperitoneally into one group, and Propolis was injected intraperitoneally into the other group at a dose of 200 μg/mouse, as shown in Fig. 4. The arrow (↑) shows the day of injection. Significant differences from the control group ($p < 0.01$) were shown 6, 10 and 14 days after injection.

Ten neonatal Swiss-Webster mice were divided into two groups. Saline was injected intraperitoneally into one group, and Paffia was injected intraperitoneally into the other group at a dose of 200 μg/mouse, as shown in Fig. 5. The arrow (↑) shows the day of injection. Significant differences from the control group ($p < 0.01$) were shown 6, 10 and 14 days after injection.

Ten neonatal Swiss-Webster mice were divided into two groups. Saline was injected intraperitoneally into one group, and ABP was injected intraperitoneal-

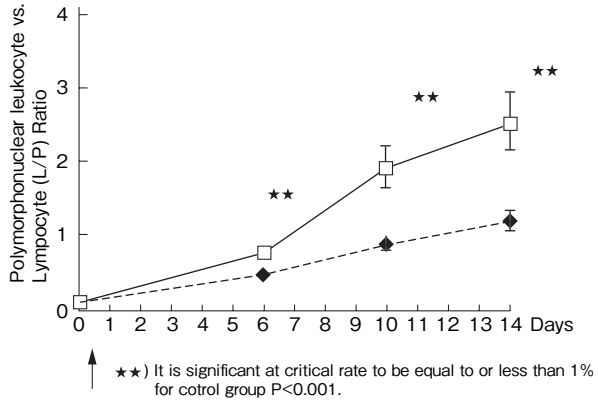

Fig. 4. Days change by *propolis*. The arrow (↑) shows the day of injection. Significant differences from the control group ($p < 0.01$) were shown 6, 10 and 14 days after injection.

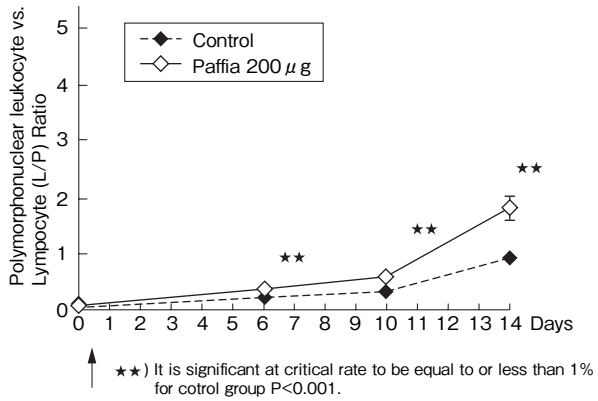

Fig. 5. Days change by *Paffia*. The arrow (↑) shows the day of injection. Significant differences from the control group ($p < 0.01$) were shown 6, 10 and 14 days after injection.

ly into the other group, as shown in Fig. 6. The arrow (↑) shows the day of injection. Significant differences from the control group ($p < 0.01$) were shown 6, 10 and 14 days after injection.

L/P ratios after the administration of Agaricus (200 μg/mouse) were 0.78 ± 0.06 (day 6), 1.49 ± 0.18 (day 10) and 2.12 ± 0.26 (day 14), whereas L/P ratios in the control group were 0.49 ± 0.04 (day 6), 0.79 ± 0.09 (day 10) and 1.26 ± 0.15 (day 14). The L/P ratio fter the administration of Agaricus was significantly elevated ($p < 0.01$), as compared to the

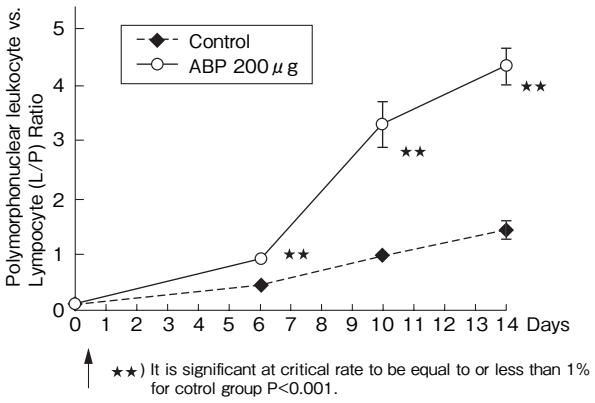

Fig. 6. Days change by ABP. The arrow (↑) shows the day of injection. significant differences from the control group ($p < 0.01$) were shown 6, 10 and 14 days after injection.

control group.

