

function during oral tolerance is naïve, because the inductive events of oral tolerance are highly orchestrated and complex.

In summary, we discovered that the continuous oral administration of OVA to OVA-specific TCR-Tg mice activates CD4⁺ IELs to produce Th1-type cytokines and induces splenic T cells to become antigen-specifically hyporesponsive. Our results also suggested that the cytokines produced from CD4⁺ IELs produce a bystander effect on the non-specific cytotoxic activities of IELs. Because another group demonstrated that the Th1 immune response is highly cytotoxic and eradicated a tumor mass by inducing cellular immunity (Nishimura et al., 1999), the present findings provide an important basis for the development of anti-cancer vaccines and for understanding the pathogenesis of cancer.

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Dietary unripe apple polyphenol inhibits the development of food allergies in murine models

Hiroshi Akiyama^{a,*}, Yuji Sato^{a,1}, Takahiro Watanabe^a, Megumi H. Nagaoka^a, Yasuo Yoshioka^a, Toshihiko Shoji^b, Tomomasa Kanda^b, Kiyoshi Yamada^c, Mamoru Totsuka^c, Reiko Teshima^a, Jun-ichi Sawada^a, Yukihiko Goda^a, Tamio Maitani^a

^a National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku Tokyo 158-8501, Japan

^b Fundamental Research Laboratory, Asahi Breweries, LTD., 1-21 Midori 1-cho, Moriya, Ibaraki 302-0106, Japan

^c Department of Applied Biological Chemistry, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

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Abstract The incidence of type I allergic disorders has been increasing worldwide, particularly, the hypersensitivity to food. We first showed that apple condensed tannin (ACT) intake would inhibit the development of the oral sensitization and that the inhibition could correlate with the rise in the population of TCR $\gamma\delta$ -T cells in the intestinal intraepithelial lymphocytes (IEL) using W/W^V mice and B10A mice which were ovalbumin (OVA)-orally sensitized. Serum OVA-specific immunoglobulin E and immunoglobulin G1 titers in the OVA-orally sensitized W/W^V and B10A mice ad libitum fed ACT were extremely inhibited compared to those of the control. The ACT intakes of OVA-sensitized W/W^V and B10A mice inhibited the immediate reduction of the body temperature or the rise in serum histamine induced by active systemic anaphylaxis. The proportions of the TCR $\gamma\delta$ -T cells in the IEL of the OVA-orally sensitized W/W^V and B10A mice ad libitum fed ACT were significantly greater than that in the controls. Furthermore, ACT feeding by itself could induce the rise in the percentage of the TCR $\gamma\delta$ -T cells among the IEL of the W/W^V and B10A mice. This suggests that the ACT intake may prevent the development of food allergies and this effect could be correlated with the rise in the percentage of TCR $\gamma\delta$ -T cells among the IEL.

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Keywords: Apple condensed tannin; IgE; Food allergy; Oral sensitization; Intestinal intraepithelial lymphocytes; TCR $\gamma\delta$ -T cell

1. Introduction

The apple contains several phenolic substances, i.e., chlorogenic acid, catechin, epicatechin, phlorizin, rutin, flavonoids, and condensed tannins [1–7]. Osada et al. demonstrated that

apple condensed tannins (ACT) are contained in unripe apples at a ten times higher level than in the ripe ones [8]. ACT is a mixture of oligomers consisting of chains of flavan-3-ol units mainly linked through C4–C8 (or C6) bonds [9]. Some of these substances have physiological and pharmacological activities. Several authors have reported that oligomeric catechins, such as ACT, showed antioxidant activities [10,11]. We have also shown that ACT had an inhibitory effect on histamine release from both rat basophilic leukemia (RBL-2H3) cells by antigen stimulation and rat peritoneal mast cells stimulated by compound 48/80 [12,13] and that ACT intake would improve the symptoms of atopic dermatitis (AD) in patients with AD [14]. These findings collectively indicate that ACT intake may have anti-allergic effects with uniquely active characteristics.

We have previously shown that mast-cell-deficient WBB6F1-W/W^V mice (W/W^V mice) and B10A mice can be efficiently sensitized by the oral administration of an antigen in the form of gavage [15,16]. These mice should be good models for studying the effect of food and drug substances on the development of oral sensitization by food allergens. In this study, we examined the effects of the ACT feeding on the oral sensitization by food allergens and on the intestinal intraepithelial lymphocytes (IEL) using flow cytometry (FCM) analysis.

