JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

*J. Agric. Food Chem.* **2006,** *54,* 5203−5207 **5203**

# Dietary Apigenin Suppresses IgE and Inflammatory Cytokines Production in C57BL/6N Mice

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Flavonoids ubiquitously exist in plants, vegetables, fruits, and teas. We evaluated the effect of dietary apigenin, one of the well-known flavonoids, on the immune system in C57BL/6N mice. Mice were fed experimental diets containing apigenin for 2 weeks. After the experimental period, there was no significant difference in body and organ weights between the control and the apigenin group. The total immunoglobulin (Ig) E levels in mice fed apigenin were significantly suppressed, whereas levels of IgG, IgM, and IgA were not affected. We also examined the effect of the apigenin diet on cytokine expression in mice sera using a cytokine array. The production of regulated upon activation normal T cell expressed and secreted (RANTES) and soluble tumor necrosis factor receptor I (sTNFRI) in mice sera was down-regulated by the apigenin diet. These results suggest that a diet containing apigenin can reduce serum IgE and inflammatory cytokines such as RANTES and sTNFRI in mice.

**KEYWORDS: Apigenin; IgE; RANTES; sTNFRI; C57BL/6N mice**

## INTRODUCTION

The flavonoids are a diverse family of chemicals commonly found in fruits and vegetables. Flavonoids are plant polyphenolic compounds, which have a diphenylpropane skeleton (C6C3C6) structure, that include monomeric flavanols, flavanones, flavones, and flavonols. Some flavonoids have been found to possess various clinically relevant properties such as antitumor, antiplatelet, antiischemic, and antiinflammatory activities (*1*−*3*); yet, they have many more biological effects. Many different enzymes involved in intracellular signaling can be affected by flavonoids (*4*). Especially the effects of flavonoids on protein kinases are of great interest since they directly influence immune functions in the host. Apigenin, one of the most common flavonoids, is widely distributed in many fruits and vegetables such as parsley, onions, orange, teas, chamomile, and wheat sprouts and in some seasonings (*5*−*8*).

Our previous report demonstrated that two flavones, chrysin and apigenin, could suppress the expression of the high affinity immunoglobulin (Ig) E receptor Fc$\epsilon$RI expression in human basophilic KU812 cells and the suppressive effect of apigenin on Fc$\epsilon$RI expression was stronger than that of chrysin (*9*). Moreover, it has been reported that apigenin has been shown to be an inhibitor for IL-4 production by basophils in vitro (*10*). However, the effect of apigenin on the production of Igs and cytokines in vivo is not examined. In the present study, we report the effect of dietary apigenin on the production of Igs and cytokines in C57BL/6N mice.

## MATERIALS AND METHODS

**Materials.** Apigenin (4′,5,7-trihydroxyflavone) was purchased from Aldrich Chemical Co. (St. Louis, MO). Phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187 were purchased from Sigma Chemical Co. (St. Louis, MO), respectively.

**Experimental Animals and Diet.** Male 8 week old C57BL/6N mice were obtained from Kyudo Co., Ltd. (Tosu, Japan). They were kept at the Biotron Institute of Kyushu University in a 12 h light/12 h dark cycle (light on 8 a.m.−8 p.m.) in an air-conditioned room (20 °C and 60% humidity under specific pathogen-free conditions). This experiment was carried out according to the guidelines for animal experiments at the Faculty of Agriculture and the Graduate Course, Kyushu University, and the Law (no. 105) and Notification (no. 6) of the Japanese government. After preliminary breeding for 1 week, the mice were divided into two groups and provided with one of the following diets ad libitum: control diet (MF diet, Oriental Inc.) or apigenin diet (MF diet containing 0.025% apigenin) for 2 weeks. At the end of the feeding period, mice were killed by drawing blood from the abdominal aorta under light anesthesia with diethyl ether. Serum was obtained by centrifugation at 1000*g* for 15 min at 4 °C and stored at −80 °C until use. The body weight was measured before the experimental feed started and after it ended. Immediately after excision, each tissue was weighed and the lymphocytes were isolated from spleen.

