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Flavonoids such as Luteolin, Fisetin and Apigenin Are Inhibitors of Interleukin-4 and Interleukin-13 Production by Activated Human Basophils

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Key Words
Allergy · Asthma · Basophils · Flavonoids · Interleukin-4 · Th2

Abstract
Background: We have previously shown that fisetin, a flavonol, inhibits IL-4 and IL-13 synthesis by allergen- or anti-IgE-antibody-stimulated basophils. This time, we investigated the inhibition of IL-4 and IL-13 production by basophils by other flavonoids and attempted to determine the fundamental structure of flavonoids related to inhibition. We additionally investigated whether flavonoids suppress leukotriene C4 synthesis by basophils and IL-4 synthesis by T cells in response to anti-CD3 antibody. ***Methods:*** Highly purified peripheral basophils were stimulated for 12 h with anti-IgE antibody alone or anti-IgE antibody plus IL-3 in the presence of various concentrations of 18 different kinds of flavones and flavonols. IL-4 and IL-13 concentrations in the supernatants were then measured. Leukotriene C4 synthesis was also measured after basophils were stimulated for 1 h in the presence of flavonoids. Regarding the inhibitory activity of flavonoids on IL-4 synthesis by T cells, peripheral blood mononuclear cells were cultured with flavonoids in anti-CD3-antibody-bound plates for 2 days. ***Results:*** Luteolin, fisetin and apigenin were found to be the strongest inhibitors of both IL-4 and IL-13 production by basophils but did not affect leukotriene C4 synthesis. At higher concentrations, these flavonoids suppressed IL-4 production by T cells. Based on a hierarchy of inhibitory activity, the basic structure for IL-4 inhibition by basophils was determined. ***Conclusions:*** Due to the inhibitory activity of flavonoids on IL-4 and IL-13 synthesis, it can be expected that the intake of flavonoids, depending on the quantity and quality, may ameliorate allergic symptoms or prevent the onset of allergic diseases.

Introduction

Th2 cytokines such as IL-4 and IL-13 play an important role in the pathogenesis of allergic diseases [1]. We have recently demonstrated that fisetin, a flavonol, inhibits the synthesis of these cytokines by activated basophils [2]. This time, we investigated whether 18 kinds of flavones and flavonols had an inhibitory effect on IL-4 and

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IL-13 synthesis or leukotriene C4 production, which is believed to be a major mediator in allergic inflammation. We also studied whether flavonoids might inhibit IL-4 production by T cells.

Methods

Culture Medium

RPMI 1640 (Nacalai Tesque, Kyoto, Japan) supplemented with 10% FCS (Dainippon Pharmaceutical, Osaka, Japan), *L*-glutamine (2 m*M*), 2-mercaptoethanol (0.05 m*M*), penicillin (100 U/ml) and streptomycin (100 μg/ml) was used as the culture medium.

Chemicals

Flavonoids were purchased from Extrasynthese (Genay, France) and dissolved in methanol at a concentration of 10 m*M*. Anti-human IgE antibody (Ab) was obtained from DAKO (Glostrup, Denmark). Anti-human CD3 Ab was purchased from Pharmingen (San Jose, Calif., USA). IL-3 was purchased from Genzyme-Techne (Boston, Mass., USA).

Purification of Basophils

Peripheral blood buffy coats from healthy transfusion donors were anticoagulated with 10 m*M* EDTA and mixed with the same amount of phosphate-buffered saline. This was then layered onto Ficoll-Paque PLUS (Amersham Pharmacia Biotech, Amersham, UK) and centrifuged at 400 *g* for 20 min. This was followed by negative selection using a MACS Basophil Isolation Kit (Miltenyi Biotech, Bergisch-Gladbach, Germany) as described before [2, 3]. The number of total cells and their viability were determined by staining with trypan blue solution and counted with a hemocytometer. The purity of the basophils in this fraction was assessed by examining 1,000 cells on cytospins treated with May-Grünwald stain. The basophil purity was 80–93% in four different experiments, and the contaminating cells consisted mainly of small lymphocytes and few monocytes. For convenience, these basophil-enriched fractions are henceforth simply referred to as basophils.

Measurement of IL-4 and IL-13 Synthesis by Basophils

Purified basophils (0.4–1 × 10^6 cells/ml) were incubated without (equivalent amount of diluted methanol) or with flavonoid at an indicated dose for 15 min and then stimulated with anti-IgE Ab (1 μg/ml) alone or plus IL-3 (20 ng/ml) for 12 h. The concentration of anti-IgE Ab has previously been determined to induce maximal synthesis of IL-4. The supernatant was harvested and the level of IL-4 and IL-13 in the culture supernatant was measured by means of ELISA (an ultrasensitive human IL-4 or IL-13 ELISA kit, Biosource International, Camarillo, Calif., USA). The measurable range of IL-4 or IL-13 was 65–25,000 fg/ml or 12–2,500 pg/ml, respectively.

Measurement of Leukotriene C4 Synthesis by Basophils

Purified basophils (0.4–1 × 10^6 cells/ml) were incubated without (equivalent amount of diluted methanol) or with flavonoid at an indicated dose for 15 min and then stimulated with anti-IgE Ab (1 μg/ml) or anti-IgE Ab plus IL-3 for 1 h. The concentration of leukotriene C4 was then measured by means of ELISA (Leukotriene C4 EIA Kit, Cayman Chemical, Ann Arbor, Mich., USA). The measurable range of leukotriene C4 was 10–1,000 pg/ml.

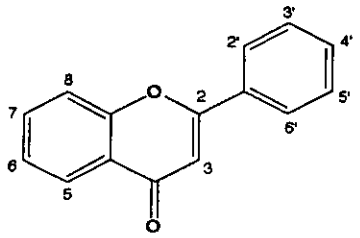
Measurement of IL-4 Synthesis by Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells from healthy volunteers were separated by Ficoll-Hypaque density centrifugation. Flat-bottom 96-well microtiter Immunlon 2 plates (Dynatech Laboratories, Alexandria, Va., USA) were coated by incubation with anti-CD3 Ab (Pharmingen, 1 μg/ml in 50 μl borate-buffered saline, pH 8.5) overnight at 4°C. Wells were washed three times with HBSS. Peripheral mononuclear cells (10^6 cells/ml) were then added to the wells in the absence or presence of the indicated doses of flavonoids and cultured for 48 h. The IL-4 concentration was measured using an ultrasensitive human IL-4 ELISA kit.

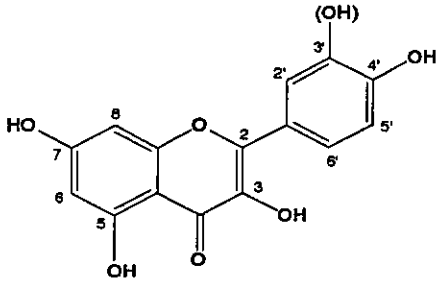
Results

Hierarchy of Flavonoids in the Inhibitory Activity of IL-4 and IL-13 Production by Basophils

It has previously been demonstrated that fisetin suppressed IL-4 synthesis by activated basophils and that its IC_{50} was 5.1 μ*M* [2]. This time, we investigated whether other flavonoids might possess such inhibitory activity. The structure of 18 selected flavonoids including flavones and flavonoids is shown in figure 1. Highly purified basophils were incubated with or without various concentrations of flavonoids for 15 min and then stimulated with either anti-IgE Ab alone or anti-IgE Ab plus IL-3 for 12 h. We performed at least three independent experiments to examine the effect of each flavonoid, and the results of three flavonoids (fisetin, luteolin and apigenin) are shown in figure 2. Depending on the donors, the stimulation of basophils with anti-IgE Ab caused various amounts of IL-4 production, and in the supernatant of some donors the IL-4 concentration was hardly detectable. However, when basophils were incubated with both anti-IgE Ab and IL-3, IL-4 synthesis was consistently increased in the culture to a detectable level, as demonstrated previously [4]. Under these conditions, luteolin and apigenin showed a marked inhibition of IL-4 production and their IC_{50} was around 2–6 μ*M* irrespective of the stimuli (anti-IgE Ab alone or anti-IgE Ab with IL-3; fig. 1). Scutellarein, 3-hydroxyflavone, kaempferol, quercetin, eriodictyol, fustin and 7-hydroxyflavone inhibited IL-4 synthesis weakly but significantly (IC_{50} = 14–26.5 μ*M*). The other flavonoids, however, showed less activity. A comparison of the structure of these various flavonoids with their inhibitory activity determined the fundamental structure for the inhibition (fig. 1, lower panel). For maximal inhibition, hydroxylation in position 7 and 4′ as well as the presence of OH in either position 3 or 5 are required. Similarly, luteolin, apigenin and fisetin suppressed IL-13 production by basophils (fig. 2) in a dose-dependent fashion.