2. Materials and methods

2.1. Mice

Female WBB6F1-W/W^V mice (6 weeks, W/W^V mice) and female B10A mice (6 weeks) were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan) and kept under pathogen-free conditions in our animal facility for 1 week before use. The mice were maintained in a temperature (23–25 °C), humidity (40–60%) and light-controlled environment with free access to a Mouse Flat (MF) diet (SLC) and water. The ACT was ad libitum administered to the W/W^V mice in the form of the solution dissolved in distilled water at levels of 0%, 0.1% ACT, 0.5% ACT and 1.0% ACT in terms of the dose-dependent study. The control group ($n = 5$, 0%), and three ACT groups (each $n = 5$, 0.1%, 0.5% and 1.0%) had free access to water and each ACT solution with the MF diet. These mice were sensitized by the administration of 1.0 mg ovalbumin (OVA; grade V, Sigma Chemical Co, St. Louis, MO) by daily gavage for 9 weeks. To assess the effect of only the ACT intake on the population of the lymphocytes in the IEL in W/W^V mice, the 1.0% ACT feeding was investigated without oral sensitization to OVA for 2 weeks or 9 weeks in W/W^V mice.

*Corresponding author. Fax: +81 03 3707 6950.
E-mail address: akiyama@nihs.go.jp (H. Akiyama).

¹ Hiroshi Akiyama and Yuji Sato contributed equally to this work.

Abbreviations: ACT, apple condensed tannins; IEL, intestinal intraepithelial lymphocytes; OVA, ovalbumin; IgE, immunoglobulin E; IgG1, immunoglobulin G1; IgG2a, immunoglobulin G2a; FCM, flow cytometry; ASA, active systemic anaphylaxis; MF, Mouse Flat

For a similar examination using B10A mice, the ACT was ad libitum administered to the mice with levels of 1.0% ACT. The control group ($n = 6$) and ACT group ($n = 6$) had free access to water and each ACT solution with the MF diet. The B10A mice were also sensitized by the administration of 1.0 mg OVA by daily gavage for 9 weeks. To assess the effect of only the ACT intake on the population of the lymphocytes in the IEL of the B10A mice, the 1.0% ACT feeding was investigated without oral sensitization to OVA for 9 weeks in the B10A mice.

For the examination of the W/W^V and the B10A mice, the blank group (each $n = 5$ –6) normally had free access to water and the MF diet without the oral sensitization of OVA for 9 weeks. The care and use of the experimental animals in this study followed “The Ethical Guidelines of Animal Care, Handling and Termination” prepared by the National Institute of Health Sciences (NIHS) in Japan.

2.2. Sample preparations

ACT from unripe apples was obtained according to the method reported by Ohnishi-Kameyama et al. [9].

2.3. ELISA for mouse serum anti-OVA IgE, IgG1 and IgG2a titer

The mouse serum titers of anti-OVA immunoglobulin E (IgE), immunoglobulin G1 (IgG1), and immunoglobulin G2a (IgG2a) were determined in triplicate in a 96-well microtiter plate by a previously reported method [17,18]. The serum OVA-specific antibody titers were calculated by the reciprocal of the serum dilution with the fluorescence intensity at 50% of the maximum level.

2.4. Induction of active systemic anaphylaxis and measurement of the body temperature changes and serum histamine level

The W/W^V or B10A mice orally immunized with OVA were intraperitoneally challenged with 0.2 ml PBS containing OVA (1 mg/mouse) to induce active systemic anaphylaxis (ASA) [15]. The body temperature changes associated with the ASA were monitored with a rectal thermometer for the mice (Shibauro Electronics Co., Ltd., Japan) without general anesthesia. Ten minutes after OVA challenge, the mice were killed and their blood was collected to obtain serum for the histamine determination. The serum histamine concentrations were measured using the post-column HPLC method described by Kawasaki et al. [19].

2.5. Isolation of mouse IEL

The IEL were isolated as previously described by Nagafuchi et al. [20].

2.6. FCM analysis

A three-color analysis of the IEL subsets was performed by a previously reported method [15].

2.7. Statistical analysis

All values are expressed as means \pm standard error of the mean. Statistical comparisons were performed by the Student's *t* test or Scheffe's method after an analysis of variances (ANOVA). In all cases, the probability (*P*) values below 0.05 were considered significant.

3. Results

3.1. Body weight and the amount of feed intake

We examined whether ACT intake affects the body weight and feed intake of the W/W^V and B10A mice. There was no significant difference in the body weight and the amount of feed intake between the control group and the ACT fed group during the experimental period. No specific symptom was observed in the ACT group during the study.

3.2. Serum OVA-specific IgE, IgG1 and IgG2a antibody titer

To investigate whether the ACT intake could affect the development of the OVA-oral sensitization of the W/W^V mice, serum OVA-specific IgE, IgG1 and IgG2a antibody titers in the OVA orally-sensitized mice were determined using indirect ELISA (Fig. 1). In the control group, we confirmed that the OVA-specific IgE, IgG1 and IgG2a antibody titers were substantially detected and the mice were sensitized by the oral administration of OVA in the form of gavage as previously reported. In contrast, the OVA-specific IgE and IgG1 titers of the 1.0% ACT feeding group (1.0% ACT group) were significantly lower than those of the control group. In addition, the OVA-specific IgE and IgG1 titers of the 0.5% ACT group were even lower than those of the control group though the OVA-specific IgE titers were not significantly different from those of the control group. These results suggested that the decrease in the OVA-specific IgE and IgG1 titers by the ACT intake appears to be dose-dependent from 0% ACT feeding to 1.0% ACT feeding. Furthermore, during the same examination of B10A mice, the OVA-specific IgE titer of 1.0% ACT group also were significantly lower than those of the control group (Fig. 2).