**Measurement of Ig Levels.** The measurement of Ig concentration in the mice sera was performed using a sandwich enzyme-linked immunosorbent assay (ELISA). Rabbit anti-mouse IgA (Zymed, San

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10.1021/jf0607361 CCC: $33.50 
Published on Web 06/13/2006

Francisco, CA), goat anti-mouse IgG (H + L) (Zymed), and rabbit anti-mouse IgM ($\mu$-chain specific) (Zymed) were used to fix each Ig. These antibodies were diluted using 1% bovine serum albumin−phosphate-buffered saline (BSA−PBS), added to a 96 well plate, and incubated for 1 h at 37 °C. Then, 300 $\mu$L of 1% BSA−PBS was added and kept at 37 °C for 2 h; samples (50 $\mu$L) were added to each well for 1 h at 37 °C. Each well was treated with a solution of either peroxidase (POD)-conjugated goat anti-mouse IgA (Zymed), POD-conjugated goat anti-mouse IgG (H + L) (Zymed), or POD-conjugated rabbit anti-mouse IgM (Zymed) to detect the respective Ig and incubated for 1 h at 37 °C. The plates were rinsed with PBS containing 0.5 mL/L polyethylene sorbitan monolaurate (Nacalai Tesque, Kyoto, Japan) between each step. Then, a 10:9:1 mixture of 1.8 mM $H_2O_2$ in 0.2 M citrate buffer (pH 4.0), $H_2O$, and 11.7 mM of 2,2′-azinobis (3-ethylbenzothiazoline sulfonic acid) was added. Finally, absorbance at 415 nm was measured after the addition of 160 mM oxalic acid to stop the coloring reaction. Measurement of total serum IgE concentration was determined using the ELISA Mouse IgE kit (Seikagaku Co., Tokyo, Japan).

**Evaluation of Cytokine Levels in Mice Sera.** Thirty-two cytokine proteins in mice sera were assessed using a commercially available mouse cytokine protein array kit (Ray Biotech, Inc., Norcross, GA) following the appended protocol. Briefly, membranes were incubated with mice sera for 1 h at room temperature, and then, the membranes were treated with biotin-conjugated antibodies. Next, membranes were treated with POD-conjugated streptavidin at room temperature for 1 h. Data were analyzed using the image analyzer ChemImager 5500 (Alpha Innotech, San Leandro, CA).

**Preparation and Stimulation of Spleen Lymphocytes.** Immediately after the excision, lymphocytes were isolated from the spleen. Cells were suspended in RPMI 1640 medium (Nissui, Tokyo, Japan) and washed two times. Then, 5 mL of the cell suspension was added to lympholyte-mouse (Cedarlane, Hornby, Canada) to isolate the lymphocytes. Red cells were lysed with the ammonium-chloride potassium carbonate buffer (155 mM $NH_4Cl$, 10 mM $KHCO_3$, and 10 mM ethylenediaminetetraacetic acid, pH 7.4), and lymphocytes were washed two times with RPMI 1640 medium. The spleen lymphocytes, $2 \times 10^6$ cells/mL, were cultured in RPMI 1640 medium containing 5% fetal calf serum (PAA Laboratories GmbH, Austria) with 0.5 ng/mL PMA and 0.15 $\mu$M A23187 and then incubated at 37 °C for 24 h.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR).** The total RNA was extracted from lymphocytes costimulated with PMA and A23187 using TRIZOL (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. First-strand cDNA was synthesized from total RNA (7 $\mu$g) with a $(dT)_{20}$ primer and 20 U of Moloney mouse leukemia virus (MMLV) reverse transcriptase (Amersham Pharmacia Biotech). Specific primer sequences for each gene were as follows: mouse IL-2, sense 5′-ATGTACAGCATGCAGCTCGCATC-3′ and antisense 5′-GGCTTGTTGAGATGATGCTTTGACA-3′; mouse IL-4, sense 5′-ATGGGTCTCAACCCCCAGCTAGT-3′ and antisense 5′-GCTCTTTAGGCTTTCCAGGAAGTC-3′; mouse IL-6, sense 5′-CTGGTGACAACCACGGCCTTCCCTA-3′ and antisense 5′-ATGCTTAGGCATAACGCACTAGGTT-3′; mouse IL-10, sense 5′-ATGCAGGACTTTAAGGGTTACTTGGGTT-3′ and antisense 5′-ATTTCGGAGAGAGGTACAAACGAGGTTT-3′; mouse IL-12p40, sense 5′-GAGGTGGACTGGACTCCCGA-3′ and antisense 5′-CAAGTTCTTGGGCGGGTCTG-3′; mouse IFN-$\gamma$, sense 5′-AACGCTACACACTGCATCT-3′ and antisense 5′-AGCTCATTGAATGCTTGG-3′; mouse TNF-$\alpha$, sense 5′-TCCCCAAAGGGATGAGAAGTTC-3′ and antisense 5′-TCATACCAGGGTTTGAGCTCAG-3′; mouse RANTES, sense 5′-GTGCCCACGTCA AGAGGATAT-3′ and antisense 5′-GGGAAGCGTATACAGGGTCA-3′; and mouse $\beta$-actin, sense 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and antisense 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′. Specific PCR fragments were separated on an agarose gel electrophoresis and visualized by ethidium bromide.