L/P ratios after the administration of Propolis (200 μg/mouse) were 0.78 ± 0.09 (day 6), 1.95 ± 0.28 (day 10) and 2.55 ± 0.39 (day 14), whereas L/P ratios in the control group were 0.50 ± 0.03 (day 6), 0.89 ± 0.07 (day 10) and 1.22 ± 0.15 (day 14). The L/P ratio after the administration of *Propolis* was significantly elevated ($p < 0.01$), as compared to the control group.

L/P ratios after the administration of *Paffia* (200 μg/mouse) were 0.38 ± 0.02 (day 6), 0.60 ± 0.02 (day 10) and 1.72 ± 0.18 (day 14), whereas L/P ratios in the control group were 0.22 ± 0.01 (day 6), 0.35 ± 0.02 (day 10) and 0.93 ± 0.03 (day 14). The L/P ratio ~~in~~ after the administration of Propolis was significantly elevated ($p < 0.01$), as compared to the control group.

L/P ratios after the administration of ABP (200 μg/mouse) were 0.91 ± 0.07 (day 6), 3.23 ± 0.39 (day 10) and 4.82 ± 0.46 (day 14), whereas L/P ratios in the control group were 0.43 ± 0.04 (day 6), 0.96 ± 0.08 (day 10) and 1.43 ± 0.39 (day 14). The L/P ratio after the administration of Propolis was significantly elevated ($p < 0.01$), as compared to the control group.

4.3 Results of anti-tumor activity

4.3.1 Anti-tumor activity in Ehrlich solid carcinoma

With respect to anti-tumor activity in Ehrlich solid carcinoma, when Agaricus (200 mg/kg B. W.), Paffia (60 mg/kg B. W.) and Propolis (80 mg/kg B. W.) were administered for 34 consecutive days, suppressive ratios were 83.1% ($p < 0.01$), 63.3% ($p < 0.05$) and 29.1% ($p < 0.05$), respectively, as shown in Fig. 7. When ABP mixture was administered at a dose of 400 μg/kg for 34 consecutive days, remarkably high anti-tumor activity (suppressive ratio: 85.1%, $p < 0.01$) was shown.

4.3.2 Anti-tumor activity in Sarcoma-180 solid carcinoma

In Sarcoma-100 solid carcinoma, when Agaricus (200 mg/kg B. W.), Paffia (60 mg/kg B. W./day) and Propolis (80 mg/kg B. W./day) were orally administered for 34 consecutive days, suppressive ratios were 60.3% ($p < 0.05$), 54.8% ($p < 0.05$) and 62.6% ($p < 0.05$), respectively, as shown in fig. 8. When the mixture, ABP was administered at a dose of 400 mg/kg B. W./day for 34 consecutive days, remarkably high anti-tumor activity (suppressive ratio: 83.5%, $p < 0.01$) was shown.

4.4 Mechanism affecting ABP-induced immunoresponse to tumor-bearing mice

4.4.1 Results of IFN-γ activity in the mice bearing Sarcoma-180

A mouse IFN-γ ELISA kit (Amersham Bioscience) was used for the measurement of IFN-γ and the results are shown in Fig. 9. The horizontal axis and vertical axis indicate each group and IFN-γ, respectively.

4.4.2 Results of TNF-α activity in the mice bearing Sarcoma 180

A mouse TNF-α ELISA kit (Pierce Biotechnology) was used for the measurement of TNF-α and the results are shown in (Fig. 10). The horizontal axis and vertical axis indicate each group and TNF-α, respectively.

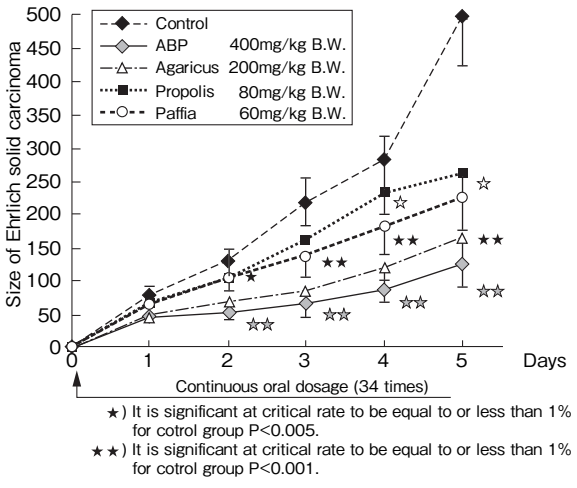

Fig. 7. Anti-tumor activity in Ehrlich solid carcinoma. When ABP mixture was administered at a dose of 400 μg/kg for 34 consecutive days, remarkably high anti-tumor activity (suppressive ratio : 85.1 %, $p < 0.01$) was shown.

