**Note**

The Soy Isoflavone Equol Enhances Antigen-Specific IgE Production in Ovalbumin-Immunized BALB/c Mice

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**Summary** Although an immunomodulatory role of the soy isoflavone genistein has been demonstrated, the effects of other soy isoflavones on induction of antigen (Ag)-specific immune responses are not known. In this study, we therefore investigated the effects of daidzein and equol on ovalbumin (OVA)-specific T cell and B cell responses in BALB/c mice. Mice that had been treated with 20 mg/kg equol showed a significantly higher level of OVA-specific IgE than control mice. Levels of interferon (IFN)-γ and interleukin (IL)-4 production were not different between the control and equol groups. However, IL-13 production level in mice administered 20 mg/kg equol was significantly higher than that in control mice. Strong induction of OVA-specific IgE production by equol was also observed in ovariectomized BALB/c mice, suggesting that the immunomodulatory effect of equol is not affected by endogenous estrogen.

**Key Words** soy isoflavone, equol, IgE, IL-13

Soy foods have been consumed for centuries in Asian countries. Human clinical and epidemiological studies have revealed many potential benefits of intake of soy products (1). For example, consumption of soy foods may contribute to lower incidences of coronary heart diseases (2), atherosclerosis (3) and type 2 diabetes (4) and to decreased risk of certain types of carcinogenesis such as breast and prostate cancers (5, 6) as well as better bone health and relief of menopausal symptoms (7). Phytoestrogens, naturally occurring hormone-like compounds in plant food, have attracted considerable attention because of their antioxidative, antiatherogenic and anticarcinogenic potentials.

Genistein is the major soy-derived phytoestrogen. Effects of genistein on humoral and cell-mediated immunity have been extensively examined, and various effects on immune responses have been found. Genistein reduced KLH-specific antibody (Ab) titers in KLH-immunized mice (8) and decreased DTH responses to a hapten 4-hydroxy-3-nitrophenyl acetyl succinimide and oxazolone (9, 10). In contrast to these immunosuppressive effects, genistein increased the activity levels of cytotoxic T cells and NK cells, conferring the cells with resistance to tumor challenge (11, 12). Thymic atrophy is observed in genistein-treated mice and the atrophy is partly estrogen receptor (ER)-dependent (8). Beneficial effects of genistein on immune functions and anti-inflammatory properties have also been found (10).

In contrast to genistein, the effects of daidzein and its metabolite equol in vivo have not been examined. Despite the limited estrogenicity of daidzein, some recent evidence suggests that its metabolite equol may play a critical role in phytoestrogen effects (11). Daidzein is converted by microbial biotransformation in the intestine to the isoflavone equol, a biologically active metabolite (12). Equol has a significantly longer half-life in circulation than that of genistein or daidzein (13–15). In this study, we examined the influence of daidzein and equol on the induction of antigen (Ag)-specific immune responses in BALB/c mice.

**Materials and Methods**

_Mice and diet._ Ovariectomized BALB/c mice (Japan SLC, Inc., Shizuoka, Japan) were maintained under specific pathogen-free conditions with a 12-h light:dark cycle at 25 ± 2°C and 55 ± 10% relative humidity. The mice were 8 wk old with body weights of 20–21 g at the beginning of the studies. All studies were performed in accordance with the ethical guidelines for animal experimentation of the Institution of Health Bioscience, The University of Tokushima and were approved by the institutional review board of the animal ethics committee.

The mice were provided with a soy isoflavone-free diet and water ad libitum. The composition of food was 20% casein, 44.7% α-starch (Oriental Yeast Co., Ltd., Chiba, Japan), 22.3% sucrose (Mitsui Sugar Co., Ltd., Osaka, Japan), 5% corn oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 2% cellulose, 5% mineral mixture and 1% vitamin mixture (Oriental Yeast Co., Ltd.).

_Daidzein and equol treatment._ Daidzein and (R,S)-
equol (LC Lab., MA, USA) solutions were freshly prepared daily in 25 mM Na₂CO₃. The mice were administered 200-μL solutions containing 4 or 20 mg isoflavone/kg body for 35 d by gavage. Control mice were treated with 200 μL of 25 mM Na₂CO₃ alone instead of isoflavone solution.