**Statistical Analysis.** The Student's *t*-test was used for the statistical analysis.

## RESULTS

**Body and Organ Weights.** We first examined whether an apigenin intake affects the weight of the body and organs. **Table 1** shows body and tissue weights after the experimental period. We set the initial body weight at 22.1 g for both experimental groups. There was no significant difference in body and organ weights during the study.

**Table 1.** Effects of Apigenin on the Growth Parameters of C57BL/6N Mice

| parameter | control | apigenin |
|---|---|---|
| | body weight (g) | |
| initial | 22.1 ± 0.1 | 22.1 ± 0.1 |
| final | 24.3 ± 0.4 | 24.1 ± 0.4 |
| | tissue weights (g) | |
| heart | 0.13 ± 0.00 | 0.12 ± 0.00 |
| kidney | 0.32 ± 0.01 | 0.31 ± 0.01 |
| liver | 1.22 ± 0.03 | 1.18 ± 0.02 |
| lung | 0.14 ± 0.00 | 0.14 ± 0.00 |
| spleen | 0.07 ± 0.00 | 0.07 ± 0.00 |

[a] Data are means ± SEM for eight mice in each group.

**Table 2.** Total Serum IgG, IgM, IgA, and IgE Levels in C57BL/6N Mice

| Ig | control | apigenin |
|---|---|---|
| IgG (mg/mL) | 4.3 ± 1.2 | 2.1 ± 0.1 |
| IgM (mg/mL) | 1.0 ± 0.2 | 1.0 ± 0.1 |
| IgA ($\mu$g/mL) | 275.7 ± 39.6 | 403.1 ± 53.0 |
| IgE (ng/mL) | 8.6 ± 1.8 | 4.3 ± 0.4* |

[a] Serum concentrations of Ig levels from five to six mice were measured by ELISA. Data are means ± SEM. Statistical analysis was performed using Student's *t*-test. *$P < 0.05$; significantly different from control diet.

**Table 3.** Effects of Apigenin on Cytokine Expression in Mice Sera

| cytokines | relative intensity as compared to the control group | cytokines | relative intensity as compared to the control group |
|---|---|---|---|
| 6Ckine | ND | IFN-$\gamma$ | 1.086 |
| CTACK | 0.815 | KC | 1.158 |
| eotaxin | 0.978 | leptin (OB) | 1.011 |
| GCSF | 0.995 | MCP-1 | 1.053 |
| GM-CSF | 1.043 | MCP-5 | 1.084 |
| IL-2 | 1.142 | MIP-1$\alpha$ | 1.115 |
| IL-3 | ND | MIP-2 | 0.876 |
| IL-4 | 1.253 | MIP-3$\beta$ | 0.813 |
| IL-5 | ND | RANTES | 0.791 |
| IL-6 | 1.148 | SCF | 0.847 |
| IL-9 | 0.952 | sTNFRI | 0.761 |
| IL-10 | 0.929 | TARC | 0.801 |
| IL-12 | 0.961 | TIMP-1 | 0.819 |
| IL-12 p70 | 0.973 | TNF-$\alpha$ | 0.954 |
| IL-13 | 1.000 | TPO | 0.848 |
| IL-17 | 1.000 | VEGF | 1.030 |