	3	5	6	7	8	2'	3'	4'	5'	IL-4 inhibition basophils IC50(μM)	IL-4 inhibition T cells IC50(μM)
Luteolin	H	OH	H	OH	H	H	OH	OH	H	2.4	10.8
Fisetin	OH	H	H	OH	H	H	OH	OH	H	5.1	19.1
Apigenin	H	OH	H	OH	H	H	H	OH	H	5.7	10.2
Scutellarein	H	OH	OH	OH	H	H	H	OH	H	14.0	13.5
3-Hydroxyflavone	OH	H	H	H	H	H	H	H	H	15.0	24.8
Kaempferol	OH	OH	H	OH	H	H	H	OH	H	15.7	>30
Quercetin	OH	OH	H	OH	H	H	OH	OH	H	18.8	>30
Eriodictyol(2-3)	H	OH	H	OH	H	H	H	OH	OH	20.8	>30
Fustin(2-3)	OH	H	H	OH	H	H	OH	OH	H	23.0	>30
7-Hydroxyflavone	H	H	H	OH	H	H	H	H	H	26.5	>30
Myricetin	OH	OH	H	OH	H	H	OH	OH	OH	>30	>30
Galangin	OH	OH	H	OH	H	H	H	H	H	>30	n.d.
Morin(dihydrate)	OH	OH	H	OH	H	OH	H	OH	H	>30	n.d.
Astragalin O-Glc	OH	H	OH	H	H	H	H	OH	H	>30	n.d.
Rutin O-Rutinose	OH	H	OH	H	H	OH	OH	H		>30	n.d.
Gossypin	OH	OH	H	H	O-Glc	H	OH	OH	H	>30	n.d.
Isoquercitrin O-Glc	OH	H	OH	H	H	OH	OH	H		>30	n.d.
Myricitrin O-Rhamnoside	OH	H	OH	H	H	OH	OH	OH		>30	n.d.



(Each OH for 3 or 5 position)

Fig. 1. Structure of 18 flavonoids and their inhibitory activity on IL-4 synthesis by human peripheral stimulated basophils and T cells. The lower figure shows the basic structure of flavonoids for inhibitory activity on IL-4 expression in basophils.

Luteolin, Fisetin and Apigenin Fail to Inhibit Leukotriene C4 Synthesis by Basophils

The important role of basophils in allergic inflammation depends on their production of chemical mediators such as histamine, leukotrienes and cytokines [5–7]. Previously, several reports have demonstrated that flavonoids (including fisetin, luteolin and apigenin) inhibited histamine release by human basophils or rat mast cells at 5–30 μ*M* [8–11]. Then we checked whether these flavonoids might inhibit another important mediator, leukotriene C4 synthesis. However, as shown in figure 2, these three flavonoids did not suppress leukotriene C4 synthesis even at a high dose (30 μ*M*).

Inhibitory Activity of Flavonoids on IL-4 Synthesis by Anti-CD3-Ab-Stimulated Mononuclear Cells

Finally, we checked whether the inhibitory activity of flavonoids on IL-4 synthesis might be specific for basophils. Since T cells are IL-4-producing cells [1], peripheral blood mononuclear cells were cultured in plate-bound anti-CD3 Ab with or without various concentrations of flavonoids for 2 days, and IL-4 concentration was measured. The inhibitory activity from four independent experiments was shown as the IC_{50} value in figure 1. Luteolin, fisetin, apigenin, scutellarein and 3-hydroxyflavone inhibited IL-4 production by stimulated T cells, but the required flavonoid dose for IL-4 suppression in T cells was higher than that in basophils (IC_{50} = 10.8–24.8 μ*M*).

Discussion

On the basis of our previous study, which has shown that fisetin possesses an inhibitory activity on IL-4 and IL-13 synthesis by normal activated basophils [2], this time we screened whether 18 kinds of selected flavonoids inhibited IL-4 production by basophils. Among these flavonoids, luteolin, apigenin and fisetin are the strongest inhibitors of IL-4 and IL-13 synthesis. Scutellarein, 3-hydroxyflavone, kaempferol, quercetin, eriodictyol, fustin and 7-hydroxyflavone also inhibited IL-4 production but more weakly than luteolin, apigenin and fisetin. The comparison of the flavonoid structure with the inhibitory activity determined the fundamental structure shown in figure 1. For maximal inhibition, hydroxylation in positions 7 and 4′ is essential and, additionally, the presence of OH in either position 3 or 5 is required. The major functions of basophils (and mast cells) in the development of allergic inflammation are reported to reside mainly in their capability to produce chemical mediators and cytokines [5–7, 12]. Fewtrell and Gomperts [8] first demonstrated the effect of flavone inhibition on histamine secretion by rat mast cells. Subsequently, flavonoids, including fisetin, apigenin, luteolin and quercetin, were reported to suppress histamine release by human basophils [9–11]. Therefore, three major flavonoids possess an inhibitory effect on Th2-type cytokines and histamine release but do not have an inhibitory activity on leukotriene C4 synthesis. Previously, it has been demonstrated that some flavonoids suppress cysteinyl leukotriene synthesis through an inhibition of phospholipase A_2 and 5-lipoxygenase [13,

