

Immunostimulatory Effects of a Polysaccharide-Rich Substance with Antitumor Activity Isolated from Black Currant (*Ribes nigrum* L.)

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The fruit juice of black currant was found to contain a polysaccharide-rich substance, which was designated cassis polysaccharide (CAPS), with macrophage-stimulating activity. Especially, its interleukin (IL)-1 β -inducing activity was remarkably high, compared with other fruit juice preparations. CAPS was found to consist of rhamnose, mannose, arabinose, galactose, xylose, and glucose in a molar ratio of 11.3:0.9:54.1:29.8:2.0:1.9. CAPS turned out to be partitioned into a soluble component (CAPS-l.m.) and a precipitable component (CAPS-h.m.) with mean MWs of 80,000 and 600,000 respectively in 45% (v/v) ethanol solution. At least *in vitro*, CAPS-l.m. rather than CAPS-h.m. appeared to play an important role in macrophage activation. Oral administration of black currant juice and CAPS to Ehrlich carcinoma-bearing mice retarded the growth of the solid tumor by 45% and 51% respectively. CAPS administration had a stimulatory effect on the release of IL-2, IL-10, interferon- γ , and IL-4 from splenocytes in comparison with PBS treatment in tumor-bearing mice. The IL-4 level was, however, still lower than that exhibited by a group of normal mice. CAPS showed a certain cytotoxicity directly against tumor cells.

Key words: black currant; polysaccharide; macrophage; cytokine; antitumor

Recently, many researchers have attempted to detect bioactive substances in plant foods, and many functions such as antitumor, antimicrobial, and antioxidative activities have been reported. Along with the movement for such phytochemical detection, the concept of preventive medicine, which influences the quality of life (QOL), has rapidly been common, especially in developed countries.^{1,2)} As for Japan today, we Japanese have been undergoing a long-term economic depression, and such a situation gives birth to many negative factors such as irregular living habits, mental stress, and improper dietary habits, which potentially cause dysfunction of our immune systems.

Many Chinese herbs including medical mushrooms

have immunostimulatory effects with antitumor activities, which are mainly due to their immunomodulatory polysaccharide components.^{3–5)} Moreover, other kinds of polysaccharides with such activities have also been isolated from seaweed and higher plants.^{6–9)} These polysaccharides exert their antitumor action mostly *via* activation of host-mediated immune mechanisms. Unlike conventional chemotherapeutics, which have many negative side effects, intake of them, at least in moderate quantities, causes no harm and places no additional stress on the host body.

From the viewpoint of preventive medicine with such functional food products, it is also important to consider the aspect of their taste as food. Fruits can be easily utilized as one of the most popular food materials because of their cheerful images and good flavor, and they have actually been used as common resources of vitamins, minerals, polyphenolic compounds, and so on.¹⁰⁾ But phytochemical studies about polysaccharides have scarcely been done with respect to fruits.

In this study, first we examined various kinds of fruits for their macrophage-stimulating activities. Macrophages play a dual role in host defense. They form the first line of defense as a component of the innate immune response, but they also act as important accessory cells in the adaptive immune response.¹¹⁾ Therefore, an appropriate activation of macrophage function, at least in part, can result in strengthening host defense mechanisms. As a result of this examination, we discovered that a polysaccharide-rich substance derived from *Ribes nigrum* L., commonly called black currant, has potent immunostimulatory activity.

Black currant, which originates from Northern Asia and Europe, is now widely cultivated in North America, Europe, and New Zealand. The fruit of black currant has been used in Chinese folk medicine over a thousand years,¹²⁾ and it is famous as a raw material of cassis liqueur, a traditional alcoholic beverage in France. Recently, a large amount of polyphenolic compounds contained in the fruit juice and the compounds' antioxidative and antimicrobial activities have been

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Abbreviations: CAPS, cassis polysaccharide; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IL, interleukin; i.p., intraperitoneally; NO, nitric oxide; PBS, phosphate-buffered saline; p.o., per os; s.c., subcutaneously; Th, T helper; TNF, tumor necrosis factor

noted.¹²⁻¹⁵ Here we report the isolation and some properties of a polysaccharide-rich substance with potent immunostimulatory and antitumor activities derived from the fruit juice of black currant.

Materials and Methods

Sample preparation. Thirty-seven kinds of fruits were separately crushed to obtain their juice. Each crude fruit juice was neutralized with 1 N sodium hydroxide and finally diluted 4 times with distilled water. All test samples were cleared of particulate matter by centrifugation at $9,000 \times g$ for 10 min and used for macrophage stimulation assay.

Partial purification of polysaccharides from black currant juice. The fruit of black currant was crushed and centrifuged at $9,000 \times g$ for 10 min. The supernatant was recovered as a crude juice and passed through a column filled with a mixture of Amberlite IR-120B resin (Organo, Tokyo) for cation exchange and Amberlite IRA-410 resin for anion exchange in order to remove the cations and anions. Effluent from the column was filtered through a SEP-PAK C-18 cartridge (Waters, Milford, MA) to remove polyphenolic compounds, and dialyzed against distilled water. The dialyzate was centrifuged and the supernatant, containing a polysaccharide-rich substance, which was designated cassis polysaccharide (CAPS), was lyophilized and stored at -20°C until use.