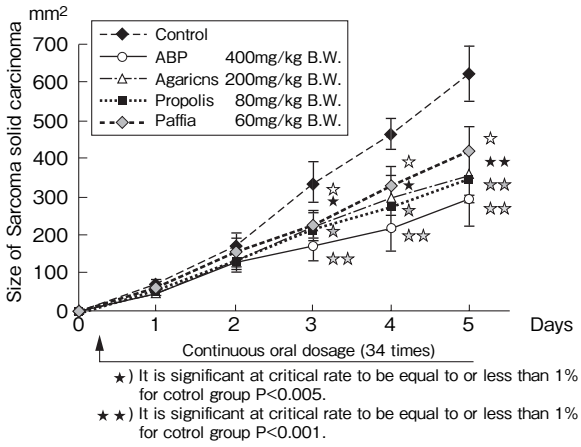

Fig. 8. Anti-tumor activity in Sarcoma-180 solid carcinoma. In Sarcoma-100 solid carcinoma, when *Agaricus* (200 mg/kg B.W.), *Paffia* (60 mg/kg B.W./day) and *Propolis* (80 mg/kg B.W./day) were orally administered for 34 consecutive days

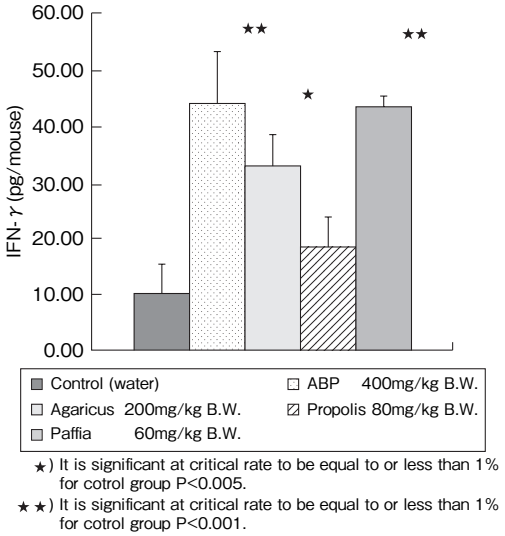

Fig. 9. IFN-γ activity in the mice bearing Sarcoma-180. The horizontal axis and vertical axis indicate each group and IFN-γ, respectively.

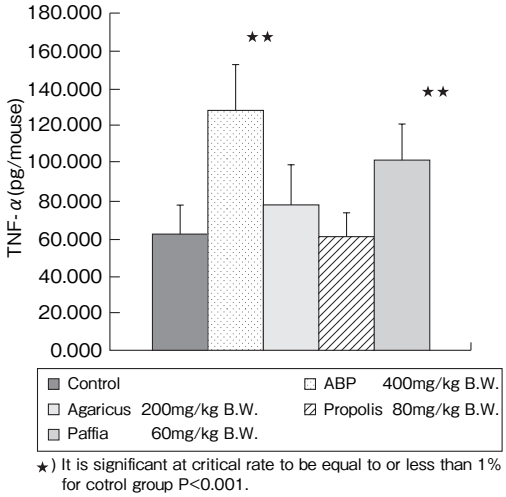

Fig. 10. TNF-α activity in the mice bearing Sarcoma 180. A mouse TNF-α ELISA kit (Pierce Biotechnology) was used for the measurement of TNF-α and the results.

4.4.3 Results of IgM activity in the mice bearing Sarcoma-180

A mouse IgM quantitation kit (Bethyl Laboratories, Inc., catalog No.: E90-101) and an ELISA starter accessory package (Bethyl Laboratories, Inc., catalog No.: E101) were used for the measurement of IgM and the results are shown in Fig. 11. The horizontal axis and vertical axis indicate each group and IgM, respectively.