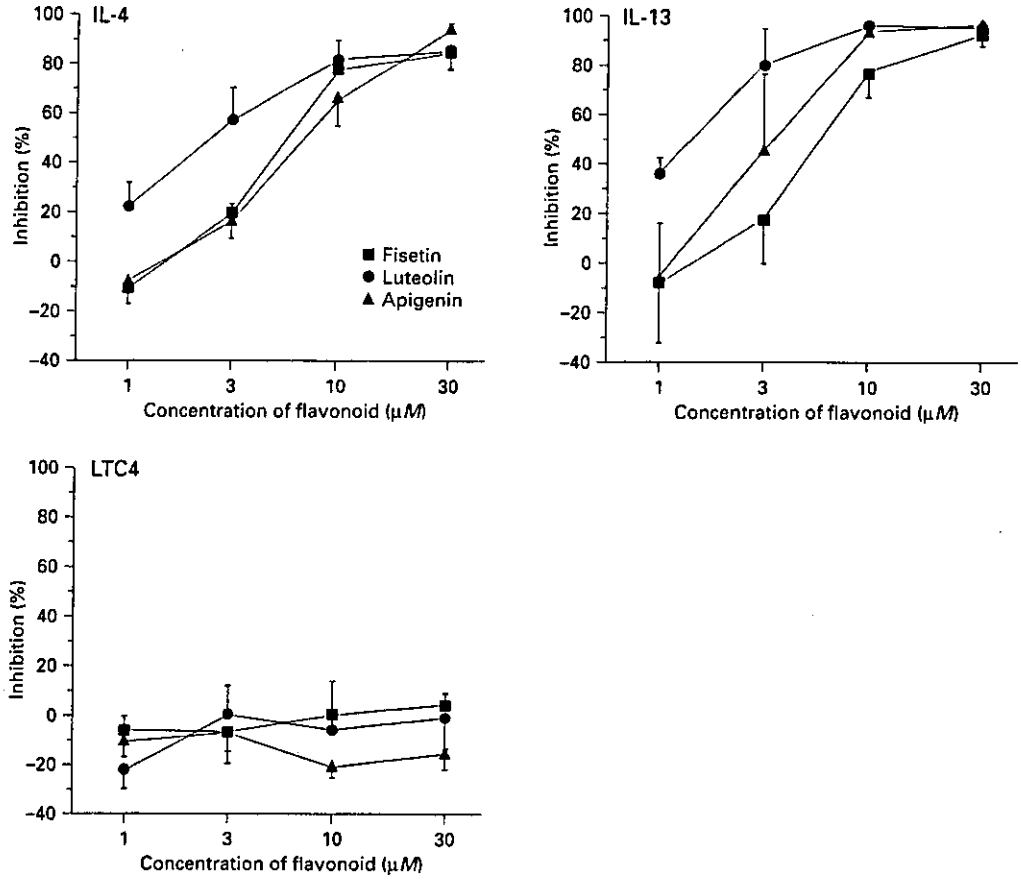


Fig. 2. Fisetin, luteolin and apigenin inhibit IL-4 and IL-13 synthesis by basophils in a dose-dependent fashion, but fail to inhibit leukotriene C4 synthesis. The data are shown as the mean inhibition from four different donors.

14]. Quercetin, as well as quercetagetin, kaempherol-3-*O*-galactoside and scutellarein were reported to be inhibitors of phospholipase A_2 [15, 16], whereas cirsiliol is shown to inhibit 5-lipoxygenase [17]. However, in human basophils, luteolin, fisetin and apigenin did not inhibit anti-IgE-Ab-induced leukotriene C4 synthesis.

The precise mechanisms through which they inhibit cytokine synthesis and histamine release in response to cross-linking of high affinity IgE receptor (FcεRI) remain to be elucidated. Previously, we demonstrated that fisetin inhibited nuclear localization of nuclear factor of activated T cells by a A23187-stimuated basophilic cell line, KU812 [2], but studies determining whether these low-molecular-weight compounds might affect other signaling cascades initiated by high-affinity IgE receptors are now in progress. It should be pointed out here that cyclosporin A or FK506, a calcineurin inhibitor, has been shown to suppress cytokine production and histamine release [18–21], but their effect on leukotriene C4 production is controversial [22]. Although T cells appear to be more resistant to these flavonoids in the synthesis of IL-4 than basophils, T cells were generally shown to be more sensitive to cyclosporin A than basophils. Thus, it is possible that in addition to inhibition of nuclear factor of activated T-cell activation, other mechanisms may contribute to flavonoid inhibition of IL-4 synthesis by basophils.

Flavonoids are naturally and ubiquitously included in plant foods and drinks and are therefore common components of our diet [23]. Whether the worldwide increase in the prevalence of allergic diseases is directly associated

with diet is unknown, but a recently reported population-based case-control study found that consumption of apples or red wine was negatively associated with asthma prevalence or severity, respectively, possibly due to the protective effect of flavonoids [24]. In addition, a cohort study in Finland demonstrated a lower asthma incidence for higher total flavonoid intakes (relative risk = 0.65, p = 0.04), mainly in the form of apples and oranges [25]. Thus these epidemiological studies raise the possibility that an intake of substantial amounts of flavonoids may ameliorate allergic symptoms as complementary and alternative medicine and prevent the onset of asthma. In this regard, we have also reported that in an atopic dermatitis mouse model, NC/Nga, administration of astragalin, a glycosylated kaempferol, showed both preventive and ameliorative effects on dermatitis [26, 27]. Based on the anti-allergic activity of flavonoids, an appropriate intake of such flavonoids as luteolin, fisetin and apigenin is strongly recommended.

In the past, the average intake of five selected flavonols and flavones, e.g. quercetin, kaempferol, myricetin, apigenin and luteolin, was 23 mg/day, of which quercetin contributed 16 mg/day in The Netherlands [28]. In 17 international subjects, intakes of these five flavonols and flavones were variable from 5.1 to 77 mg/day [29]. Quercetin is found in abundance in onion, kale, broccoli, apples and oranges, while kale, endive and other leafy vegetables have been shown to contain substantial amounts of kaempferol [30]. It should also be noted that celery contains exceptionally large quantities of apigenin (17–750 mg/kg) and luteolin (22–200 mg/kg) [31], both of which strongly inhibit Th2 cytokine expression by basophils. Luteolin is also included in bird chili (1,035 mg/kg), onion leaves (391 mg/kg), belimbi fruit (202 mg/kg) and belimbi leaves (464 mg/kg) in Malaysian edible plants [32]. Apigenin is detected in substantial amounts (272–579 mg/kg) in bell pepper, belimbi fruit and guava [32]. Recently, it has been reported that fisetin is found in strawberries (160 mg/kg) and apples (27 mg/kg) [33].

So far, diet therapy for allergic diseases has not been established except for avoidance of food allergens in cases where patients were sensitized to foods. Lactobacillus GG is promising since it was shown to be effective in the prevention of early atopic eczema in children at high risk, when it was administered prenatally to mothers and postnatally to their infants [34]. Similarly it is expected that, depending on the quantity as well as the quality, the intake of flavonoids may alleviate allergic symptoms and even prevent allergic diseases [35].

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Calorie Restricted Diet and Urinary Pentosidine in Patients with Rheumatoid Arthritis

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Abstract Low-energy diets and fasting have suppressive effects on rheumatoid arthritis. It was reported recently that urine levels of pentosidine (i.e., an advanced glycation end product formed by glycosylation) is associated with the activity of rheumatoid arthritis. We conducted a regimen of caloric restriction combined with fasting in patients with rheumatoid arthritis, and then evaluated urinary pentosidine levels. Ten patients with rheumatoid arthritis underwent a 54-day caloric restriction program. Urinary pentosidine levels were measured and the Lansbury Index were determined by examining the clinical features, blood biochemistry and the inflammation activity of rheumatoid arthritis on days 0, 25 and 54. On day 0, the mean urinary pentosidine level of patients with rheumatoid arthritis was significantly higher than that of the control subjects. On day 54, the mean body weight had reduced due to caloric restriction. The mean values of the erythrocyte sedimentation rate and the Lansbury Index of patients both significantly decreased during the study. In addition, although the urinary pentosidine levels showed no significant difference between day 0 and 25, it was significantly decreased at the end of the study (day 54). The study showed that under a low energy diet a reduction of disease activity in rheumatoid arthritis was accompanied with a reduction of the urinary pentosidine. *J Physiol Anthropol Appl Human Sci 23 (1): 19–24, 2004* http://www.jstage.jst.go.jp/en/

Keywords: caloric restriction, fasting, rheumatoid arthritis, advanced glycation end-product (AGE), pentosidine

Introduction

It is well acknowledged that nutritional stress, such as dietary restriction or fasting, activates various responses. The dietary restriction extends life span and retards the development of chronic diseases (Weindruch and Walford, 1988); it also has beneficial effects on various inflammatory diseases (Fernandes G, 1978; Hishinuma, 1990; Kubo, 1987; Ogura, 1989). Fasting also has suppressive effects on inflammation (Nakamura, 2001); and the immediate consequences of fasting include marked increases in plasma cortisol, ACTH, beta-endorphin, beta-lipotrophic hormone, adrenaline, noradrenaline and dopamine (Becker, 1992; Beer, 1989; Brady, 1990; Komori, 1996).

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease, resulting in the destruction of multiple joints (Firestein GS, 1992; Harris, 1990). Dietary regimens such as fasting, caloric restriction, or a vegetarian diet have the beneficial effects of improving the symptoms of patients with RA (Beri D, 1988; Forre, 1991; Hafström, 1998; Kjeldsen-Kragh J, 1991; Sköldstam et al., 1979; Stroud, 1983; Udén et al., 1983).

