Augmentation of Antigen-Specific Antibody Production and IL-10 Generation with a Fraction from Rooibos (Aspalathus linearis) Tea

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Rooibos tea was extracted with boiling water. The aqueous extract was chromatographed in a Diaion HP20 column eluted stepwise with water, 25%, 50% and 75% (v/v) aqueous methanol, and 100% methanol. The water eluate (fraction A) showed an augmenting effect on anti-ovalbumin (anti-OVA) immunoglobulin M (IgM) production in OVA-stimulated murine splenocytes in vitro. Fraction A also showed a strong augmenting effect on interleukin-10 generation in murine splenocytes. Furthermore, continuous ingestion of fraction A was found to increase the anti-OVA IgM level in the sera of OVA-immunized mice.

Key words: Rooibos tea; HP20 column; immunoglobulin M (IgM); interleukin-10 (IL-10)

Rooibos (Aspalathus linearis) is a shrubby legume that is indigenous to the Cederberg region of the Western Cape of South Africa. Rooibos tea has traditionally been taken as a health drink for more than a century in the Republic of South Africa and in Europe.1,2) It is known that this tea contains abundant flavonoids3) and has potent antioxidative activity,4) but interestingly contains no caffeine and alkaloids, and has a low contents of tannins.1,2) It has also been reported that Rooibos tea had diverse physiological and pharmacological actions such as a protective effect on DNA strand scission,5) inhibitory effect on tumour promotion in mouse skin,6) hepatoprotective effect on CCl4-induced liver damage in rats,7) protective effect against mutagenesis,8) and anti-HIV activity in vitro.9) In addition, some attempts have been made to clarify whether Rooibos tea is effective in the control of allergic diseases.10) However, very little information has been published concerning the immunological properties of this tea.

We found in our previous study that a Rooibos tea extract increased antigen-specific antibody production and interleukin (IL)-2 generation in murine splenocytes in vitro and restored reduced antigen-specific antibody production in immunosuppressive rats.11) We examined in this present study the effects of a Diaion HP20 column-fractionated Rooibos tea extract on antigen-specific antibody production and cytokine generation in murine splenocytes in vitro. The effect of the water eluate on the antigen-specific antibody levels in mice in vivo was also investigated.

A Rooibos tea extract was prepared by boiling Rooibos leaves and stems (17.5 g; Kirin Well-Foods Co., Tokyo, Japan) with water (1 liter) for 15 min. The extract was filtered and filled up to 1000 ml with distilled water. A 100-ml aliquot of the extract was lyophilized to produce 0.28 g of a dry solid, and then used for the subsequent experiments as a crude extract. The remaining solution (900 ml) was concentrated to approximately 200 ml under reduced pressure, and then loaded into a Diaion HP20 (Mitsubishi Chemical Co., Tokyo, Japan) column (ø4 cm × 28.0 cm; bed volume of 352 cm3) that had been equilibrated with distilled water. The column was eluted stepwise with 500 ml of distilled water, 25%, 50% and 75% (v/v) aqueous methanol, and 100% methanol (MeOH). After removing MeOH, the water, 25% MeOH, 50% MeOH, 75% MeOH and 100% MeOH eluates were lyophilized to produce 0.72 g of a dry solid (fraction A), 0.21 g of a dry solid (fraction B), 0.61 g of a dry solid (fraction C), 0.69 g of a dry solid (fraction D) and 0.16 g of a dry solid (fraction E), respectively. These fractions were kept at room temperature until needed.

Specific pathogen-free female BALB/c mice (7 weeks old) were purchased from Charles River Japan (Yokohama, Japan). The care and use of the animals in this study followed the guidelines of Okayama University. These mice were kept in our animal facility for at least 1 week before use. All mice were used at 8 to 12 weeks of age. They were housed in a room with controlled temperature (23–25°C), humidity (50–60%), and a preset light–dark cycle (12 h:12 h), given sterile

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wood-chip bedding, and provided with food and water *ad libitum* under specific pathogen-free conditions. These mice were sacrificed, and their spleens were removed. The spleens were minced, and a single-cell suspension was obtained in a minimum essential medium (MEM).