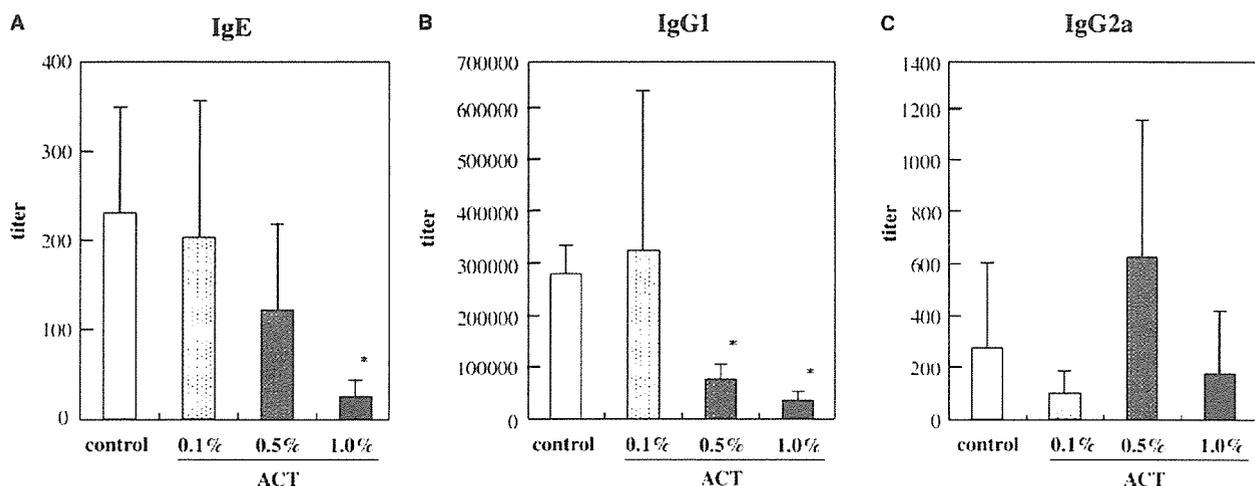


Fig. 1. Effects of ACT intake on serum OVA-specific IgE (A), IgG1 (B) and IgG2a (C) titers in W/W^V mice orally sensitized by OVA. ACT solutions were dissolved in distilled water at levels of 0.1%, 0.5% and 1.0% ACT. Bars represent mean values \pm S.E.M. for five mice. The asterisk indicates significant difference from the control group value (**P* < 0.05).

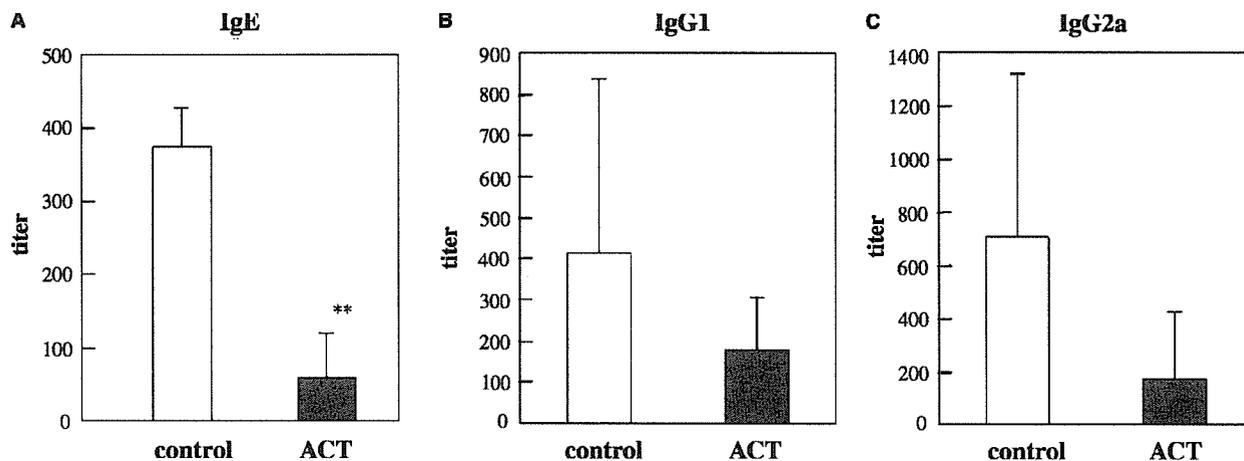


Fig. 2. Effects of ACT intake on serum OVA-specific IgE (A), IgG1 (B) and IgG2a (C) titers in B10 A mice orally sensitized by OVA. ACT solutions were dissolved in distilled water at levels of 1.0% ACT. Bars represent mean values \pm S.E.M. for six mice. The asterisk indicates significant difference from the control group value (** $P < 0.01$).