It has been reported recently that the serum, urine and synovial fluid levels of pentosidine, correlated with the activities of RA (Chen, 1999; Chen, 1998; Takahashi, 1997). Urinary pentosidine, an advanced glycation end product (AGEs), is a glycoxidation damage biomarker (Chen, 1999; Takahashi, 1997; Takahashi, 1996). The nonenzymatic glycation and oxidation (glycoxidation) reaction of protein is thought to contribute to the cross-linking of tissue proteins, gumming up tissues, making them stiffer and less elastic, and so causing connective tissues to become leathery.

In the present study, we conducted a regimen of caloric

restriction combined with fasting in patients with RA, and evaluated the disease activity by measuring urinary pentosidine levels.

Methods

Subjects

Ten Japanese female patients with RA (age; 58.2±4.8 year; range 48–77) were enrolled in this study. All of them were outpatients of Kouda Clinic located in Osaka. All participants gave informed consent, and the study procedures were in accordance with the Declaration of Helsinki. The patients were diagnosed on the American Rheumatism Association 1987 revised criteria (Arnett, 1988). The mean duration of RA was 6.6 years (range, 1–20). None of the patients had complications of cancers, diabetes mellitus, renal diseases, inflammatory disease or other autoimmune diseases, but one had hypertension.

Six patients took a non-steroidal anti-inflammatory drug (NSAID) and pre-donisolone daily. Their drug therapy had not changed for at least 3 months preceding the study. In five patients, doses of these drugs were diminished according to the degree of improvement in symptoms during the study. None needed an increase in their drugs during the clinical trial.

The control group consisted of 15 healthy females aged 43–80 year (60.2±6.0). They were selected from the group of medical examination in our institution. There was no significant difference in age between RA patients and control subjects. All control subjects had no previous history of diabetes mellitus and renal disease, and were currently receiving no medication.

Trial design and Diet arrangements

Ten RA patients underwent a caloric restricted diet from days 0 to 10, 14 to 24, 31 to 43, and 49 to 54. From day 11 to 13, 25 to 30, and 44 to 48, fasting was conducted (Fig. 1). On a calorie restriction day, the patients were given fresh vegetable juice (corresponding to 250 g of fresh vegetables) at breakfast to avoid any shortage of micronutrients that might accompany with dietary restriction. For lunch and dinner, they were given brown rice porridge (corresponding to 80 g of brown rice) sprinkled with 5 g of kelp powder, bean curd (tofu: wet weight 200 g) and 10 g of sesame paste. The daily requirement of 2.5 g of non-refined salt was also added to the diet. The energy intake (1,085 kcal) was 55% constituted by nutritional requirements, while was protein 75%, calcium 180%, iron 130%, vitamin A 150%, vitamin C 250%, and vitamin E 110% of daily requirements. On a fasting day, only vegetable soup (720 kcal/day) was served.

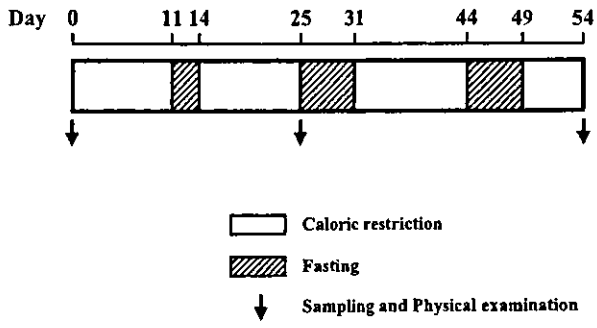

Fig. 1 The study of the caloric restriction program.

Collection of serum and urine samples

To measure blood urea nitrogen (BUN), Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT), albumin, haemoglobin, erythrocyte sedimentation rate (ESR), and pentosidine, blood and urine samples were collected on the same day from all subjects between 7:00 and 9:00 a.m. on days 0, 25 and 54. For an age-matched control group, 15 healthy volunteers (15 females), aged 35 to 68 years (56.2±2.0) with no history of RA or the known disease also gave urine.

Disease activity in RA

The Lansbury Index (LI) was noted by a rheumatologist on days 0, 25 and 54. LI was determined based on the duration of morning stiffness, erythrocyte sedimentation rate (ESR) (value at 1 hour), grip strength (mmHg) and joint score (Lansbury, 1966; Lansbury, 1957). The joint score was determined as the total sum for painful, tender or swollen joints.

Measurement of urinary pentosidine

The measurement of urinary pentosidine was performed as described (Takahashi, 1993). We identified the pentosidine in a hydrolysate of urine as the total form of pentosidine. After thawing, urine samples were centrifuged at 3000×*g* for 10 minutes. A 0.5-ml aliquot was hydrolyzed with an equal volume of 12 mol/l hydrochloric acid at 110°C for 17 h in a sealed glass tube. Hydrolysate of urine 0.25-ml was mixed with 15 ml of water and applied to an SP-Sephadex C-25 column (H^+ form, 0.8×2.0 cm; Pharmacia LKB Biotechnology AB, Uppsala, Sweden) that had been equilibrated with water. The column was washed with 20 ml of 0.15 mol/l hydrochloric acid. The elute evaporated under vacuum, and the residue dissolved in 200 μl of 1% heptafluorobutyric acid. The solutions were stored at −30°C for an analysis.

High-performance liquid chromatography (HPLC)

The HPLC system consisted of a Model LC-6A pump (Shimazu, Kyoto, Japan), a Model 474 spectrofluorometer (Waters Associates, Inc., Milford, MA), a Model AS-8020 autosampler (TOSOH, Tokyo, Japan), and a Model Chromatocorder 12 data processor (SIC, Tokyo, Japan). A column (8 mm×10 cm) prepacked with Radial-Pak C18, of 10-μm particle size, type 8C 1810 μ (Water Associates Inc., Milford, Mass, USA) was used. The flow rate was 1.0 ml/min. The volume of each sample injected was 160 μl. For the detection of pentosidine, the fluorescence at 385 nm was measured on excitation at 335 nm. The level of the pentosidine

content in urine samples is expressed as the micromoles of the pentosidine per 1 mol of urinary creatinine. The urinary creatinine content was measured by a routine method. The standard pentosidine was donated by Dr.V. M. Monnier, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106, U.S.A.

Statistical analysis

Values are expressed mean±standard error. The unpaired *t* test was used to compare the mean values of urinary pentosidine between the control subjects and RA patients. The paired t test was used to compare the mean values of pentosidine, LI and other variables of RA patients.

Results

None of the participants dropped out of the study. Table 1 shows pathological and laboratory findings. A mean body weight of 2.4 kg of was lost by day 25 of the study, and at the end of the study 4.9 kg (day 54). But, the mean hemoglobin and serum albumin concentrations showed no significant changes during the study. The ESR decreased in 9 patients and the mean ESR showed a significant decrease by day 25 and at day 54 from baseline. There were no statistical differences in serum concentrations of BUN, or liver enzymes such as AST and ALT.

Before caloric restriction (day 0), the mean value of LI was 46.2±7.9%. It decreased significantly during the study: 39.7±6.4% ($P<0.05$ vs. day 0) on day 25, and 37.6±7.5% ($P<0.05$ vs. day 0) on day 54 (Fig.2).

The mean value of urinary pentosidine in RA patients was significantly higher than that in control subjects: 4.20±

Table 1

	Before Caloric restriction		After Caloric restriction
Day	0	25	54
Body weight (kg)	51.2±3.0	48.8±2.6‡	46.3±2.3‡
S-Albumin (g/dl)	4.3±0.1	4.4±0.1	4.5±0.1
S-Hemoglobin (g/dl)	12.5±0.4	12.7±0.4	13.1±0.2
S-AST (IU/dl)	17.8±1.7	17.9±1.4	18.3±0.8
S-ALT (IU/dl)	10.0±1.7	8.8±1.4	10.3±1.6
S-BUN (mg/dl)	8.5±0.8	8.6±1.1	8.8±1.0
ESR (mm/h)	48±11.8	33.3±10.4†	23.3±8.0

Values are expressed mean±SEM (n=10), S: Serum, AST: Aspartate Aminotransferase, ALT: Alanine Aminotransferase, BUN: Blood urea nitrogen, ESR: Erythrocyte sedimentation rate, †$p<0.05$, ‡$p<0.01$. The statistical significance of differences was evaluated using paired t-test. The statistical analysis refers to the comparison with the values from day 0.