[a] The relative levels of cytokine were determined by intensity. The densities of signals were normalized with background and positive control. CTACK, cutaneous T-cell-attracting chemokine; GCSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; IL, interleukin; IFN, interferon; KC, CXC ligand 1; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated upon activation normal T cell expressed and secreted; SCF, stem cell factor; sTNFR, soluble tumor necrosis factor receptor; TARC, thymus and activation-regulated chemokine; TIMP, tissue inhibitor of metalloprotease; TNF, tumor necrosis factor; Tpo, thrombopoietin; VEGF, vascular endothelial growth factor; and ND, not detected.

**Ig Productions in Mice Sera.** We investigated whether dietary apigenin could affect Ig productions, and serum antibody levels were measured by ELISA. As shown in **Table 2**, we could not detect any significant deference between the control and the apigenin group for IgG, IgM, and IgA. However, the levels

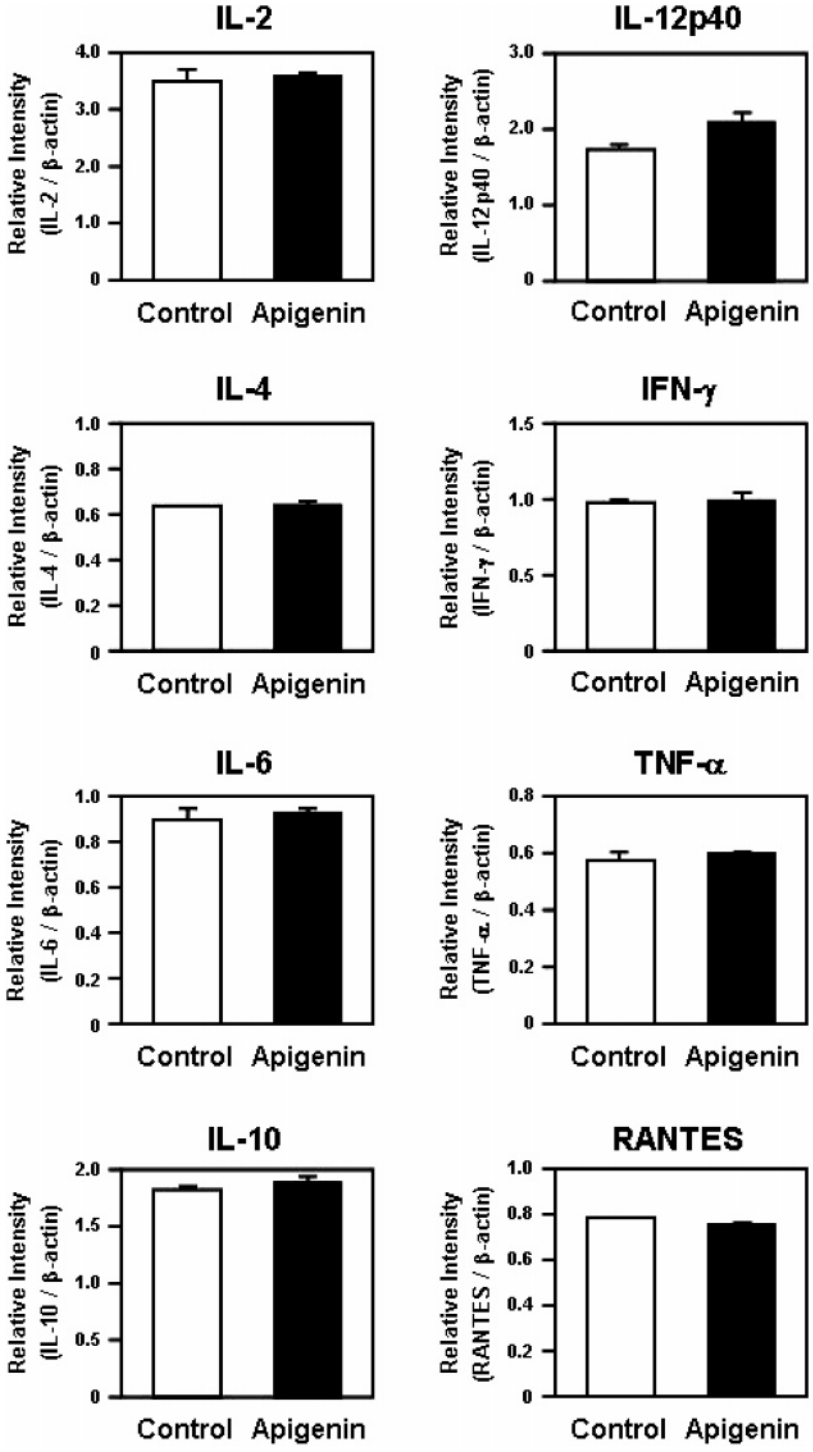


**Figure 1.** Effect of the apigenin diet on the expression of cytokines mRNA in spleen lymphocytes. Total mRNA was isolated from spleen lymphocytes stimulated with PMA and A23187 in C57BL/6N mice fed apigenin. Each cytokine and $\beta$-actin mRNA was analyzed by RT-PCR. Data are means ± SEM for four mice in each group.

of IgE in mice fed with apigenin diet were significantly lower than those in mice fed with control diet.

**Cytokines Levels in Mice Sera.** As dietary apigenin decreased the IgE level in mice sera, we hypothesized that apigenin can regulate cytokines production in mice sera. To identify cytokines affected by feeding apigenin, a cytokine protein array assay was performed as shown in **Table 3**. There were no cytokines up-regulated or down-regulated over two times in the apigenin group as compared with