**Immunization.** Mice were intraperitoneally immunized with 10 μg of ovalbumin (OVA) (Sigma Chemical Co., MO, USA) absorbed in 2 mg of Aluminium Hydroxide Gel Adjuvant (HCl Biosector, Denmark) on days 7 and 21 after the start of genistein administration.

**Proliferation assay.** To prepare single cell suspensions, the spleen from mice administered vehicle, daidzein or equol was squeezed with two slide glasses in RPMI-1640 medium (Sigma) and filtered through mesh. To remove red blood cells, the cells were treated with 0.83% NH₄Cl (pH 7.5) at 37˚C for 10 min. Then the cells were washed two times with RPMI-1640 medium and suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 100 μg/mL streptomycin and 100 U/mL penicillin. Five×10⁶ splenocytes were stimulated with 400 μg/mL OVA or plate-bound anti-CD3 mAb (coated overnight at 1 μg/mL) in a 48-well flat-bottom plate at 37˚C under 5% CO₂ for 72 h. After the culture, culture supernatants were collected and stored at −40˚C until used. Interferon (IFN)-γ, interleukin (IL)-4 and IL-13 in the supernatants were quantified using a mouse IFN-γ, IL-4 or IL-13 ELISA kit (eBioscience, CA, USA) according to the manufacturer’s instructions.

**Determination of Ag-specific Ab levels.** On day 35 after the start of isoflavone administration, blood collected from mice was centrifuged, and sera were stored at −20˚C until used. Serum levels of OVA-specific Abs were measured by ELISA. Ninety-six-well ELISA plates were coated with 10 μg/mL OVA in 50 mM carbonate buffer (pH 9.6) and left overnight at 4˚C. The plates were washed with 0.05% Tween 20-PBS (PBS-T) and blocked with 1% bovine serum albumin (BSA)-PBS-T. All washing steps were done with PBS-T. After washing, various dilutions of the samples were added and incubated for 2 h at room temperature. Then the plates were washed and incubated with alkaline phosphatase (AP)-conjugated anti-mouse IgG Ab, AP-conjugated anti-mouse IgG1 Ab and AP-conjugated anti-mouse IgG2a Ab (Southern Biotechnology Associates, Inc., AL, USA) for 2 h at room temperature. In the case of OVA-specific IgE determination, plates that had been coated with
OVA were incubated with diluted serum and then biotin-conjugated anti-mouse IgE mAb (Becton Dickinson, CA, USA) for 1 h at room temperature. Then streptavidin-conjugated AP (Beckton Dickinson) was added after washing and incubated for 1 h at room temperature. After the plates had been washed, 1 mg/mL p-nitrophenyl phosphate (Sigma) in 10% diethanolamine buffer was added to the wells. The reaction was terminated by adding 3 M NaOH, and the OD was determined at 405 nm.

Statistical analysis. Data were analyzed using one-way analysis of variance followed by the Scheffe post hoc test for multiple comparisons. Data are expressed as means±SD. Differences were considered significant at \( p<0.05 \).

Results

There were no differences between the groups in mean weight, weight gain, spleen weight or lymphocyte subsets expressing CD4, CD8, B220 or DX5 molecule (data not shown).

We first analyzed humoral immune response in OVA-immunized and isoflavone-administered BALB/c mice. Serum OVA-specific IgG, IgG1, IgG2a and IgE levels were evaluated using serially diluted serum. Significant differences were not found in OVA-specific IgG, IgG1 or IgG2a production between control mice and mice administered different doses of daidzein or equol. In contrast, mice that received 20 mg/kg equol showed a significantly higher level of OVA-specific IgE than the control mice and mice that received 4 mg/kg daidzein (Fig. 1).

To address the mechanism of IgE induction by equol, production levels of cytokines from OVA-stimulated splenocytes were measured by ELISA. Levels of IFN-\( \gamma \), IL-4 and IL-13 were examined, but a significant difference was not observed among the 5 groups. However, IL-13 production level in mice administered 20 mg/kg equol was significantly higher than that in control mice (Fig. 2).

It has been reported that endogenous estrogen interfaces with genistein, thus affecting immune response (16). Equol has weak estrogenic activity because of its structural similarity to estrogen. To avoid the effect of endogenous estrogen, ovariectomized BALB/c mice were administered 20 mg/kg equol and immunized with OVA. In our preliminary experiment, equol administration enhanced Ag-specific IgE response in sham operated BALB/c mice (data not shown). OVA-specific IgE was significantly higher in the equol group than in the control group as in the case of non-ovariectomized mice (Fig. 3).