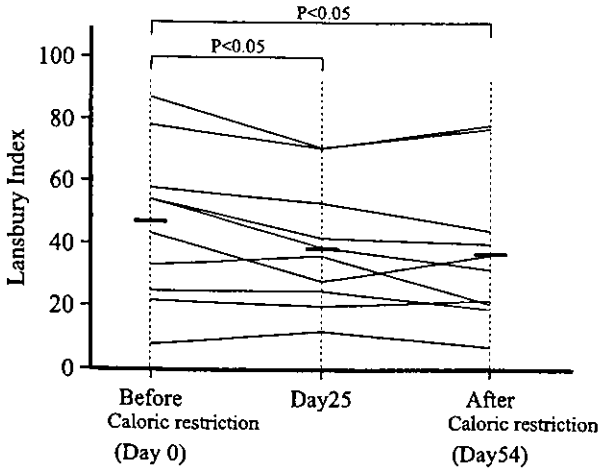

Fig. 2 Effects of the caloric restriction on the Lansbury index (LI) of 10 RA patients on days 0, 25 and 54. Horizontal bars indicate mean values. Significant difference was determined by paired t test.

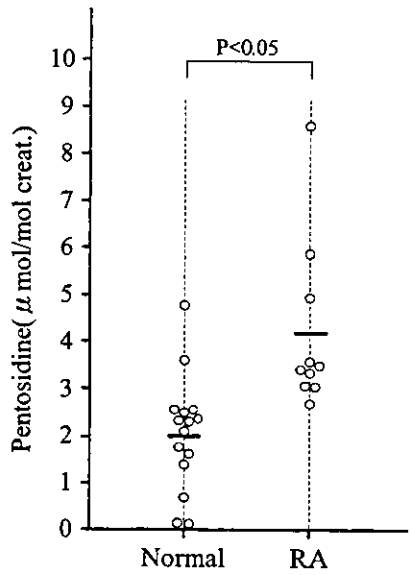

Fig. 3 Comparison between the levels of urinary pentosidine in 10 patients with rheumatoid arthritis (RA) and 15 control subjects on day 0. Horizontal bars indicate the mean values. Significant difference was determined by the unpaired t test.

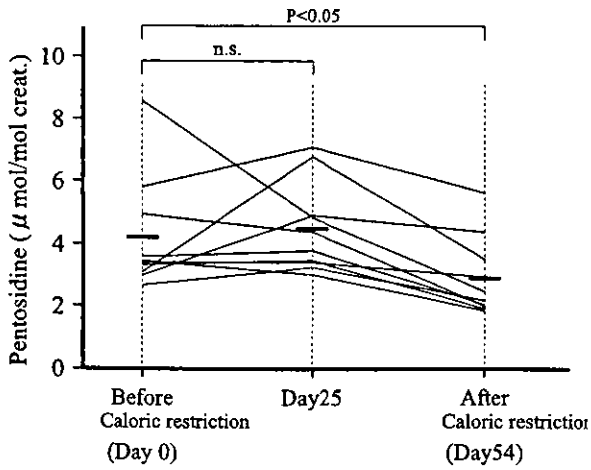

Fig. 4 Effects of caloric restriction on urinary pentosidine levels of 10 RA patients on days 0, 25 and 54. Horizontal bars indicate the mean values. Significant difference was determined by the paired t test.

0.57 μmol/mol creatinine vs. 2.04±0.31 μmol/mol creatinine ($p<0.01$) (Fig. 3). In RA patients, there was no significant difference in the urinary pentosidine levels between days 0 and 25 (4.20±0.57 vs. 4.50±0.46 μmol/mol creatinine, $p=0.60$). However, the urinary pentosidine levels significantly decreased at the end of the study (day 54) compared with the baseline (2.87 ±0.36 vs. 4.20±0.52 μmol/mol creatinine, $P<0.05$) (Fig. 4).

Discussion

The pentosidine is one of the advanced glycation endproducts (Monnier, 1992; Sell, 1989; Uchiyama, 1991), and formation of pentosidine was accelerated in the increased sugars concentrations. Accelerated formations of pentosidine in diabetes mellitus (Takahashi, 1993) and in atherosclerotic cardiovascular disease (Uchiyama, 1991) have been reported. Recently, Takahashi et al. (1997) reported that the serum and urine levels of the pentosidine are correlated with the RA activity and then proposed that the serum and urine pentosidine might be a significant novel marker for evaluating RA disease status. In addition, dietary regimens such as fasting, caloric restriction, or vegetarian diet have the beneficial effects by improving symptoms of patients with RA (Beri D, 1988; Hafström, 1998; Kjeldsen-Kragh J, 1991). In the present study, the urine level of pentosidine in patients was significantly higher than that of control females. In addition, we demonstrated a reduction of the pentosidine in patients with RA by calorie restriction, with a simultaneous reduction of the Lansbury Index representing RA disease activity. Furthermore, a reduction of the pentosidine was accompanied by a significant reduction in body weight. This is consistent with previous reports (Kouda et al., 2000; Tanaka et al., 2001). However, the serum albumin, hemoglobin, BUN, AST and ALT were not significantly changed during calorie restriction, although ESR was significantly reduced. These results indicated that a low-energy diet does not malnourish participants, but has a suppressive effect on inflammatory diseases.

The actual mechanism for the reduction of the pentosidine by calorie restriction is unclear. In our study, we combined 3–6 days fasting after the 9–13 days caloric restriction. Previously, it has been hypothesized that the pentosidine formation is accelerated in pathological conditions accompanied with oxidative stress (Kouda et al., 2001; Oya et al., 1997; Suzuki et al., 1999). In animal and clinical studies, the calorie restriction decreases in oxidative damage to tissues (Yu, 1996). The previous reports have demonstrated that the calorie restriction reduces inflammations of dermatitis and the oxidative DNA damage derived from inflammation (Fan et al., 2001: Kouda et al., 2000; Tsuboi et al., 1998). In addition, the caloric restriction results in a decrease in the age-dependent accumulation of glycoxidation products, such as the pentosidine, in tissue (Cefalu et al., 1995; Iqbal et al., 1999; Reiser et al., 1994; Sell et al., 1997; Sell et al., 1996). Furthermore, the fasting reduces food intolerance (Panush, 1986), diminishes the gastrointestinal permeability (Sundqvist, 1982) and decreases the intake of inflammatory mediators, prostaglandins and leukotrienes (Darlington, 1986). Further, the foodstuffs used in this study were rich in antioxidant. It has been reported that a diet antioxidant and fruites may have an antioxidant effects (Fan WY et al., 2000; Pool-Zobel et al., 1997; Thompson et al., 1999; Verhagen et al., 1997). A complex combination of these mechanisms might relate to the present results.

In conclusion, under a low energy diet the reduction of a RA disease activity was accompanied with a reduction of the urinary pentosidine.

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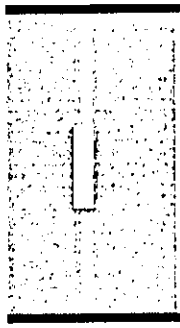

Is an appropriate intake of flavonoids a prophylactic means or complementary and alternative medicine for allergic diseases?