3.3. Body temperature and serum histamine levels after antigen stimulation

To investigate the effects of ACT on the OVA-induced hypersensitivity reaction in this model, the body temperatures of the sensitized W/W^V or B10A mice were measured every minute for 10 min after intraperitoneal challenge with 1 mg of OVA. As shown in Fig. 3, intense hypothermia developed in the control group after challenge for both the W/W^V and B10A mice. However, there was no hyperthermia in the 1.0% ACT group for both W/W^V and B10A mice. Furthermore, a marked increase in serum histamine occurred in B10A mice undergoing antigen challenge. The serum histamine level in the 1.0% ACT group was significantly lower than that in the control group after antigen stimulation of the B10A mice (Fig. 4).

These results suggest that 1.0% ACT intake could prevent the development of the food allergies in these models and could be involved in the inhibition of ACT feeding on the oral sensitization.

3.4. Flow cytometric analysis of the IEL in W/W and B10A mice

To examine the mechanisms involved in the inhibition of oral sensitization by the ACT intake, IEL were isolated from the small intestine of the normal W/W^V and B10A mice and

their lymphocytes were analyzed by FCM. As previously reported [15], the proportion of the TCR $\gamma\delta$ (TCR $\alpha\beta^- \gamma\delta^+$)T cells in the IEL of the W/W^V mice without oral sensitization was approximately 3.0–5.0% of the blank group at the same experimental point (blank in Table 1). As shown in Table 1, the

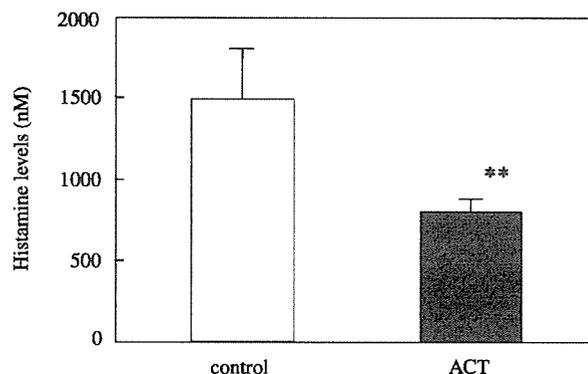


Fig. 4. Determination of serum histamine level of B10A mice after active active systemic anaphylaxis. Asterisk indicates significant difference from control value (** $P < 0.01$). Bars represent mean values \pm S.E.M of four mice.

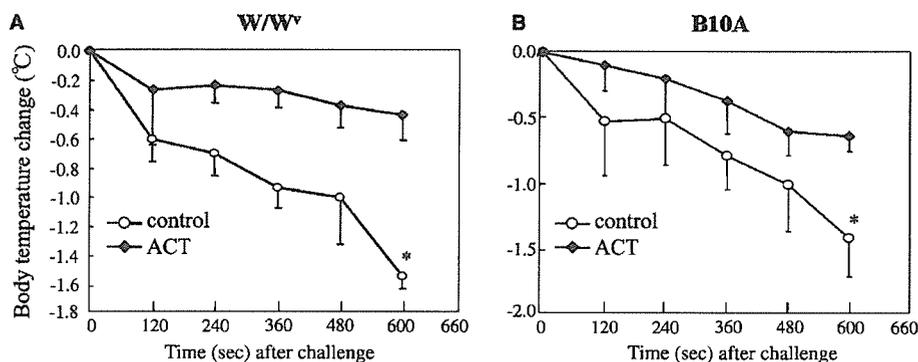


Fig. 3. Changes in body temperature on the sensitized W/W^V mice (A) and B10A mice (B) after the antigen stimulation. *Significantly different from control at $P < 0.05$. Each value represents mean values \pm S.E.M of five mice.