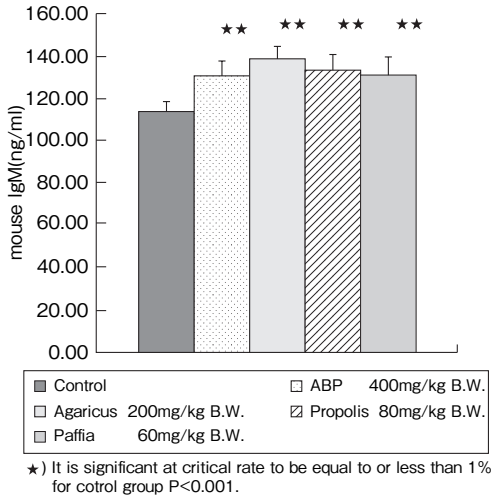

Fig. 11. IgM activity in the mice bearing Sarcoma-180. The horizontal axis and vertical axis indicate each group and IgM, respectively.

4.4.4 Results of IgG activity in the mice bearing Sarcoma-180

A mouse IgG ELISA quantitation kit (Bethyl Laboratories, Inc., catalog No.: E90-101) and an ELISA starter accessory package (Bethyl Laboratories, Inc., catalog No.: E101) were used for the measurement of IgG and the results are shown in Fig. 12. The horizontal axis and vertical axis indicate each group and IgG, respectively.

5. Discussion

When the antioxidant activities of ABP, Propolis and Agaricus were examined by the DPPH and Rhoudan iron methods, all three samples showed radical scavenging activity. Paffia did not show antioxidant activity, whereas Propolis showed antioxidant activity. Chen et al. reported that fravonoids showed high antioxidant activity[37]. Since Propolis

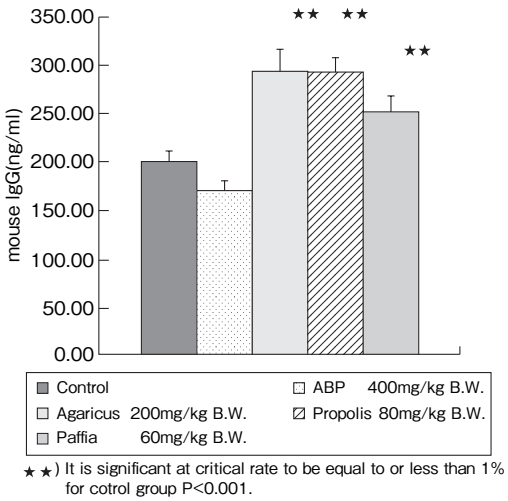

Fig. 12. IgG activity in the mice bearing Sarcoma-180. The horizontal axis and vertical axis indicate each group and IgG, respectively.

contains high levels of fravonoids, it is thought that fravonoids may be responsible for the antioxidant activity in this study. In addition, we found that Agaricus also showed antioxidant activity. Ekaterini et al. have reported that glucan shows antioxidant activity[38], suggesting that β-glucan in Agaricus may be rsesponsible. On the contrary, Paffia did not show antioxidant activity, has high levels of saponin which lacks antioxidant activity. There is also the possibility that the major constituents of Propolis and Agaricus are responsible for the antioxidant activity of ABP, and that the high antioxidant activity represents a synergistic action.

With respect to lymphocyte versus polymorphoculear leukocyte enhancement activity, it is known that lymphocytes act to specifically recognize pathogenic bacteria and induce an adaptive immunoresponse. B cells recognize particular antigens via receptors on the cell surface, and produce antibodies to eliminate the antigens. T cells undergo antigen presentation, and are classified into helper T cells (Th1 and TH2 are involved in cellular immunity and humoral immunity, respectively), cytotoxic T cells, inflammatory T cells and suppressor T cells. In the examination of immunoenhancement activity, we measured lymphocyte versus polymorophonuclear leukocyte ratios (L/P activity). The number of lymphocytes was significantly increased in all groups treated with ABP, Agaricus, Proplolis and Paffia. The reason that the immature immunoresponse of neonatal mice was activated to the same level observed in mature mice might be because various constituents in the samples used in this experiment activated the neonatal immune system.

IFN-γ is a multi-functional factor which shows antiviral effects, suppression of cell growth, antitumor effects, activation of macrophages, enhancement of NK cell activation, regulation of immunoresponse, and Specifically, high levels of IFN-γ were measured in mice bearing the S-180 carcinoma, after administration of ABP, Paffia and Agaricus. This strongly suggests that cellular immunity is especially activated by treatment with ABP, Paffia and Agaricus, because production of IFN-γ is limited to the T cells and NK cells stimulated by mitogen and sensitized antigen.