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Abstract

The prevalence of allergic diseases such as asthma, atopic dermatitis and allergic rhinitis is increasing all over the world and now one-third of the population in the developed countries is estimated to be suffering from allergic diseases. Although both genetic factors and environmental factors have contributed to this increase, environmental factors are believed to be more significantly responsible for the onset and development of allergic diseases. Diet appears to be one of environmental factors that have caused such an increase. Indeed, it has been reported that an appropriate intake of probiotics, Lactobacillus

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GG or fish oil reduced the onset of infant atopic dermatitis or their severity, respectively. Starting from a clinical study that evaluated whether a certain traditional diet regime might be effective for adult patients with atopic dermatitis, we found that some kinds of flavonoids exerted a novel anti-allergic activity. Luteolin, fisetin and apigenin have an inhibitory effect on IL-4 and IL 13 synthesis as well as on histamine release by human activated basophils and mast cells. Quercetin and kaempferol, flavonoids taken daily substantially, also have a similar but weaker effect. The mechanism through which these flavonoids exert their suppression of human basophils is thought to be partially related to their inhibitory effect on the activation of the nuclear factor of activated T cells. Moreover, oral administration of a flavonoid into an atopic dermatitis-model mouse NC/Nga prior to the clinical onset had a striking preventive effect on the onset of dermatitis, scratching behavior and serum IgE elevation and showed an ameliorative effect on the dermatitis even after the onset. A preliminary clinical study through a randomized controlled trial demonstrated the effectiveness of flavonoids for adult patients with atopic dermatitis. In addition, a recent epidemiological study has reported that a higher intake of flavonoids correlates with a lower incidence of asthma.

These findings thus raise the possibility that an appropriate intake of flavonoids, depending on the quality and quantity, may have a prophylactic effect or serve as complementary and alternative medicine for allergic diseases.

Introduction

The worldwide prevalence of allergic diseases has increased dramatically during the last two or three decades (1-3). It is assumed that one-third of the population of the developed countries is now suffering from allergic diseases such as asthma, atopic dermatitis, allergic rhinitis or conjunctivitis. The interaction between genetic and environmental factors is believed to be responsible for sensitization to allergens and the onset of allergic diseases (4-8). However, the recent increase in the prevalence of these diseases strongly suggests that the environmental factors have contributed more to this increase than the genetic factors. In order to slow down the acceleration of the increase in allergic diseases, it is essential to identify both genetic and environmental factors and how they interact with each other to sensitize people to allergens and to cause the onset of allergic diseases (9, 10). To this purpose, polymorphisms of genes have been studied for about ten years and more than twenty candidate polymorphisms have been reported (11, 12). It can be expected that analysis of the polymorphisms of these genes will lead to the development of specific treatments and a means for selecting individuals who are likely to develop allergic diseases. Among environmental factors that influence susceptibility to the development of asthma in predisposed individuals, allergens, occupational

sensitizers, air pollution, respiratory infections, parasitic infections, socioeconomic status, family size and obesity have been identified (4).

Diet is also considered to be an environmental factor (4, 13), since it includes not only allergy-promoting substances such as salicylates, food preservatives, monosodium glutamate and omega-6 polyunsaturated fatty acids but also anti-allergic and anti-inflammatory constituents such as omega-3 polyunsaturated fatty acids, vitamin C and a probiotics, Lactobacillus GG. Moreover, we have recently found that flavonoids contained in vegetables, fruits and teas have an inhibitory effect on the production of Th2 type cytokines such as IL-4 and IL-13 and of histamine by stimulated basophils and mast cells (14). At present, however, diet management has not been established for patients with allergic disorders except for avoidance of food allergens (15, 16). This review summarizes a recent progress in this field and discusses whether an appropriate intake of flavonoids may have a prophylactic effect and serve as complementary and alternative medicine for allergic diseases.

Increase in prevalence of allergic diseases

Allergic diseases such as asthma, atopic dermatitis, allergic rhinitis and allergic conjunctivitis are among the most common chronic diseases and are characterized by the presence of allergic inflammation, hyper-production of IgE and hyper-reactivity of organs to various stimuli (17-19). Accumulating evidence from both molecular biological studies of allergic inflammation and from clinical studies has led to the establishment of guidelines for the management of allergic diseases (4, 20-24). In addition to steroids, which are powerful inhibitors of allergic inflammation, a number of other anti-allergic drugs have been developed (25), and it has become clear that the appropriate use of these drugs, depending on the severity of allergic diseases, has significantly improved patients' symptoms and quality of daily life.

However, the prevalence of allergic diseases has increased worldwide (1-4). For instance, the current incidence of asthma in Australia and New Zealand is estimated at more than 10% (26) and that of atopic dermatitis in children in North America at 10-20% (27). The prevalence of these diseases in industrialized countries has doubled or even tripled during the past two or three decades. In Japan, the first cases of allergic rhinitis due to Japanese cedar pollen were reported in 1964, but at present it is reported that more than 40% of the population may be sensitized to this pollen and the prevalence of cedar pollen induced allergic rhinitis is estimated at 16% (28). This striking increase has led not only to a significant increase in patient morbidity but also to a rise in costs for the patients and their families and a major burden for society (29-33). The interaction between genetic and environmental factors is generally accepted as a key factor in the development of allergic diseases (4-8). Since it seems unlikely that genes would change over two or three generations, it is believed that recent

changes in the environment have contributed more to the increase in prevalence. It is therefore essential to determine what environmental factor(s) cause such a high prevalence through their interaction with genetic determinants and to find strategies to counteract their development (9, 10).

Among environmental factors that influence the susceptibility to the development of asthma, the Global Strategy for Asthma Management and Prevention lists indoor and outdoor allergens, occupational sensitizers, air pollution, respiratory infection, parasitic infections, socioeconomic status, family size, drugs and obesity (4). Diet is also mentioned as one of these factors, since there are allergy-promoting or anti-allergic substances in foods and there has been a significant change in diet over the last few decades. Some ingested substances, including salicylates, food preservatives, monosodium glutamate, omega-6 polyunsaturated fatty acids and others have been reported to cause allergic symptoms in some patients. On the other hand, it remains controversial whether omega-3 polyunsaturated fatty acids that are plentiful in fish oil are associated with a low prevalence of asthma (34-36). A Cochrane review concluded that there is little evidence to recommend that people with asthma supplement or modify their dietary intake of marine omega-3 fatty acids in order to improve their asthma control (37). The consumption of fruit rich in vitamin C may reduce wheezing symptoms in childhood (38), while other nutrients, including vitamins B6 and B12, magnesium and zinc, are also reportedly associated with a reduction in asthma symptoms. However, no definitive conclusions have been reached with regard to supplementation with vitamins C, B6 and B12 and with zinc, or intravenous injection of magnesium sulfate for asthmatic patients (39). Caffeine is chemically related to theophylline and has a bronchodilator effect. A Cochrane review reported that a modest clinical improvement in lung function parameters was found in response to caffeine compared to a placebo for up to four hours after ingestion, but whether this effect is clinically relevant could not be assessed with the data (40). At present, therefore, there is limited evidence that some dietary components constitute complementary and alternative medicine for allergic patients (4, 41, 42). The only recommendation that can be made with confidence is for allergic patients to avoid certain foods to which they are sensitized (15, 16). However, it has been demonstrated that some dietary components are effective for prevention. When the probiotics Lactobacillus GG was administered prenatally to mothers and postnatally to their infants, it was effective for the prevention of early atopic eczema in children at high risk (43, 44). Fish oil supplementation during pregnancy compared with a placebo also resulted in less severe symptoms of atopic dermatitis, although there was no difference in the frequency of atopic dermatitis at 1 year of age (45).

Flavonoids as potential anti-allergic substances

We previously reported the clinical effect of one traditional dietary component on adult patients with atopic dermatitis (46). After a two-month period of treatment, the severity score of dermatitis decreased from 49.9+18.6 to 27.4+16.8. This diet regime consisted of a low energy intake (1085 kcal) of traditional Japanese foods, fresh vegetable juice and persimmon leaf tea (1-2 l/day). We also examined whether vegetables and persimmon leaf possessed anti-allergic substances. HPLC analysis of the constituents of persimmon leaf extract showed that it included a large amount of flavonoids (0.45-0.72% astragalin, 0.32% toripholin and 0.38-0.55% isoquercitrin) (47).