For further fractionation, CAPS was dissolved in a moderate volume of phosphate-buffered saline (PBS), and ethanol was added to the solution at a final concentration of 45% (v/v). After centrifugation at $9,000 \times g$ for 10 min, the supernatant and precipitate were recovered separately. The former, containing one group of polysaccharides (CAPS-low molecular: l.m.) was dialyzed and lyophilized. The latter, including the other group of polysaccharides (CAPS-high molecular: h.m.) was dissolved in PBS and further purified by 3 times-repeated ethanol precipitation and finally lyophilized.

Molecular weight distribution. The molecular weight of CAPS was analyzed by gel-filtration chromatography on HPLC (Shimadzu, Kyoto, Japan) equipped with a Shodex OHpak SB-805(4) HQ column (Showa Denko, Tokyo) equilibrated with PBS (40°C) at a flow rate of 1 ml/min. Detection was done with a refractive index detector (Shimadzu RID-10A). A calibration curve was made with a dextran standard solution consisting of T-2000 (MW 2,000,000), T-500 (MW 473,000), T-70 (MW 67,200), T-40 (MW 43,000), T-10 (MW 10,000), saccharose (MW 342), and glucose (MW 180). These marker dextrans were purchased from Pharmacia (Uppsala, Sweden).

Enzymatic digestion. CAPS dissolved in 0.1 M phosphate buffer (pH 5.0) at a polysaccharide concentration

of 2 mg/ml was incubated at 50°C with Sumizyme ACH (100 units/ml), a hemicellulase product derived from *Aspergillus niger* (Shin Nihon Chemical, Anjo, Japan). The digestion process of CAPS was monitored by gel-filtration chromatography on HPLC. After complete digestion, the residual cytokine-inducing activity of the enzyme-treated CAPS was measured by ELISA, as described in "Determination of cytokine and NO production".

Carbohydrate analysis. The sugar composition of CAPS, which had previously been subjected to acid-hydrolysis, was characterized by ion-exchange chromatography using HPLC equipped with a TSK-gel Sugar AXG column (Tosoh, Tokyo) in combination with the post-column method, with slight modifications.¹⁶ Briefly, as pretreatment, a moderate amount of CAPS was incubated in 2 N trifluoroacetic acid (TFA) at 100°C for 6 h, followed by vacuum-drying. The preparation was dissolved in distilled water and injected into the column equilibrated with 0.5 M potassium borate (pH 8.7, 70°C) at a flow rate of 0.4 ml/min. Eluate from the analytical column was mixed with 1% (w/v) L-arginine dissolved in 3% (w/v) boric acid solution and heated to 150°C in a reaction coil at a flow rate of 0.5 ml/min. Due to reaction of reducing sugars in the eluate, fluorescent light was detected (Ex = 320 nm, Em = 430 nm) with a fluorometric detector (Shimadzu RF-10AXL).

Preparation of effector cells. Mice were purchased from Nihon SLC (Shizuoka, Japan). Peritoneal exudate cells were aseptically collected from ICR female mice (6-8 weeks old) inoculated 4 d prior with 2.7 ml of thioglycollate medium. The cells were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum (complete medium) and incubated in a 96-well tissue culture plate for 2 h. Non-adherent cells were removed by washing the plate twice, and adherent cells were used as macrophages. To obtain splenocytes from ICR mice, spleens were pressed between two sterile glass microscope slides to release cells. Macrophages and splenocytes were finally cultured at 1×10^6 cells/ml and 2.5×10^6 cells/ml respectively in complete medium with 100 units/ml of penicillin and 100 $\mu\text{g}/\text{ml}$ of streptomycin at 37°C in 5% CO_2 air.

Determination of cytokine and NO production. For macrophage stimulation, various test samples (20 $\mu\text{l}/\text{well}$) were incubated with macrophages (180 $\mu\text{l}/\text{well}$) in a 96-well plate. After 16-20 hours, the amounts of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-12 p70 produced from macrophages were measured, respectively, by ELISA using a commercially available kit from R&D Systems (Minneapolis, MN). Moreover, nitric oxide (NO) production was detected by assaying for the presence of nitrites (NO_2) in the culture medium using a Griess Reagent Kit obtained from Molecular Probes (Eugene, OR). A standard curve was prepared by

dissolution of sodium nitrite into the culture medium. After 30 min of development time, the absorbance of each sample was read at a wavelength of 540 nm.

For the sake of measuring the release of IL-2, IL-4, IL-10, and interferon (IFN)- γ , splenocytes were prepared from ICR female mice and cultured for 96 h, and then each cytokine level was determined by ELISA in the same way.