the control group. Only regulated upon activation normal T cell expressed and secreted (RANTES) and soluble tumor necrosis factor receptor I (sTNFRI) levels in mice sera derived from the apigenin group tended to decrease as compared with those of the control group (relative intensity of RANTES and sTNFRI in mice sera from the apigenin group as compared to the control group were 0.791 and 0.761, respectively).

**Quantitation of Cytokines mRNA Levels from Spleen Lymphocytes.** PMA is mitogenic to T and B cells activating several cytokine-encoding genes. Ionophore A23187 increases calcium permeability across the cellular membrane to the cytosol of lymphoid cells and is considered a comitogen of T lymphocytes (*11*). Practically, on costimulation with PMA and A23187,

some cytokines mRNA expression in spleen lymphocytes was significantly up-regulated (data not shown). After costimulation with PMA and A23187, mRNA expressions of IL-2, IL-4, IL-6, IL-10, IL-12p40, IFN-$\gamma$, TNF-$\alpha$, and RANTES in spleen lymphocytes from mice fed apigenin were detected by RT-PCR. No significant differences were found in these cytokines mRNA expression between the control group and the apigenin group (**Figure 1**).

## DISCUSSION

IgE is one of the major mediators of the immediate hypersensitivity reaction that underlies atopic conditions such as seasonal allergy, food allergy, asthma, and anaphylaxis (*12*). In many individuals, the level of total serum IgE correlates roughly with the severity of these allergic diseases (*13*). As shown in **Table 2**, we demonstrated that in vivo apigenin selectively down-regulates the production of IgE. IL-4 and IL-13 are key molecules related to IgE production, Th2 differentiation, and allergic inflammation. However, **Table 3** shows that IL-4 and IL-13 levels in mice sera were not modulated by the apigenin diet.