Table 1
Flow cytometric analysis of the IEL in W/W^V mice

	Blank	Control	0.1% ACT	0.5% ACT	1.0% ACT	1.0% ACT (non-oral sens. for 9 weeks)	1% ACT (non-oral sens. for 2 weeks)
CD3 ⁺ β7 ⁺ (T cell)	86.6 ± 6.0	82.6 ± 5.1	84.5 ± 5.5	88.9 ± 3.6	90.1 ± 2.2*	90.7 ± 2.5*	89.4 ± 3.8*
CD4 ⁺ CD8 ⁻ (TCRαβCD4)	13.1 ± 2.9	15.8 ± 5.9	12.9 ± 5.9	16.4 ± 4.4	10.7 ± 2.0	7.9 ± 3.0*	8.0 ± 2.4*
CD4 ⁺ CD8 ⁺ (TCRαβCD4CD8αα)	24.1 ± 9.2	29.1 ± 5.7	29.0 ± 8.1	33.8 ± 5.1	25.3 ± 7.5	24.7 ± 12.4	10.3 ± 4.2
CD8α ⁺ CD8β ⁻ (CD8αα)	34.9 ± 7.0	37.2 ± 6.1	38.3 ± 4.7	44.6 ± 5.4	49.9 ± 6.1**	50.7 ± 6.0**	41.7 ± 2.5**
CD8α ⁺ CD8β ⁺ (TCRαβCD8αβ)	39.2 ± 9.8	32.5 ± 8.4	33.9 ± 10.9	30.5 ± 7.1	31.7 ± 8.4	36.5 ± 8.6	43.2 ± 5.1
TCRαβ ⁻ γδ ⁺ (TCRγδ)	3.4 ± 1.2	2.0 ± 0.7	2.2 ± 1.2	3.6 ± 1.0*	10.1 ± 1.7**	9.5 ± 3.9**	11.5 ± 2.9**
TCRαβ ⁺ γδ ⁻ (TCRαβ)	89.4 ± 4.1	90.30 ± 1.45	90.9 ± 1.6	90.7 ± 2.3	84.7 ± 1.9*	85.0 ± 4.8*	80.2 ± 4.6*

ACT was ad libitum administered to the W/W^V mice in the form of a solution dissolved in distilled water at levels of 0%, 0.1% ACT, 0.5% ACT and 1.0% ACT in terms of the dose-dependent study. The 1.0% ACT with non-oral sens. for 9 weeks (*n* = 5) and 1.0% ACT with non-oral sens. for 2 weeks (*n* = 5) were investigated without the oral sensitization of OVA for 9 weeks and 2 weeks, respectively. The values represent mean ± S.E.M. (% (gated)), *, **Significant difference from control at *P* < 0.05 and *P* < 0.01, respectively.

Table 2
Flow cytometric analysis of IEL in B10A mice

	Blank	Control	1.0% ACT	1.0% ACT (non-oral sens. for 9 weeks)
CD3 ⁺ β7 ⁺ (T cell)	88.9 ± 2.0	84.6 ± 7.6	85.4 ± 3.7	85.7 ± 2.3
CD4 ⁺ CD8 ⁻ (TCRαβCD4)	7.1 ± 2.9	8.5 ± 5.2	6.0 ± 3.1	4.0 ± 1.6
CD4 ⁺ CD8 ⁺ (TCRαβCD4CD8αα)	15.9 ± 7.4	15.4 ± 7.7	8.5 ± 4.4	9.1 ± 4.1
CD8α ⁺ CD8β ⁻ (CD8αα)	58.0 ± 4.8	54.6 ± 6.0	59.8 ± 6.1	66.7 ± 2.7**
CD8α ⁺ CD8β ⁺ (TCRαβCD8αβ)	23.0 ± 6.3	22.8 ± 2.7	18.0 ± 2.1**	17.8 ± 2.8*
TCRαβ ⁻ γδ ⁺ (TCRγδ)	27.0 ± 7.1	25.9 ± 5.6	34.6 ± 6.8**	34.5 ± 4.9**
TCRαβ ⁺ γδ ⁻ (TCRαβ)	61.9 ± 8.1	60.6 ± 5.0	52.0 ± 6.7**	53.3 ± 6.1**

The control group (*n* = 6) and ACT groups (1.0% ACT, *n* = 6) had free access to water and 1.0% ACT solution, respectively. The 1.0% ACT with non-oral sens. for 9 weeks (*n* = 6) were investigated without the oral sensitization of OVA. The values represent mean ± S.E.M. (% (gated)), *, **Significantly different from control at *P* < 0.05 and *P* < 0.01, respectively.

decrease in the proportion of TCRγδ-T cells (2.0%) by oral sensitization shown as the control group (Control in Table 1) was also confirmed as previously reported [15]. On the other hand, the proportions of TCRγδ-T cells (10.1%) and CD8α⁺T cells (49.9%) of the 1.0% ACT group (1.0% ACT in Table 1) were significantly higher than those of the control group. The proportion of TCRγδ-T cells in the 0.5% ACT group was slightly increased compared to that of the control group. These results indicated that the increase in the proportion of the TCRγδ-T cells could appear to be ACT dose-dependent up to the 1.0% ACT feeding. Also, in the B10A mice, we found that the proportions of TCRγδ-T cells (34.6%) of the 1.0% ACT group were significantly higher than those (25.9%) of the control group (Table 2, Fig. 5).