Assay of antitumor activity. The fruit of black currant was crushed and the crude juice was recovered by centrifugation, as described above. Antitumor activities of the crude juice and CAPS against Ehrlich carcinoma were examined in comparison with Picibanil (OK-432) (Chugai Pharmaceutical, Tokyo) and doxorubicin hydrochloride, which used to be called Adriamycin (Kyowa Hakko, Tokyo). CAPS was dissolved in a moderate volume of PBS so that it had appropriately the same level of macrophage-stimulating (cytokine-inducing) activity *in vitro* as the crude juice. ICR female mice (5 weeks old) were per os (p.o.) given the crude juice and CAPS solution (10 ml/kg body wt.) respectively, daily for 21 d. Control mice were p.o. given PBS in a similar manner. Seven d after the first administration, *i.e.*, on day 0, Ehrlich ascites tumor cells (2×10^6) were subcutaneously (s.c.) inoculated into the inguinal region of mice. From day 1, Adriamycin (0.5 mg/kg body wt.) was intraperitoneally (i.p.) injected into mice for 9 consecutive d.¹⁷⁾ From day 2, OK-432 (1 KE/mouse) was also i.p. injected 5 times every other day.¹⁸⁾ The growth of tumor cells with the elapse of days was observed by measuring the size of the carcinoma. On day 14, tumors were removed and weighed, and splenocytes were simultaneously prepared according to the method described above and used to assay cytokine production.

Cytotoxicity test of CAPS. Ehrlich ascites cells (1×10^5 cells/ml) were incubated with a serial dilution of CAPS in a 96-well microtiter plate to determine the degree of cytotoxicity. After 72 h, cell viability was assayed using a Cell Proliferation Kit purchased from Chemicon International (Temecula, CA). This assay was based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases.¹⁹⁾ An aliquot of the WST-1 solution was added to each well and incubated for an additional 2 h, and then absorbance was measured at 450 nm.

Other measurements and procedures. The total phenol content of the fruit juice was measured by the Folin-Ciocalteu method, with gallic acid as a standard.²⁰⁾ The phenol-sulphuric acid method was used to quantitate the carbohydrate content of CAPS with glucose as a standard, and absorbance was measured at 490 nm.²¹⁾ The protein concentration was measured using a Bio-Rad Protein Assay Kit with BSA as a standard.

Statistical analysis. All values are presented as the mean \pm standard error (S.E.). The significance of differences was determined by Student's *t*-test. *P*-values less than 5% ($p < 0.05$) were considered statistically significant.

Results

Effects of various fruit juices on cytokine production

Cytokine production from peritoneal macrophages stimulated by test samples which had been prepared from 37 kinds of fruit juice was assayed (Fig. 1). The total phenol content of the fruit juice preparations did not appear to be correlated directly with their macrophage-stimulating activities. Among the fruits tested, the juice of black currant drastically enhanced the production of the inflammatory cytokines from macrophages. Especially, its IL-1 β -inducing activity was remarkably high, compared with the other fruit juice preparations. Next, the addition of ethanol to the test sample from black currant fruit juice at a final concentration of 70%

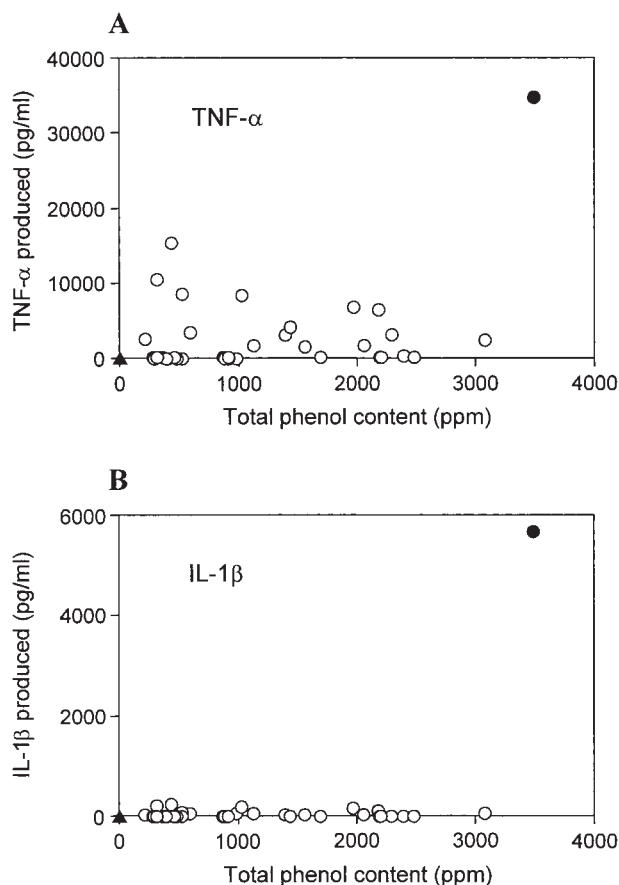


Fig. 1. Macrophage Activation by Various Fruit Juices.

Macrophages (1×10^6 cells/ml) were incubated with 37 kinds of fruit juice preparations (final concentration: 2.5% [v/v]) in a 96-well plate. The induction levels of TNF- α (A) and IL-1 β (B) were measured by ELISA. The total phenol content of the fruit juice was measured by the Folin-Ciocalteu method with gallic acid as a standard. \blacktriangle , control (PBS); \bullet , black currant; \circ , other fruits.