On the other hand, in this study, the apigenin diet tended to decrease the RANTES level in sera of mice. RANTES is a key signaling chemokine involved in the initiation of physiologic inflammation (*14*). Chemokines including RANTES are relevant in allergy and asthma not only for their role in regulating leukocyte recruitment but also for other activities, such as cellular activation, inflammatory mediator release, promotion of Th2 inflammatory responses, and regulation of IgE synthesis (*15*). The reduction of serum IgE by apigenin diet may result in part from the decrease serum RANTES. Recently, it has been reported that peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$) ligands decrease RANTES protein secretion in human endometrial stromal cells (*16*). It was reported that some flavonoids including apigenin exhibited weak PPAR$\gamma$ agonist activities in an in vitro competitive-binding assay (*17*). Moreover, we previously reported that PPAR$\gamma$ ligand inhibits IL-4-induced IgE class switching in human B cell line DND39 (*18*). Apigenin acting as PPAR$\gamma$ ligands may suppres both RANTES production and thus IgE production in vivo. For the future, it is necessity to confirm whether apigenin suppresses the RANTES as well as PPAR$\gamma$ ligands and affects directly IgE production by B cells.

We also found that dietary apigenin was down-regulated sTNFRI levels in mice sera as shown in **Table 3**. Soluble TNFRI, which circulates in many body fluids (*19*−*21*), represents the extracellular domains of the TNFRI (*22*). Elevated sTNFRI levels have been found in serum, plasma, ascites, or urine in association with endotoxinemia, infections, and malignancies as well as chronic autoimmune disorders including inflammatory bowel disease (*23*−*27*). Our results conclude that the apigenin diet can decrease the inflammatory cytokines level in sera.

In spleen lymphocytes, there were no effects of dietary apigenin on IL-2, IL-4, IL-6, IL-10, IL-12, IFN-$\gamma$, and TNF-$\alpha$ mRNA expression induced by PMA and A23187. These results were consistent with the results obtained by cytokine array. On the other hand, dietary apigenin reduced the RANTES level in mice sera without down-regulating its mRNA expression from spleen lymphocytes. RANTES expresses in various cells including T lymphocytes, monocytes, basophils, and eosinophils (*28*). We presume that apigenin diet might attenuate the RANTES expression in tissues except spleen lymphocytes although further studies are required.

We showed here that dietary apigenin reduced serum IgE, RANTES, and sTNFRI levels in C57BL/6N mice. A wide range of phenolic compounds are present in plants, vegetables, fruits, and teas, and their chemical characteristics such as the type of glycosylation, esterification, or polymerization have a great influence on their bioavailability and metabolism (*29*−*32*). Apigenin is detected in substantial amounts in parsley, celery, bell pepper, belimbi fruit, guava, and the chamomile anthodium (*33*−*35*) and accumulates in a bound form as apigenin 7-*O*-glucoside and various acylated derivatives. After ingestion, the apigenin glucosides are hydrolyzed by both intestinal mucosal and bacterial $\beta$-glucosidases releasing the aglycone. In a recent study, absorption and excretion of apigenin after the ingestion of apigenin-rich food, i.e., parsley, were tested; in the result, apigenin is enriched in the human circulation. However, maximum plasma concentrations were comparably low (0.34 $\mu$mol/L), and on average, only 0.22% of the ingested apigenin dose was found in the 24 h urine samples. The main part of the ingested apigenin was either excreted unabsorbed or was rapidly metabolized after absorption (*36*).

In case of intake flavonoids as dietary supplements, we ingested at 1−3 g/day. Moreover, in the normal U.S. diet, flavonoids are unavoidably consumed daily with an estimated total consumption of 1 g/day (*37*). This quantity corresponds to apigenin contents (0.025%) comprised of the diet in our experiments. Diet therapy for allergic diseases has not been established except for avoidance of food allergens in cases when patients are sensitized to foods. The intake of apigenin may alleviate allergic symptoms and even prevent allergic diseases. On the other hand, a vegetable very rich in apigenin such as parsley and celery is also very often involved in adverse reactions to foods. Thus, the results coming from this study need to be deeply investigated in the future.

## ACKNOWLEDGMENT

We thank S. Kobayashi, H. Onda, M. Sumida, and A. Tajima for technical assistance.

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**Received for review March 15, 2006. Revised manuscript received May 11, 2006. Accepted May 17, 2006. This work was supported in part by grants from the Kanpou Science Foundation and from the Integrated Research on Safety and Physiological Function of Food, MAFF, Japan, to H.T.**

JF0607361