Furthermore, to examine the effect of ACT on the population of IEL, only 1.0% ACT was ad libitum administered to the W/W^V or B10A mice without the oral sensitization with OVA. Surprisingly, the proportions of TCRγδ-T cells (9.5%) and CD8α⁺T cells (50.7%) of the 1.0% ACT group without oral sensitization were significantly higher than those of the blank group (3.4% for TCRγδ-T cells and 34.9% for CD8α⁺T cells) that were not being orally sensitized, as shown in Table 1. Also, in the B10A mice, the proportions of TCRγδ-T cells (34.5%) and CD8α⁺T cells (66.7%) of the 1.0% ACT group without oral sensitization were significantly higher than those of the blank group (27.0% for TCRγδ-T cells and 58.0% for CD8α⁺T cells). These results suggest that only a 1.0% ACT intake induced an increase in the percentage of

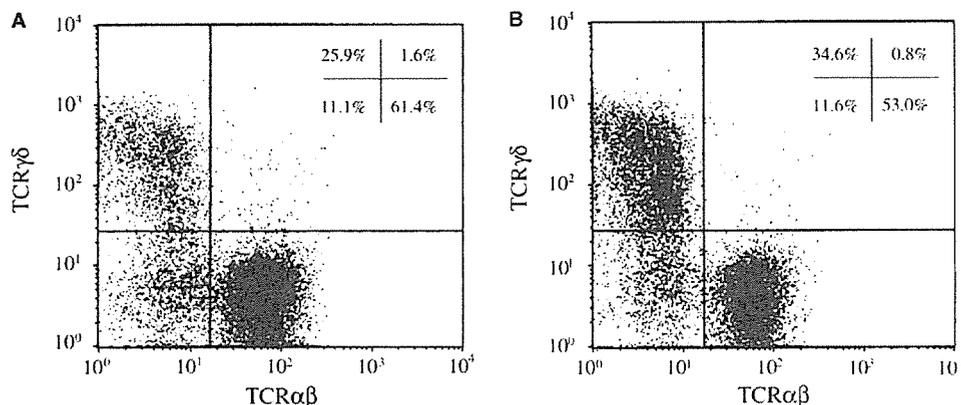


Fig. 5. Expression of TCRαβ and TCRγδ on IEL from B10A mice. IEL were isolated from control group (A) and 1.0% ACT group (B) orally sensitized with OVA. The histograms are representatives of six independent experiments.

TCR $\gamma\delta$ -T cells in the IEL of the W/W^V and B10A mice. Upon the further examination of feeding only 1.0% ACT in the W/W^V mice for two weeks, the proportions of TCR $\gamma\delta$ -T cells (11.5%) and CD8 $\alpha\alpha$ T cells (41.7%) of the 1.0% ACT group with non-oral sensitization were significantly higher than those of the control group for this examination though the proportions of the TCR $\gamma\delta$ -T cells and CD8 $\alpha\alpha$ ⁺T cells in the control group of this examination was 4.2% and 32.3%, respectively (approximately 3–5% for TCR $\gamma\delta$ -T cells and 30–35% for CD8 $\alpha\alpha$ ⁺T cells, respectively, data not shown) and was same as those of the blank group because of not being orally sensitized (Table 1).

3.5. Association of the proanthocyanidin polymerization of the ACT structure for the rise of population of the TCR $\gamma\delta$ -T cells of IEL in the W/W^V mice

In addition, to elucidate the association of the proanthocyanidin polymerization of the ACT structure on the increase in the percentage of the TCR $\gamma\delta$ -T cells of IEL in the W/W^V mice, only a 1.0% monomer proanthocyanidin solution in the mixture of (+)-catechin (14.4%) and (–)-epicatechin (83.6%) extracted from apple was ad libitum administered to the W/W^V mice without the oral sensitization with OVA for two weeks. Interestingly, there was no significant difference between the proportions of the TCR $\gamma\delta$ -T cells (4.8%) and CD8 $\alpha\alpha$ ⁺T cells (33.3%) for only the 1.0% mixture of (+)-catechin and (–)-epicatechin feeding group and those (5.7% and 32.7%, respectively) of the control group.

4. Discussion

In the present study, we first showed that ACT intake would inhibit the development of oral sensitization and that the inhibition could correlate with the rise in the population of TCR $\gamma\delta$ -T cells in the IEL of the W/W^V mice and B10A mice, which are highly susceptible to oral sensitization without using an adjuvant as previously reported [15,16]. In addition, we showed that the ACT intake of OVA-orally sensitized W/W^V and B10A mice inhibited the immediate reduction of the body temperature or the rise of serum histamine levels induced by the antigen stimulation.

Some researchers have developed allergic orally sensitization mouse models using C3H/HeJ mice [21,22]. However, these mice have used cholera toxin as an oral adjuvant in order to be orally-sensitized. Therefore, we considered that the W/W^V and B10A mice should be more appropriate to assess the effect of food substances on the development of oral sensitization and the intestinal mucosal immunity system because these mice can be orally sensitized without using any adjuvants.