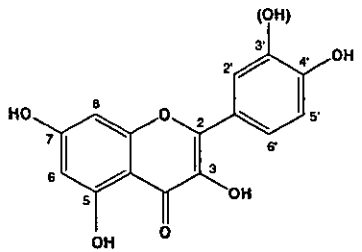
Flavonoids are comprised of a large group of low molecular weight polyphenolic secondary plant metabolites and are found in fruits, vegetables, nuts, seeds, stems, flowers, roots, bark, tea, wine and coffee and are thus common substances in our daily diet (48, 49). Flavonoids have been shown to exert antioxidant, anti-bacterial and anti-viral activities, to have anti-inflammatory, anti-angionic, analgestic, hepatoprotective, cytostatic, apoptotic, estrogenic or anti-estrogenic properties as well as anti-allergic effects (50). Based on their skeletal structure, flavonoids are categorized into eight groups: flavans, flavanones, isoflavanones, flavones (shown in Fig.1), isoflavones, anthocyanidines, chalcones and flavonolignans and more than 8,000 different flavonoids have been identified so far (50).

I. Anti-allergic activity of flavonoids

The anti-allergic effect of flavones as inhibitors of ATPase transport in histamine secretion from rat mast cells was firstly demonstrated by Fewtrell and Gomperts (51). The effect of six flavonoids (fisetin, kaempferol, morin, quercetin, myricetin and rutin) on antigen-induced histamine secretion was measured. Fisetin, quercetin, myricetin and kaempferol inhibited histamine release while morin and rutin showed little effect. Subsequently, quercetin was reported to inhibit histamine release by allergen-stimulated human basophils (52, 53), while Cheong et al. identified the relationship between the structure of flavonoids and their anti-allergic actions through analyses of their inhibitory activity on hexosaminidase release from rat mast cells (54). Apigenin, luteolin, 3.6-dihydroxy flavones, fisetin, kaempferol, quercetin, and myricetin with an IC50 value of less than 10 μM were found to inhibit this release.

Mast cells or basophils have been shown to release chemical mediators such as histamine and leukotriene and to produce cytokines and chemokines (55). Among the cytokines produced by these cells, IL-4, IL-13 and IL-5 are believed to be key molecules related to IgE production, Th2 differentiation and allergic inflammation (56). Therefore, studies were conducted to determine whether flavonoids could inhibit production of these cytokines and leukotriene C4 (57, 58).

	3	5	6	7	8	2'	3'	4'	5'	IC50(μM)
Luteolin	H	OH	H	OH	H	H	OH	OH	H	2.4
Fisetin	OH	H	H	OH	H	H	OH	OH	H	5.1
Apigenin	H	OH	H	OH	H	H	H	OH	H	5.7
Scutellarein	H	OH	OH	OH	H	H	H	OH	H	14.0
3-Hydroxyflavone	OH	H	H	H	H	H	H	H	H	15.0
Kaempferol	OH	OH	H	OH	H	H	H	OH	H	15.7
Quercetin	OH	OH	H	OH	H	H	OH	OH	H	18.8
Eriodictyol(2-3)	H	OH	H	OH	H	H	H	OH	OH	20.8
Fustin(2-3)	OH	H	H	OH	H	H	OH	OH	H	23.0
7-Hydroxyflavone	H	H	H	OH	H	H	H	H	H	26.5
Myricetin	OH	OH	H	OH	H	H	OH	OH	OH	> 30
Galangin	OH	OH	H	OH	H	H	H	H	H	> 30
Morin(dihydrate)	OH	OH	H	OH	H	OH	H	OH	H	> 30



(Each OH for 3 or 5 position)

Figure 1. Inhibitory activity of 13 flavonoids on IL-4 production by basophils. Thirteen kinds of flavonoids were tested for their inhibition of IL-4 synthesis by human basophils in response to anti-IgE antibody plus IL-3. The lower panel shows basic structure of flavonoids for the inhibition.

In the human basophilic cell line, KU812, reverse-transcription (RT)-PCR analyses showed that fisetin at a dose of 30 μM suppressed IL-4, IL-5 and IL-13 expression but not IL-1β, IL-6 or IL-8 expression in response to A23187. Fisetin also inhibited IL-4, IL-5 and IL-13 production by A23187+PMA-stimulated KU812 cells, but not IL-6 or IL-8 production (57). The addition of fisetin to a culture of allergen-stimulated or anti-IgE antibody-stimulated purified peripheral basophils resulted in a decrease in IL-4 and IL-13 synthesis but did not reduce the amount of leukotriene C4 in the supernatant (58). The IC50 of fisetin for the inhibition of IL-4 expression by KU812 cells was 19.1+4.9 and 4.8+0.4 μM for that of normal basophils. Further screening of other flavonoids, assayed in terms of IL-4 production in peripheral blood basophils by means of ELISA, showed that luteolin, fisetin and apigenin with an IC50 of 2.4 to 5.7 μM showed at most an inhibitory activity, while flavonoids such as scutellarein, 3-hydroxyflavone, kaempferol, quercetin, eriodictyol, fustin and 7-hydroxyflavone

substantially inhibited IL-4 production with an IC50 of 14 to 26.5 μM (58). However, other flavonoids including myricetin and morin did not suppress its production. A comparison of the activity with the structure has resulted in a basic structure of flavonoids that exert an inhibitory activity on Th2 type cytokine expression by basophils (shown in Fig 1). For maximal inhibition, hydroxylation in positions 7 and 4' is essential and the presence of OH in either position 3 or 5 is required (58).

II. Mechanism through which flavonoids inhibit Th2 type cytokine production by basophils

The mechanisms by which these flavonoids suppress Th2 type cytokine expression were analyzed with the aid of KU812 cells. The specific inhibitory effect of flavonoids on IL-4, IL-5 and IL-13 suggests that they suppress the activation of certain common specific nuclear factors that positively regulates IL-4, IL-5 and IL-13 gene activation or induce repressor proteins. The nuclear factor of the activated T cells (NFAT) family plays a key role in inducing cytokine genes in T cells, including IL-2, IL-4, IL-5, IL-13, IFN-γ, tumor necrosis factor (TNF)-α and granulocyte/macrophage colony stimulating factor (GM-CSF) (59). An electrophoretic mobility shift assay demonstrated that the presence of fisetin inhibited activation of NFAT1 by A23187-stimulated KU812 cells (57). Our finding that flavonoids including fisetin, apigenin and luteolin did not reduce intracytoplasmic concentration of calcium in response to calcium ionophore, and a previous finding that quercetin directly interacted with a calmodulin, the Ca^{2+}-sensing protein (60 and our unpublished data), suggest that flavonoids may modulate the function of calmodulin and affect some Ca/calmodulin dependent enzymes, which regulate transcriptional activation of these cytokines in addition to NFAT inhibition. However, T cells appear to be more resistant to these flavonoids than basophils in terms of IL-4 synthesis (58), so that other unknown mechanisms may also contribute to this inhibition.

III. Clinical effect of flavonoids on allergic diseases

The *in vivo* effect of flavonoids was examined in the NC/Nga mouse, which spontaneously develops scratching behaviour, severe eczema and serum IgE elevation with aging under nonspecific pathogen free circumstances (61). In addition, the histology of its dermatitis mimics human atopic dermatitis, so that the NC/Nga mouse is considered an atopic dermatitis-model mouse. The mice were orally given a control diet without or with persimmon leaf extract (250 mg/kg) or its major constituent of flavonoid, astragalin (1.5 mg/kg). Oral intake of persimmon leaf extract or astragalin markedly inhibited the appearance of the skin symptoms as well as scratching behaviour, transepidermal water loss and serum elevation of IgE (47, 62). This preventative effect of persimmon leaf extract on the onset and development of eczema was dose-response (62).