(v/v) partitioned the constituent substances of the juice preparation into a soluble fraction and precipitable fraction. The soluble fraction had no effect on cytokine production from macrophages, whereas the precipitable fraction showed a certain cytokine-inducing activity, at approximately 55% of the juice preparation. In general, polysaccharide-including substances within their molecular structures are known occasionally to possess such macrophage-stimulating activities, and they can be easily precipitated by adding ethanol above certain concentrations. Therefore, the existence of an immunostimulatory polysaccharide-rich substance in the juice of black currant was anticipated. Nevertheless, as mentioned in "Discussion" below, no such ethanol-precipitation method was employed to obtain polysaccharides from black currant juice.

Partial characterization of CAPS

Freeze-dried CAPS was obtained with a typical yield of approximately 9.1 g per liter of juice prepared by centrifugation of the crushed fruit of black currant. CAPS was found to contain 479.9 μg (approximately 48.0%) of carbohydrate and 15.0 μg (about 1.5%) of protein per mg of freeze-dried CAPS. Prior to sugar composition analysis, it was investigated whether or not the carbohydrate (polysaccharide) component of CAPS was indeed necessary for its macrophage-stimulating activity. CAPS was completely digested with hemicellulase as described in "Materials and Methods", and residual TNF- α -inducing activity was assayed (Fig. 2). The production level of TNF- α from macrophages was drastically decreased, suggesting that the key component of CAPS was a polysaccharide moiety, which was considered to be derived mainly from hemicellulose polysaccharides. Subsequently, HPLC analysis revealed that CAPS was composed of rhamnose, mannose,

arabinose, galactose, xylose, and glucose in a molar ratio of 11.3:0.9:54.1:29.8:2.0:1.9.

CAPS dissolved in PBS was partitioned into two fractions, consisting of a soluble component (CAPS-l.m.) and a precipitable component (CAPS-h.m.), by adding a moderate amount (45% [v/v]) of ethanol, as described in "Materials and Methods". A phenol-sulphuric acid test revealed that the polysaccharides contained in CAPS were distributed between CAPS-l.m. and -h.m. at a gravimetric ratio of 1:9–10. Also, the average molecular weight of each preparation was analyzed by HPLC. The elution profiles of CAPS-l.m. and -h.m. showed major peaks indicating mean MWs of 80,000 and 600,000 respectively.

Effect of CAPS on macrophage stimulation

The effect of CAPS on macrophage activation was assessed by measuring the amount of TNF- α , IL-1 β , IL-12 p70, and NO respectively produced from thioglycollate-elicited macrophages, and the dose-dependent induction levels of these biomarkers were compared with those exhibited by CAPS-l.m. and -h.m. (Fig. 3). CAPS consisting of CAPS-l.m. and -h.m. induced these biomarkers peaking at a carbohydrate concentration of 300 $\mu\text{g}/\text{ml}$, and then declined. On the whole, CAPS-l.m. rather than CAPS-h.m. appeared to play an important role in macrophage activation because the induction levels exerted by CAPS-l.m. itself were relatively and predominantly high in comparison with those indicated by CAPS-h.m. In the case where IL-1 β was induced at a polysaccharide (carbohydrate) concentration of 300 $\mu\text{g}/\text{ml}$, however, the coexistence of the two components appeared to show a synergistic effect on cytokine production from macrophages (Fig. 3B).

Effect of CAPS against Ehrlich carcinoma

Prior to administration, CAPS was dissolved in PBS at a carbohydrate concentration of 3.9 mg/ml. This polysaccharide solution showed approximately the same level of macrophage-stimulating activity as crude black currant juice (data not shown). Oral intake of the crude juice and the CAPS solution retarded the growth of Ehrlich carcinoma by 45% and 51% respectively, with statistical significance (Fig. 4). These results suggest that a polysaccharide-rich substance with potent macrophage-stimulating activity, *viz.*, CAPS, was the most important component of black currant fruit juice in exhibiting its antitumor effect. Administration (i.p.) of OK-432, a streptococcal preparation and well-known immunopotentiator, and Adriamycin, a type of antibiotic used specifically in the treatment of cancer, inhibited solid tumor growth by 64% and 62% respectively.

Most polysaccharides with antitumor activities act through host-mediated mechanisms, and are known as a group of cytokine inducers. Hence the effect of CAPS on splenocytes was evaluated by measuring the induction levels of the cytokines IL-2, IL-4, IL-10, and IFN- γ . Splenocytes were prepared from ICR mice implanted

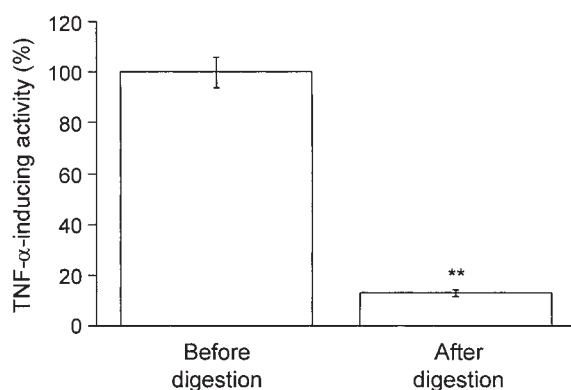


Fig. 2. Cytokine-Inducing Activity of Hemicellulase-Digested CAPS.