Some studies have demonstrated that anaphylactic reactions can be induced in normal and mast-cell-deficient mice, W/W^V mice, after passive transfer of antigen-specific IgG1 antibodies and that these reactions reflect the binding of these antibodies to Fc γ RIII, which can be expressed on the surface of mast cells as well as other cell types such as macrophages, platelets and eosinophils in the mouse [23]. Furthermore, it has been shown that the anaphylactic reactions in mast-cell-deficient mice are involved in the rise of the plasma platelet-activating factor (PAF) [15,24]. In the present study, we are considering that the inhibition of specific IgG1 antibody production by ACT feeding could inhibit the immediate reduction of the body tem-

perature and anaphylactic reactions induced by the reaction among antigen, specific IgG1 antibody and Fc γ RIII expressed on some cells, such as macrophages, platelets and eosinophils in the W/W^V mice. While B10A mice normally have mast cells, the anaphylactic reaction has been thought to be induced by antigens, antigen-specific IgE or IgG1 antibody and Fc ϵ RI expressed on the mast cells because of the immediate rise in the serum histamine levels induced by antigens. Therefore, we conclude that ACT feeding could inhibit specific IgG1 and IgE antibody production induced by oral sensitization and the specific IgG1- or IgE-dependent anaphylaxis on the mast cells in B10A mice.

It has also shown that the percentage of TCR $\gamma\delta$ -T cells in the IEL of the W/W^V mice should be much lower than those of the BALB/c mice, and that the W/W^V mice are also highly susceptible to oral sensitization [15]. Furthermore, we have shown that an additional decrease in the percentage of TCR $\gamma\delta$ -T cells in the IEL cells of the W/W^V mice was also observed in the orally sensitized W/W^V mice [15]. The proportion of TCR $\gamma\delta$ -T cells in the IEL of the W/W^V mice reconstituted with bone marrow cells from C57BL/6J increased much more than that in the untreated W/W^V mice and further extremely inhibited the development of oral sensitization [25]. These findings suggested that the lowering of the population of TCR $\gamma\delta$ -T cells in the IEL of the W/W^V mice could be associated with the high oral sensitivity of the W/W^V mice. In addition, we have shown that ACT intake can induce the proportion of TCR $\gamma\delta$ -T cells and CD8 $\alpha\alpha$ ⁺T cells in the IEL of the W/W^V or B10A mice. Most TCR $\gamma\delta$ -T cells in the IEL are thought to express CD8 $\alpha\alpha$ ⁺ and differentiate into cryptopatches (CPs) [26–28]. Therefore, the present study suggests that the ACT intake mainly increases the population of the TCR $\gamma\delta$ -T cells expressed with CD8 $\alpha\alpha$ ⁺ in the IEL.

Although the function of the TCR $\gamma\delta$ -T cells in the IEL has not been fully elucidated, several reports have suggested the involvement of TCR $\gamma\delta$ -T cells in the IEL in oral tolerance [29–31] and the allergy crisis [32–34]. McMenamin et al. showed that systemic tolerance that is induced by exposure of the respiratory mucosa to OVA activates the TCR $\gamma\delta$ -T cells and CD8⁺ splenic T cells that specifically suppress IgE responses to OVA in adoptive recipient rats and mice [35]. These reports suggest that TCR $\gamma\delta$ -T cells in the IEL would contribute to the induction of oral tolerance.

Considering these findings and our studies, it can be fairly assumed that ACT intake could prevent the development of food allergies through the stimulation of the intestinal mucosal immunity system, and the gut-associated lymphoid tissue correlated with oral tolerance, not due to the physical inhibition of OVA-oral sensitization by ACT.

Condensed tannins are called proanthocyanidins, and have a wide distribution in plants as secondary metabolites. We consume small amounts of these compounds in daily life from fresh fruits such as apple and the processed foods made from these fruits. Rather than nutrients, plant proanthocyanidins are known as the functional food factors that possess a variety of physiological activities such as antioxidant [3–6], anti-allergy, and inhibition against the activities of some physiological enzymes and receptors [36,37]. Most of these parts of the activities of proanthocyanidins appear to depend on their structures and particularly on their degree of polymerization. We showed that the mixture of (+)-catechin and (–)-epicatechin intake in the W/W^V mice for two weeks have no effects

on the percentage of TCR $\gamma\delta$ -T cells in the IEL of the W/W^V mice. This result suggests that the proanthocyanidin polymerization of the ACT structure would be a critical factor for the rise in the percentage of TCR $\gamma\delta$ -T cells of the IEL in the W/W^V mice. The inhibition mechanism of the ACT intake on the oral sensitization in the W/W^V and B10A mice still remains unclear. However, we presumed that the unique structure of the catechin polymerization of ACT should contribute to this activity.

Several clinical medicines prescribed for allergy symptoms, such as corticosteroids, epinephrine, histamine antagonists, and leukotriene synthesis inhibitors, interfere with some steps and lead to the attenuation of the allergic symptoms [6]. However, the effects of these medicines are often short-lived to reduce the allergic responses and induce some side effects. Therefore, a more fundamental means of preventing the development of food allergies should be desirable. Since it has been thought that IgE-mediated food allergies to egg or cow's milk could be developed in early infancy by failing to induce oral tolerance [38], we postulate that the ACT intake during early infancy might prevent the development of food allergies and may continue to inhibit allergic responses to food proteins for a lifetime.