For antigen-specific antibody production *in vitro*, the spleen cells (1 x 10^6 cells/200 μl/well), in which the red blood cells had been lysed by adding an ammonium chloride solution (144 mM NH₄Cl, 16.5 mM Tris–HCl, pH 7.2), were cultured with OVA (500 ng/ml) and the various Rooibos tea fractions in 96-well round-bottom microplates (163320; Nunc, Roskilde, Denmark) at 37 °C in an atmosphere of 5% CO₂ and 95% air. Each sample was used at a concentration equivalent to 100 μg/ml of the crude extract. On day 3 of the culture, the cells were washed twice with MEM, and then the culture was continued in the presence of the various Rooibos tea fractions under the antigen-depleted condition for another 7 days. An RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 μM 2-mercaptoethanol, 100 U/ml of penicillin G, and 100 μg/ml of streptomycin was used for the culture. The antigen-specific IgM levels in each cultured supernatant were measured by an enzyme-linked immunosorbent assay (ELISA). An ELISA plate (Maxisorp 442404, Nunc) was coated with 50 μl of anti-mouse IgM Ab (10 μg/ml; Organon Teknika, Durham, NC, USA) at 4 °C overnight, and then blocked with 100 μl of 5% FBS-PBS at room temperature for 1 h. The plates with 50 μl of the culture medium or appropriately diluted standard sera added were incubated at room temperature for 2 h. The plates with 50 μl of biotin-conjugated OVA (1 μg/ml) added were incubated at room temperature for 2 h. The plates with 50 μl of HRP-conjugated streptavidin added (diluted 1:2500; Zymed Lab, San Francisco, CA, USA) were further incubated at room temperature for 1 h. The wells were washed, and 100 μl of a 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was added to each well. The reaction was stopped by the addition of 50 μl of 0.18 M H₂SO₄/well, and the reaction products were measured with an ELISA reader at 450 nm. Pooled anti-OVA serum (640000 U/ml) obtained from OVA-immunized BALB/c mice was used as a standard. In the case of the *in vivo* experiments, the mice were immunized twice at 2-week intervals by means of an intraperitoneal injection of 100 μl of a PBS solution containing OVA (100 μg/body) with aluminum hydroxide as the adjuvant. The crude extract and fraction A were each dissolved in sterile water to form 0.56 g/l and 0.16 g/l solutions, respectively. These concentrations are equivalent to the concentration drunk as tea by steeping 3.5 g of Rooibos in 1 liter of boiling water. The solutions were ingested by the mice *ad libitum* (3–5 ml/day) starting from 1 week before the first immunization to the end of the experiment for 5 weeks. Sample sera were obtained at 0, 1, 2, 3, and 4 weeks after immunization and stored at −80 °C until an assay of anti-OVA IgM. The antigen-specific IgM levels were measured by ELISA. The ELISA process was the same as that just described.

For cytokine generation, spleen cells (1 x 10⁶ cells/200 μl/well) were cultured with OVA (500 ng/ml) and various concentrations of fraction A in 96-well round-bottom microplates for 3 days. The cytokine level in each cultured supernatant was measured by ELISA. For IL-2, IL-4 and IL-10 measurement, an ELISA plate was coated with 50 μl of anti-mouse IL-2, IL-4 (Pharmingen, San Diego, CA, USA) or IL-10 (Genzyme, Cambridge, MA, USA) Ab (2 μg/ml) at 4 °C overnight. The coated plates were blocked with 100 μl of 1% BSA-PBS at room temperature for 1 h. The plates with 50 μl of the culture medium or standard IL-2 or IL-4 (Genzyme) added were incubated at 4 °C overnight, while the plates with 50 μl of the culture medium or standard IL-10 (Genzyme) added were incubated at room temperature for 2 h. The plates with 50 μl of biotin-conjugated anti-mouse IL-2 or IL-4 Ab (500 ng/ml; Pharmingen) added were incubated at room temperature for 1 h, while the plates with 50 μl of biotin-conjugated anti-mouse IL-10 Ab (400 ng/ml; Genzyme) added were incubated at room temperature for 2 h. The plates with 50 μl of HRP-conjugated streptavidin (diluted 1:2500) added were further incubated at room temperature for 30 min. For IFN-γ measurement, an ELISA plate was coated with 50 μl of anti-mouse IFN-γ Ab (1 μg/ml; Endgen, Woburn, MA, USA) at 4 °C overnight. The coated plates were blocked with 100 μl of 4% BSA-PBS at room temperature for 1 h. The plates with 50 μl of culture medium or standard IFN-γ (PeproTech EC, London, UK) added were incubated at room temperature for 1 h. The plates with 50 μl of biotin-conjugated antimouse IFN-γ Ab (500 ng/ml; Endgen) added were incubated at room temperature for 1 h. The plates with 50 μl of HRP-conjugated streptavidin added (diluted 1:8000) were further incubated at room temperature for 30 min. The wells were washed, and 100 μl of a TMB substrate solution was added to each well. The reaction was stopped by the addition of 50 μl of 0.18 M H₂SO₄/well, and the reaction products were measured with an ELISA reader at 450 nm.