CAPS (2 mg/ml) dissolved in 0.1 M phosphate buffer (pH 5.0) was incubated at 50 °C with hemicellulase (Sumizyme ACH, 100 units/ml). After complete digestion, residual TNF- α -inducing activity was assayed by ELISA. The activity of CAPS incubated with the same amount of the enzyme previously denatured by boiling for 5 min was taken as 100%. Data represent the mean \pm S.E. (** $p < 0.01$).

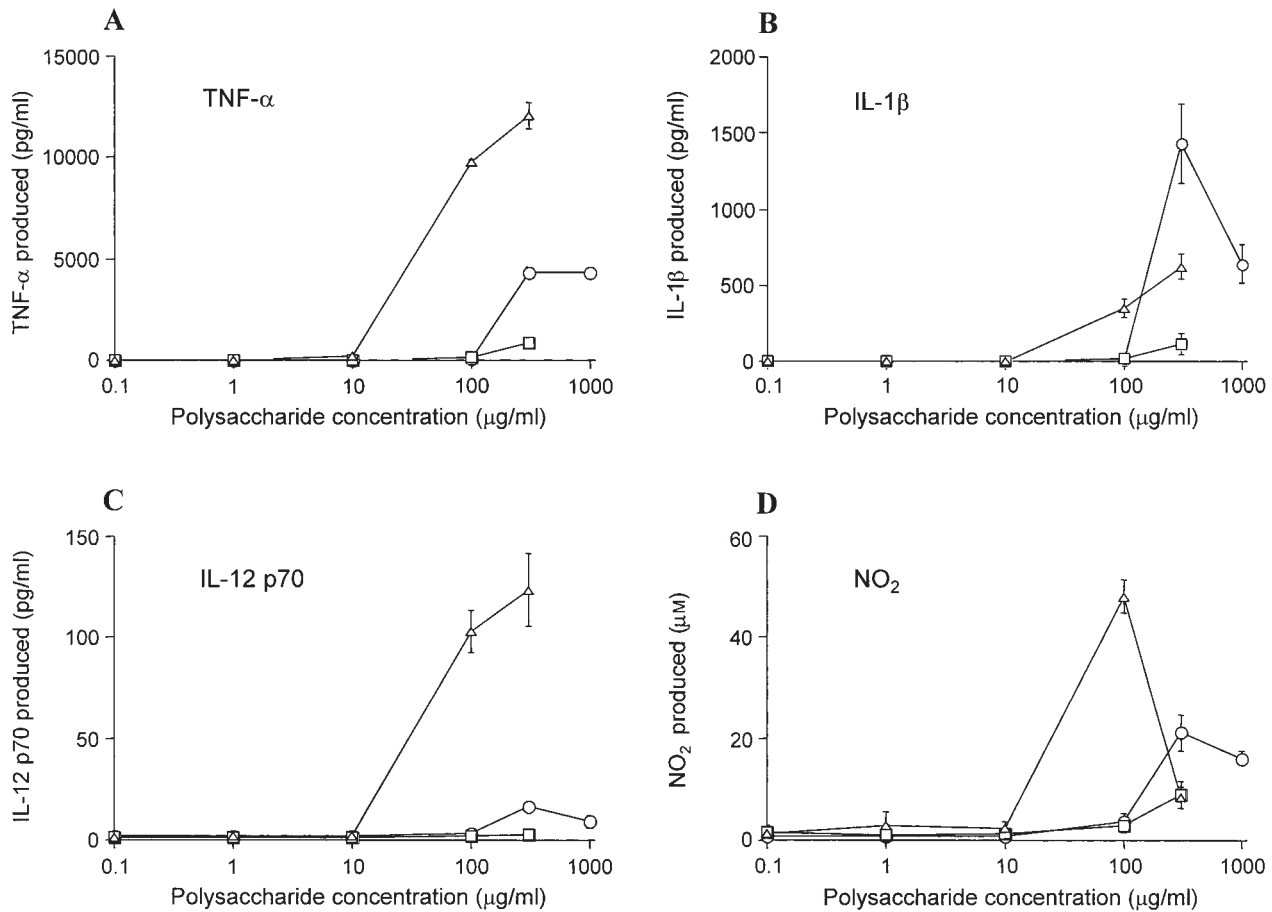


Fig. 3. Dose-Response of Macrophage Activation by CAPS and Its Component Parts.

Macrophages were incubated with serial dilutions of CAPS, CAPS-l.m. (the soluble component of CAPS in 45% [v/v] ethanol solution) and CAPS-h.m. (the precipitable kind at the same ethanol concentration) respectively. The production levels of TNF- α (A), IL-1 β (B), and IL-12 p70 (C) were determined by ELISA. Production of NO was detected as NO₂ (D) using a Griess Reagent Kit. \circ , CAPS; Δ , CAPS-l.m.; \square , CAPS-h.m.