Moreover, these substances significantly diminished the severity of dermatitis even after onset without any significant adverse effects. These results indicate that, at least in an atopic dermatitis-model mouse, these substances exert a prophylactic action against the development of atopic dermatitis and have a substantial anti-allergic effect leading to amelioration of skin symptoms. Since astragalin is absorbed after being converted into kaempferol by intestine β-glucosidase, these effects are thought to be partly due to the inhibition of basophil (and mast cell) activation.

On the basis of this evidence, we used randomized controlled trials to examine the clinical effect of persimmon leaf extract on adult patients with atopic dermatitis (Takigawa et al., unpublished data). Patients were treated with a placebo or with 1g or 2g of persimmon leaf extract (including 1% flavonoid) for 4 weeks without any change in medication, and the severity of their skin lesions was measured with the SCORAD index. In addition, serological markers, such as the number of peripheral eosinophils, and serum IgE levels, were also monitored. The intake of persimmon leaf extract at a dose of 1g and 2g per day produced a significant lowering of the SCORAD index, accompanied by a decrease in the number of peripheral eosinophils. However, serum IgE levels did not change significantly. In order to verify that flavonoid is effective for patients with atopic dermatitis, further studies involving a large number of participants are required, but we believe that these preliminary findings are promising for the establishment and development of complementary and alternative medicine for atopic dermatitis

IV. Flavonoids content of foods and daily intake of flavonoids

Flavonoids are naturally and ubiquitously included in plant foods and drinks and are therefore common components of our diet. Hertog in the Netherlands was the first to examine the content of flavonoids such as quercetin, kaempferol, myricetin, apigenin and luteolin in 28 vegetables and nine fruits after their extract had been acid-hydrolyzed (63). Subsequent studies examined the content of flavonoids in vegetables, fruits, and others types of food (64-70). Table I summarizes the content of flavonoids such as luteolin, apigenin, fisetin, quercetin and kaempferol in representative vegetables and fruits. Quercetin levels in the edible parts of most vegetables are less than 5 mg/kg, except for onions (185-634 mg/kg), kale (110-120 mg/kg), broccoli (10-68 mg/kg), lettuce (5-30 mg/kg), apples (5-72 mg/kg) and oranges (18 mg/kg). Kaempferol is found in kale (211-470 mg/kg), broccoli (16-100 mg/kg), and other vegetables. The content of luteolin and apigenin is less than 1 mg/kg in most of vegetables and fruits, but celery contains exceptional amounts of luteolin (22-200 mg/kg) and apigenin (17-750 mg/kg), both of which show a strong inhibitory effect on Th2 cytokine expression by basophils. Parsley reportedly contains a large

Table I. Flavonoid contents in representative vegetables and fruits

Product (mg/Kg)	Luteolin	Apigenin	Fisetin	Quercetin	Kaempferol
Onion	2	<1	5	185-634	<2-14
Kale	<1	<1	n.d.*	110-120	211-470
Broccoli	<1-75	<1	<1	10-68	16-100
Lettuce	<1- 5	<1	<1	5-30	<2
Cerely	22-200	17-750	<1	<1	<2
Parsley	<1-11	1850	3	7	11-45
Apple	<1	<1	27	5-72	<2-27
Strawberry	<1	<1	160	6- 9	8-19
Orange	1	<1	<1	18	32
Grape	<1	<1	4	<1-12	<2-17

Contents of luteolin, apigenin, fisetin, quercetin and kaempferol in representative vegetables and fruits are summarized from the litteratures. n.d*; not determined

amount of apigenin (1850 mg/kg), while substantial quantities of fisetin have been identified in apples and strawberries.

The daily intake of flavonoids, based on their content in foods and beverages, was examined in several countries (65, 71-79). The average levels of flavonoid intake are summarized in Table II. The total amount of flavonoids varied from 2.6 to 68.2 mg/day in nine different countries. In every country the major flavonoid was consistently quercetin (14-100% of total flavonoids), followed by kaempferol and myricetin with an oral intake of 0.1-5.9 mg/day. Luteolin, apigenin and fisetin, which are strong inhibitors of IL-4 synthesis by basophils, are also ingested but in average amounts of less than 1 mg/day.

V. Epidemiological studies regarding relationship of flavonoid intake to allergic diseases

From the viewpoint of the hierarchy of inhibitory effects of flavonoids on cytokine production and histamine release, we strongly recommend that allergic patients ingest flavonoids such as luteolin, apigenin, fisetin, quercetin and kaempferol from the most suitable vegetables, fruits and other foods. However, are these flavonoids truly effective? Two studies have suggested that a high intake of fresh fruit (80) and vegetables (81) may protect against asthma, but neither could determine which specific foods or nutrients were responsible.

Table II. Daily intake of flavonoids

Country	Total flavonoids (mg/day)	Luteolin	Apigenin	Fisetin	Quercetin	Kaempferol	Myricetin
Croatia	40.2-58.2	n.d.*	n.d.	n.d.	21.0-38.2	n.d.	n.d.
Denmark	26.0	n.d.	n.d.	n.d.	12.4	2.4	2.0
Finland	3.4	n.d.	n.d.	n.d.	3.2	n.d.	n.d.
	2.6-9.6	n.d.	n.d.	n.d.	2.6-9.6	n.d.	n.d.
	24.7	n.d.	n.d.	n.d.	3.3	0.6	0.1
Greece	15.6-15.7	n.d.	n.d.	n.d.	14.1-15.0	n.d.	n.d.
Italy	23.1-33.9	n.d.	n.d.	n.d.	17.2-26.8	n.d.	n.d.
Japan	60.8-68.2	n.d.	n.d.	n.d.	7.2-34.6	n.d.	n.d.
	16.7	0.3	n.d.	0.8	9.3	5.9	0.4
	16.3	0.3	n.d.	0.4	8.3	4.9	0.6
The Netherlands	33.1	n.d.	n.d.	n.d.	13.1	n.d.	n.d.
	23.0	0.9	0.7	n.d.	16.0	3.9	1.4
	25.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	28.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Serbia	9.0-13.6	n.d.	n.d.	n.d.	7.7-13.1	n.d.	n.d.
United States	12.9	n.d.	n.d.	n.d.	11.0	n.d.	n.d.
	20.1	n.d.	n.d.	n.d.	15.4	3.6	0.9
	20.0-22.0	n.d.	n.d.	n.d.	14.6-16.7	n.d.	n.d.

Daily intake of total flavonoids, luteolin, apigenin, fisetin, quercetin, kaempferol or myricetin in each country is summarized from litteratures.
n.d*; not determined

Recently, however, Shaheen et al. reported that the results of a population-based case-control study in South London indicated that apple consumption and red wine intake were negatively associated with, respectively, asthma prevalence and severity, perhaps due to the protective effect of flavonoids (82). Moreover, an epidemiological study in Finland found that asthma incidence was lower for higher flavonoid intakes and that the relative risk was 0.65 (83). These epidemiological findings thus indicate a strong possibility that an appropriate intake of flavonoids may constitute complementary and alternative medicine for

allergic diseases and prevent the onset of allergic diseases. To confirm this hypothesis, further intervention studies are needed.

Conclusion

Allergy, a common worldwide disease, has become the subject of growing concern because of its increasing rate of prevalence. In order to reduce the prevalence of allergic diseases, intervention strategies for the primary prevention of these diseases are essential. Although diet management for allergic diseases has not yet been established, it can be expected that the development of appropriate diets for their prevention and treatment is only a matter of time, just as it was for other diseases such as hypertension, hyperlipidemia and diabetes mellitus. Flavonoids appear be strong candidates as components of such diets, and we can expect that flavonoid-rich dietary regimens will be established, both for prevention and as complementary and alternative medicine for allergic diseases.

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