In our previous study, it was found that a Rooibos tea extract increased the antigen-specific antibody production in murine spleenocytes *in vitro*. We examined whether the Diaion HP20-fractionated extracts of Rooibos tea could enhance the antigen-specific antibody production in murine spleenocytes. As shown in Fig. 1, fraction A as well as the Rooibos tea crude extract markedly increased anti-OVA IgM production. The augmenting effect of fraction A on antigen-specific antibody production was at the same level as that of the crude extract. Fraction A also had an enhancing effect on anti-OVA IgM production in a concentration-dependent manner between 10 μg/ml and 300 μg/ml (data not shown). Fractions C and D decreased anti-OVA IgM production, while fraction B and E had no...
influence. These results suggested that the crude extract included both active and suppressive compounds on anti-OVA IgM production. Polyphenol-enriched plant extracts are used as functional food ingredients to enhance the health-promoting properties of food products. The Rooibos tea extract contains a large quantity of such flavonoids as aspalathin, rutin and orientin, but contains no caffeine and alkaloids, and has a low content of tannins. It has also been reported that oligosaccharides and polysaccharides from rooibos tea showed anti-HIV activity in vitro. The active ingredient of the Rooibos tea extract was not adsorbed and passed through the Diaion HP20 column. The HP-20 resin is a synthetic adsorbent of cross-linked polystyrene and well used for the separation of flavonoids. Therefore, these results suggested that the active substances in fraction A were not flavonoids, but might have been highly polar compounds such as oligosaccharides and polysaccharides.

It is well known that IL-10 is a kind of cytokine generated by T cells (Th2 and Tr1), B cells, mast cells and macrophages, and suppresses the production of IL-2 and IFN-γ by T cells (Th1). It is also known that IL-10 induces the proliferation and differentiation of B cells, regulates immunoglobulin synthesis by B cells, and suppresses the delayed allergy such as graft-versus-host disease. Fraction A significantly increased the IL-10 generation in a concentration-dependent manner between 10 μg/ml and 300 μg/ml in murine splenocytes in vitro (Fig. 2A). The enhancement profile of IL-10 production was nearly in accordance with that of the anti-OVA IgM production (data not shown). This result suggested that the enhancement of IL-10 production by fraction A contributed to the augmentation of anti-OVA IgM production. In contrast, fraction A decreased the IL-2 and IFN-γ generation in a concentration-dependent manner between 10 μg/ml and 300 μg/ml in murine splenocytes in vitro (Fig. 2B and C). The level of IL-4 was not affected by fraction A at 10 or 30 μg/ml but was decreased at 100 and 300 μg/ml (Fig. 2D). The crude extract also showed similar profiles for IL-2, IL-4, IL-10 and IFN-γ generation (data not shown). However, the decrease of IL-2 is contradictory to our previous report that the Rooibos tea crude extract increased the antigen-specific antibody production with IL-2 augmentation in murine splenocytes. The timing of IL-2 induction seems to have been altered by the use of Rooibos tea from a different supplier, although the anti-OVA IgM augmentation was not influenced. The present results suggest that IL-10 induced by fraction A suppressed the production of IL-2 and IFN-γ. IL-4, which was secreted in a small amount, may be considered to have been consumed for the proliferation and differentiation of B cells in the antibody production process at the concentration of more than 100 μg/ml.

We next examined whether fraction A, which showed strong augmentation of anti-OVA IgM production in vitro, would enhance the anti-OVA IgM production in vivo. Fraction A and the crude extract showed a strong effect in augmenting the anti-OVA IgM level in the serum (Fig. 3). The animals receiving fraction A and the crude extract solution as drinking fluid appeared to be healthy, showing no pathological signs or abnormalities during the ingestion period. No difference in the intake amount and growth rate was apparent in all of the groups tested (data not shown). Therefore, a routine intake of fraction A from Rooibos tea can be expected to be applicable to the prevention of infectious diseases.
Augmentation in vitro and that continuous ingestion of fraction A augmented the anti-OVA IgM level in the sera of OVA-immunized mice. These results indicate that the immunological activities of fraction A would be clinically useful. We propose in the near future to determine the structure of the active substance(s) in fraction A and to investigate the mechanism for the fraction A-induced antigen-specific antibody production and IL-10 generation.

Fig. 2. Effects of Fraction A on INF-γ (A), IL-2 (B), IL-10 (C) and IL-4 (D) Generation in Cultured Murine Splenocytes.
Splenocytes (1 x 10^6 cells/200μl/well) were cultured with OVA (500 ng/ml) and various concentrations of fraction A for 3 days. The cytokine levels were determined by ELISA. Each value is the mean ± SD for triplicate cultures. Statistical significance (*) was determined by Student’s t-test. *, P < 0.05; **, P < 0.01; ***, P < 0.001, as compared with the value of the control.

Fig. 3. Effects of the Crude Extract and Fraction A on the OVA-Specific IgM Level in Murine Sera.
The crude extract and fraction A solutions were ingested by mice ad libitum starting from 1 week before the first immunization to the end of the experiment for 4 weeks. The serum anti-OVA IgM level was determined by ELISA. Each value is the mean ± SE, n = 3. Statistical significance (*) was determined by Student’s t-test. *, P < 0.05; **, P < 0.01; ***, P < 0.001, as compared with the value of the vehicle.

References