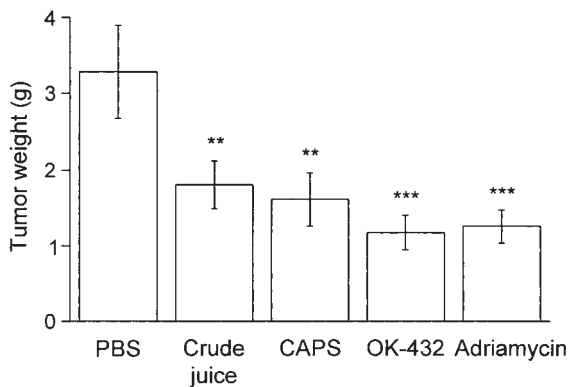


Fig. 4. Antitumor Effect of CAPS against Ehrlich Carcinoma.

CAPS and crude black currant juice were p.o. administered to ICR mice implanted s.c. with Ehrlich tumor cells. OK-432 and Adriamycin were used as positive controls and i.p. administered. Fourteen d after tumor inoculation, the tumors were removed and weighed. Data represent the mean \pm S.E. ** p < 0.01, *** p < 0.001 compared with the group of PBS-treated tumor-bearing mice.

s.c. with Ehrlich carcinoma cells and given the CAPS solution or PBS orally. Splenocytes were also prepared from non-implanted mice, that is, normal ones (control

group). The results are shown in Fig. 5. Statistical analyses of these results were done in comparison with the group of PBS-treated tumor-bearing mice. Production of IL-2, IL-10, and IFN- γ from splenocytes was increased significantly by implantation of Ehrlich carcinoma cells. Especially, the induction level of IFN- γ , which was negligibly low in the control group, was drastically enhanced by implantation of tumor cells. On the other hand, IL-4 production was remarkably decreased by tumor implantation. Oral administration of the CAPS solution had a stimulatory effect on the release of these cytokines with statistical significance, compared with PBS treatment in tumor-bearing mice. The IL-4 level induced by CAPS administration was, however, still lower than that exhibited by the control group.

Cytotoxicity of CAPS against Ehrlich ascites cells

In order to examine whether CAPS had a direct cytotoxic action on Ehrlich ascites cells, the tumor cells were cultured for 72 h with or without CAPS and cell viability was assessed by WST-1 assay. As shown in Fig. 6, CAPS showed a certain cytotoxicity directly

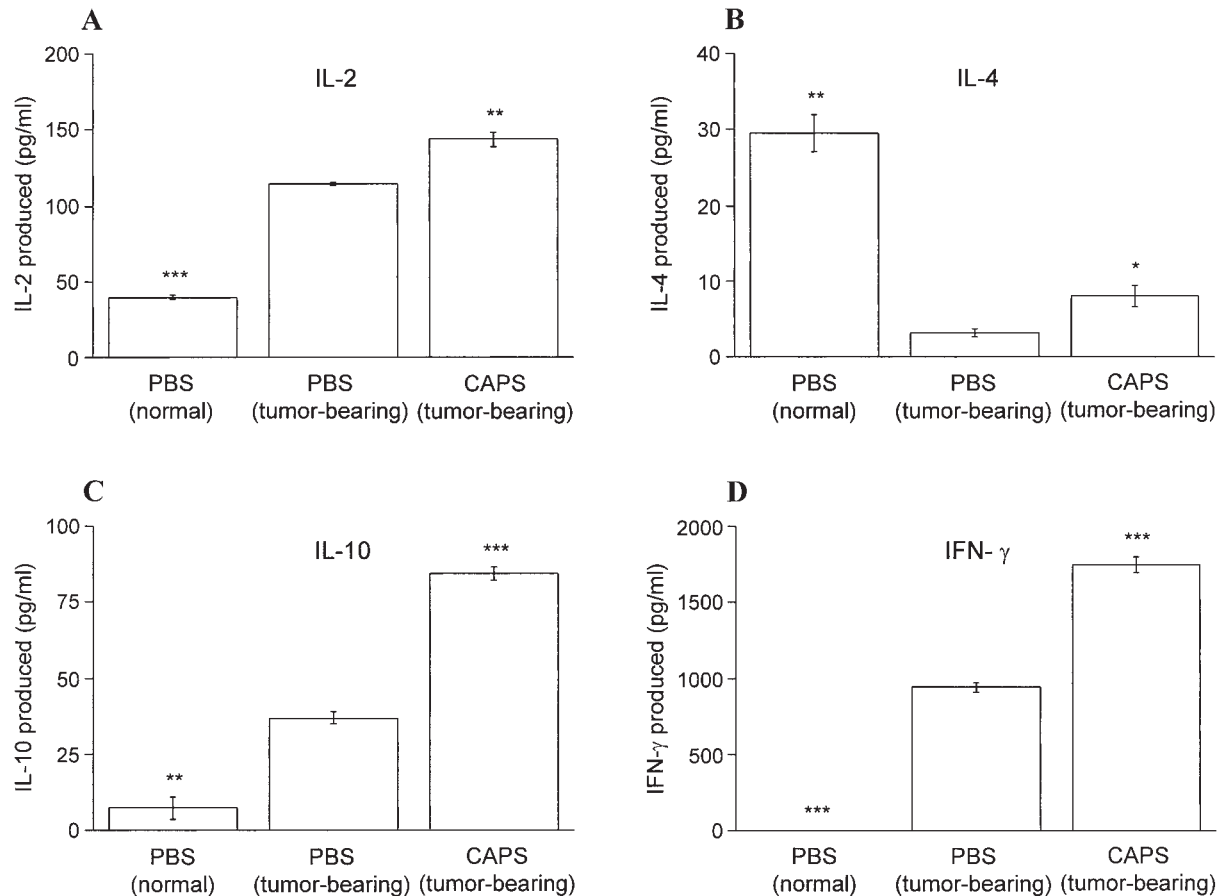


Fig. 5. Cytokine Production from Splenocytes.