Discussion

In this study, we demonstrated that equol significantly increased Ag-specific IgE production from B cells...
and Ag-specific IL-13 production from T cells in BALB/c mice. Soy isoflavones have been shown to have potential beneficial effects, including enhancement of immune function. However, most studies on soy isoflavones and immune function have been performed using genistein, and information on the effects of other soy isoflavones on immune functions has been limited. To our knowledge, this is the first study showing equol affects Ag-specific immune response and induces Ag-specific IgE production in vivo.

The identification of a functional polarization of mouse CD4+ Th cell clones based on their cytokine secretion profiles has provided a molecular insight into the regulation of immune response (17, 18). Th1 cells producing IL-2, IFN-γ and LTβ are a subset that is important for enhancing cellular immune responses, while Th2 cells, characterized by their secretion of IL-4, IL-5, IL-6, IL-10 and IL-13, evoke production of IgE and accumulation of eosinophils (19, 20). Th1 and Th2 reciprocally regulate their function and maintain immune homeostasis. Breakdown of Th1/Th2 balance leads to the development of allergies or autoimmune diseases. In this study, we demonstrated that equol enhances Ag-specific IgE production (Fig. 1) and we investigated the molecular mechanisms underlying the enhancement of Ag-specific IgE production. It has been shown that both IL-4 and IL-13 are crucial for induction of IgE (21, 22). The results showing that equol increased IL-13 production provide evidence that the enhancement of IgE production by equol is mediated by IL-13 rather than by IL-4. IgG1 level is known as a physiological marker for Th2 response, but its levels was not different between the control and equol groups (Fig. 1). In IL-13 gene-deficient mice, the level of OVA-specific IgE was significantly decreased by OVA immunization, but the OVA-specific IgG1 level was not different from that in control mice. Therefore, IgE production is more sensitive to IL-13 than is IgG1 production.

It has been shown that the soy isoflavones formononetin, daidzein and equol enhance IL-4 production by mediating AP-1 DNA binding activity (23). Although IL-4 production but not IL-13 production was observed in the study by Park et al. (23), equol did not influence the level of IL-4 in our study (Fig. 2). Different experimental designs may account for the discrepancy. In Park’s study, the effect of equol on cytokine production was investigated by using PMA-stimulating EL-4 lymphoma cells, while the effect was investigated by using OVA-immunized mouse spleen cells in our study.

Equol is a nonsteroidal estrogen of the isoflavone class. It is exclusively a product of intestinal bacterial metabolism of dietary isoflavones and it possesses estrogenic activity, having affinity for both estrogen receptor (ER)α and ERβ. Maret et al. found that OVA-specific Th1 responses were increased in estrogen-administered mice but were diminished in estrogen-administered ERα-deficient mice (24). This indicates that ERα is essential for the induction of Th1 response by estrogen. Considering these studies, we examined the possibility that equol competes with endogenous estrogen for binding to the estrogen receptor, thus resulting in a suppression of Th1 response. To avoid the effect of endogenous estrogen, ovariectomized BALB/c mice were given equol and immunized with OVA, and the serum levels of OVA-specific IgE were determined. Levels of OVA-specific IgE were increased in mice administered equol, suggesting that enhancement of IgE by equol is not due to competition with endogenous estrogen.

We previously demonstrated that the soy isoflavone genistein suppresses Ag-specific immune response. The mechanism underlying the suppression by genistein is competition of the endogenous estrogen with the estrogen receptor (16). The soy isoflavones equol and genistein structurally resemble estrogen. At present, we do not know the reason why immunity-modulating action is different between equol and genistein. The binding affinity of equol for ERα and ERβ was found to be similar to that of genistein, but equol induced transcription more strongly than any other isoflavone, especially with ERα (25). In addition, equol has been shown to have the greatest antioxidant activity of all isoflavones tested when measured in vitro in the ferric reducing ability of plasma (26, 27). These unique properties of equol might account for the different immune regulations by isoflavones.

In summary, in this study, we found that the soy isoflavone equol enhanced Ag-specific IgE production and that IL-13, but not IL-4, contributes to the enhancement of Ag-specific IgE production.

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