TNF-α shows a different extent and mechanism of action depending on the target cells. TNF-α causes impairment and suppression of growth in tumor cells, enhancement of growth, expression of IL-2 receptor and enhancement of IFN-γ and CSF production in T cells, and induction of antibody production in B cells. In addition, TNF-α increases cytotoxic activity in NK cells and LAK cells. In the macrophage, TNF-α increased cytotoxicity and enhances production of PGE2, IL-1, 6, 8, CSF and active oxygen. When TNF-α was measured in mice bearing the S-180 carcinoma, mice treated with ABP and Paffia showed higher TNF-α levels as compared to the control group. This suggests that activated macrophages produce TNF-

α in mice treated with ABP or Paffia, activated macrophages and lymphocytes are the source of most TNF-α.

When macrophages act as antigen-presenting cells (APC), they present antigens to helper T cells which give an auxiliary signal of stimulation to B-cells via the CD40 ligand. At the same time, helper T cells activate B-cells by producing cytokines. At this time, the direction of class switching of B cells is determined and differentiation to plasma cell occurs. IgM is an antibody with a pentamer structure and its production precedes IgG production in response to an antigen. Distinctive features of IgM include 10 antigen binding sites, easy production in response to granular antigens such as red blood cells, bacteria and viruses, and strong aggregation activity and activation of the complement system. Mice bearing a carcinoma and treated with ABP, Propolis, Agaricus or Paffia showed higher levels of IgM than the control group. This could be the result of enhancement of B cell activation in all treated groups, because immature B cells express antigen receptor, IgM on cell surface by binding of CD40 ligand located on the membrane of activated helper CD4 T cell to CD40 molecule located on the membrane of B cell.

IgG is abundant in the blood, and causes a marked enhancement of phagocytosis by phagocytes and macrophages, although the extent is different in each subclass. In mice bearing S-180 carcinoma, groups treated with Propolis, Agaricus or Paffia showed higher IgG production than control groups. IL-4 is mainly associated with a class switch of IgM to IgG. IFN-γ and IL-4 are related to differentiation of Th1 and TH2, respectively, and these cytokines suppress each other. Therefore, after treatment with ABP, which resulted in a high production of IFN-γ, differentiation to Th2 is suppressed, and a significant class switch to IgG doesn't occur. Thus, Th1-related cellular immunity might be activated.

When anti-tumor action was examined using two kinds of sarcoma (Ehrlich solid carcinoma and Sarcoma-180 carcinoma), tumor-suppressive ratios after treatment with ABP, Agaricus, Propolis and Paffia were 85, 83, 29.1 and 63%, respectively. When Sarcoma-180 solid carcinoma was used, tumor-suppressive ratios were 83, 60, 62 and 54%, respectively. Thus, ABP showed strong anti-tumor activity against two kinds of solid carcinoma. Taken altogether, this strongly suggests that a mixture of three kinds of materials (Agaricus, Propolis and Paffia) enhances original functions of macrophages and NK cells, and as a result, secondarily enhances the immune reaction and suppresses tumor growth. On the other hand, significant increases in TNF-α suggest an enhancement of T cell growth and induction of cytotoxic activity towards tumor cells and a suppression of tumor growth. Thus, high anti-tumor activity would be expressed more effectively due to direct activity towards tumor cells and activation of a host-mediated immunoreaction. However, in the group treated with Paffia, despite the activation of IFN-γ and TNF-α with activation of cellular immunofunction, strong tumor suppressive ratios were not observed. One possible reason is the balance between NF-kB and IkB, which are closely related to apoptosis, is lost. In general, there are two conflicting biological functions, namely, growth by cell division and programmed cell death, and the healthy living body grows by keeping a balance[39]. However, this balance is lost and tumors or mutated cells grow abnormally when there is a suppression of cell death in the process of transformation of normal cells to the tumor cells. NF-kB suppresses apoptosis by combination with some specific proteins[40]. It ~~is~~ has been reported that substances with antioxidant activity suppress the binding of NF-kB to specific proteins[41]. Therefore, there is the pos-

sibility that after treatment with Paffia, in which no antioxidant activity is found, the balance between NFkB and IkB is lost, and the apparent tumor-suppressive ratio is low due to suppression of apoptosis of tumor cells. By contrast, Propolis shows no increase in IFN-γ and TNF-α levels as compared with control, and it is not thought that cellular immunofunction is activated. However, since Propolis shows anti-tumor activity, there is the possibility that Propolis restores the balance between NF-kB and I-kB, and induces the apoptosis and tumor suppression secondarily.

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