Splenocytes (2.5×10^6 cells/ml) separately prepared from the three groups of ICR mice, *viz.*, normal, PBS-treated tumor-bearing, and CAPS-treated tumor-bearing, were incubated in a 96-well microtiter plate for 96 h. Release of the cytokines IL-2 (A), IL-4 (B), IL-10 (C), and IFN- γ (D) was measured by ELISA. Data represent the mean \pm S.E. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the group of PBS-treated tumor-bearing mice.

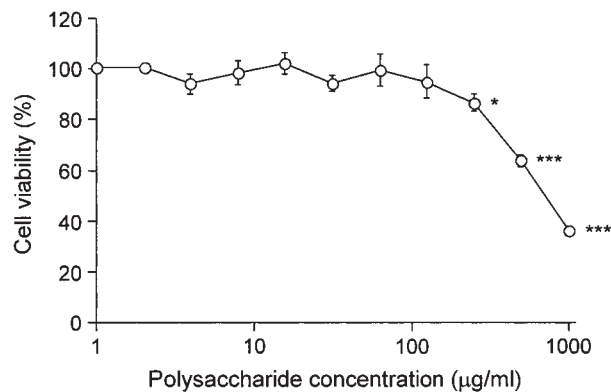


Fig. 6. Cytotoxicity of CAPS against Ehrlich Ascites Cells.

A serial dilution of CAPS was incubated with Ehrlich tumor cells (1×10^5 cells/ml) in a 96-well microtiter plate for 72 h and cell viability was determined by WST-1 assay. The viability of cells incubated with PBS instead of CAPS was taken as 100%. Data represent the mean \pm S.E. * $p < 0.05$, *** $p < 0.001$ compared with the control.

against the tumor cells. This cytotoxicity was found to be statistically significant above $250 \mu\text{g/ml}$, and the IC_{50} value of CAPS was estimated to be about $760 \mu\text{g/ml}$.

Discussion

We have described the development and some properties of a polysaccharide-rich substance with potent immunostimulatory and antitumor activities derived from black currant fruit juice. First, 37 kinds of fruit juice were examined for their macrophage stimulation activities by measuring TNF- α and IL-1 β levels in the cell culture medium. TNF- α directly causes hemorrhagic necrosis of tumor cells.²²⁾ A marked increase in the release of IL-1 β results in maturation, differentiation, and proliferation of immunocompetent cells responsible for host defense mechanisms.²³⁾ It is generally known, however, that thioglycollate-elicited macrophages tend to be susceptible to immunostimulatory substances such as lipopolysaccharide (LPS), as compared with naive cells, in their TNF- α production. Therefore, IL-1 β rather than TNF- α might be a suitable biomarker in such a macrophage stimulation test. As can be seen from Fig. 1B, black currant fruit juice drastically induced IL-1 β in comparison with the other fruit juice preparations, and hence the fruit of black currant is perhaps useful for developing dietary or pharmaceutical

products with potent immunostimulatory activity. By the way, red currant (*Ribes rubrum* L.), the wild type of black currant, and gooseberry (*Ribes uva-crispa* L.) are classified into the same genus category with black currant. Each fruit juice showed a similar level of stimulatory effect on the production of cytokines from macrophages *in vitro* (data not shown). It is likely that a similar or common polysaccharide-rich substance with powerful immunostimulatory activity is contained in fruits belonging to the genus *Ribes*.

With regard to polysaccharide fractionation, the primary purification step for naturally occurring polysaccharides frequently entails the process of ethanol precipitation.²⁴⁾ The precipitable fraction obtained from ethanol-treated (70% [v/v]) black currant juice, however, showed about 55% of the macrophage-stimulating (cytokine-inducing) activity of the crude juice preparation. This ethanol concentration appears to be insufficient for complete precipitation of the component parts of CAPS. This is probably due to the highly water-soluble property of CAPS-l.m., which is presumed to be more important, at least *in vitro*, than CAPS-h.m. in the induction of macrophage-derived cytokines. Therefore, when preparing CAPS, no such ethanol-precipitation method was used in this study. It has not been clarified whether CAPS-l.m. is derived from CAPS-h.m., *i.e.*, whether it is originally a constituent part of it or is unrelated to CAPS-h.m. at the molecular level.

A number of polysaccharides from medical mushrooms have been reported to possess immunostimulatory effects with antitumor activities. Such fungal polysaccharides are known to belong mainly to the group of β -glucans.²⁴⁾ On the other hand, it has been reported that the key active component of seaweed is the sulfated polysaccharide fucoidan.²⁵⁾ In the case of higher plants, the unique structures and immunostimulatory effects of certain arabinogalactan moieties, which are derived chiefly from hemicellulose polysaccharides, have been reported.^{8,9,26,27)} In this study, CAPS was also found to contain a relatively large amount of arabinose and galactose as its sugar component. Thus CAPS may have its own unique arabinogalactan structure, responsible for exerting its powerful immunostimulatory activity. Further characterization of the polysaccharide component of CAPS is now under way.

CAPS effectively enhanced the production of inflammatory cytokines and NO from thioglycollate-elicited macrophages. These macrophage-derived products are considered important mediators for host defense because the antitumor activities of certain polysaccharides are inhibited by pretreatment with antimacrophage agents.²⁸⁾ Furthermore, CAPS-l.m. rather than CAPS-h.m. might play an important role in the induction of antitumor action by CAPS because of its potent stimulatory effect on IL-12 p70 production *in vitro* (Fig. 3C). IL-12 p70 is known as a significant initiator that indirectly induces antitumor effects through a T-helper 1 (Th1) cellular immune response, generally with enhanced production

of IFN- γ and decreased production of IL-4.²⁹⁾ However, thus far, it has not been clarified whether CAPS-l.m. alone eventually exhibits sufficient antitumor activity *in vivo*. Therefore the antitumor test in this study was done with CAPS, *i.e.*, a mixture of CAPS-l.m. and -h.m. components. Oral administration of CAPS to tumor-bearing mice enhanced the production of IL-2, IL-10, IFN- γ , and IL-4 with statistical significance, compared with PBS treatment (Fig. 5). IL-2 is regarded as a T-cell growth factor, which is very important for priming Th1 cells.³⁰⁾ IL-10, a Th2 cytokine, has a variety of suppressive effects, including inhibition of Th1 cytokine production, down-regulation of macrophage and natural killer (NK) cell functions, and an overall lowering of T-cell proliferation.³¹⁾ In the present antitumor test, IL-10 might act in such a negative feedback mechanism to prevent excessive macrophage activation by CAPS. On the other hand, IFN- γ , a Th1 cytokine that is the final end product of the Th1 immune response, acts as a stimulator that promotes the Th1 immune response including further activation of macrophages, and finally shifts the Th1/Th2 balance toward Th1 dominance.^{7,29)} IL-4, another Th2 cytokine, serves as a stimulator for a Th2 immune response.³²⁾ Thus IL-4 and IFN- γ generally have opposing effects on cell-mediated immune responses. In this antitumor test, the induction levels of IL-4 and IFN- γ were certainly decreased and increased respectively by implantation of Ehrlich carcinoma cells, and both cytokine levels were further enhanced by CAPS administration. The IL-4 level was, however, lower than that exhibited by the group of normal mice. With respect to a series of these phenomena, CAPS appeared to prevent oversuppression of IL-4 production in immune mechanisms somewhat damaged by implantation of tumor cells. Taking into account this effect of CAPS on the IL-4 or IL-10 induction level in the antitumor test, CAPS is supposed to have potential as a biological response modifier (BRM) that restores immune systems in a wrong condition to their normal state.³³⁾

CAPS showed a certain cytotoxicity directly against Ehrlich ascites cells, and the IC₅₀ value of CAPS was estimated to be about 760 μ g/ml. The direct effect of CAPS on the proliferation of normal cells has not yet been examined. It has been reported that extracts from crude natural materials are routinely designated as nontoxic when they are non-cytotoxic at concentrations of 1–2 mg/ml.³⁴⁾ Based on the report, CAPS may be considered to have weak cytotoxicity against Ehrlich tumor cells. However, oral administration of CAPS in this study was performed at a carbohydrate concentration of only about 4 mg/ml. As a rule, it appears difficult to conclude that the rather high molecular polysaccharide components constituting CAPS are able directly to attack solid tumor cells while maintaining such a high concentration level. From these results, it is reasonable to conclude that the antitumor effect of CAPS is due to activation of host-mediated mechanisms rather than to

direct cytotoxicity against tumor cells. The mechanisms of action by CAPS are now under study.

As described above, oral administration of CAPS certainly retarded the growth of Ehrlich carcinoma. There are several factors such as strain of mice, type of tumor, and suitable dosage that are essential to achieve the antitumor effect of a polysaccharide component.^{35–37} With regard to dosage in such an antitumor test, there usually exists an optimum one and it probably depends on the biochemical properties of the polysaccharides used.^{37,38} Further studies are needed to characterize a variety of antitumor effects of CAPS. During the antitumor test, the group of CAPS-treated tumor-bearing mice always showed the lowest level of average weight among all the groups of tumor-bearing mice tested (data not shown). We assume that this is not due to a negative side effect, but is a function of CAPS as dietary fiber, generally known as an antiobesity effect.³⁹

Taken together, we developed CAPS consisting of a polysaccharide-rich substance from black currant fruit juice. CAPS acts as an immunopotentiator with powerful antitumor activity and does not appear to be inferior to OK-432, a well-known one. CPAS might become an excellent material for new pharmaceutical products or dietary supplements not only because of its potent immunostimulatory activity but also because of cheerful and tasty images imagined from the fruit of black currant.

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