Sampson-HA reported that quantification of food-specific IgE is a useful test for diagnosing symptomatic allergy to egg, milk, peanut, and fish in the pediatric population [38]. Therefore, our present mouse models seem to be useful for examining the effect of bioactive materials to humoral responses of those food allergens.

In conclusion, we showed that ACT intake could inhibit oral sensitization with the rise in the population of TCR $\gamma\delta$ -T cells in the IEL and may fundamentally be used to prevent food allergies. In addition, we believe that ACT can be used as a tool to clarify the role of TCR $\gamma\delta$ -T cells in the IEL. Further studies will be necessary to clarify the mechanism of the induction of oral tolerance by ACT.

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腸管免疫系:アレルギーあるいは免疫寛容の 選択的誘導部位としての役割

Gut Immune System as a Pivotal Site to Direct the Antigen-Specific
Immune Response to either Allergy or Tolerance

戸塚 護

Mamoru Totsuka

東京大学大学院農学生命科学研究科

Department of Applied Biological Chemistry,
The University of Tokyo

Gut Immune System as a Pivotal Site to Direct the Antigen-Specific Immune Response to either Allergy or Tolerance

Mamoru Totsuka

Department of Applied Biological Chemistry, The University of Tokyo
1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Summary

The gut is constantly exposed to various luminal antigens, resulting in the development of the unique immune system called gut-associated lymphoid tissues (GALT). We focused on two kinds of cells comprising GALT, intestinal intraepithelial lymphocytes (IEL) and intestinal epithelial cells (IEC). Their immunological functions and the effects of food ingredients on these cells were investigated. Since the physiological functions and characteristics of IEL are not yet decisively clarified, first the gene expression profile of IEL was analyzed by a DNA microarray analysis. We found that gene expression of interleukin-10 (IL-10), a key inhibitor

of the immune responses, was enhanced in IEL when ovalbumin-specific T-cell receptor (TCR) transgenic mice were fed ovalbumin. When mice were fed nucleotides, a proportion of an IEL subset expressing $\gamma\delta$ TCR increased. Feeding nucleotides also enhanced secretion by IEC of IL-7, which is essential for the development of $\gamma\delta$ TCR⁺ IEL, suggesting that this caused the increase in the number of $\gamma\delta$ TCR⁺ IEL. We also found that IEC-like Caco-2 cells secrete IL-8 in response to yeast cells only in the presence of butyric acid, and that butyric acid enhanced the gene expression of Toll-like receptors recognizing yeast cell components in Caco-2 cells.

1. Intestinal epithelial cells and intestinal intraepithelial lymphocytes as important components of gut-associated lymphoid tissues (GALT)

The gut is constantly exposed to various kinds of antigens and pathogens, and thus, the unique immune system called gut-associated lymphoid tissues (GALT) develops (Fig. 1). GALT consist of many tissues and immune cells, such as Peyer's patches, mesenteric lymph nodes, lamina propria lymphocytes. The intestinal epithelium consists of a monolayer of intestinal epithelial cells (IEC) and intestinal intraepithelial lymphocytes (IEL) existing between IEC, both of which are important components of GALT. We focused on these two kinds of

Gut-associated lymphoid tissues (GALT)

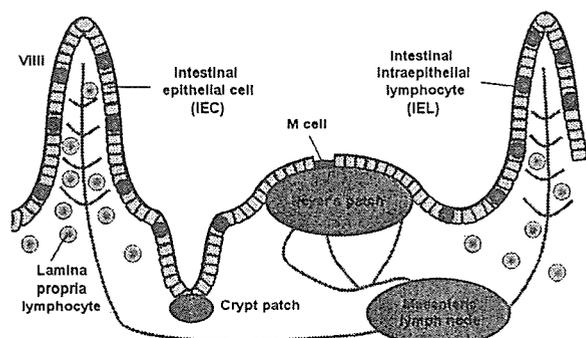


Fig. 1. Scheme of gut-associated lymphoid tissues (GALT).

cells, IEC and IEL, and investigated the effect of food ingredients including microbial components on the function of these cells.

IEC are essential for digestion and absorption of nutrients, and also play an important role in the gut immune system by producing an array of cytokines and chemokines¹. Moreover, IEC are reported to have the capacity to present antigens to T cells, and therefore the interaction between IEC and CD4⁺ T cells in the epithelium and/or the lamina propria has been suggested².

IEL are a kind of T cell but different from typical systemic T cells in many aspects. IEL do not consist of a unique population of cells but can be classified into several subsets according to the expression of different types of T cell receptors (TCR) and the co-receptor molecules (Fig. 2). The most unique feature of IEL is that there are many cells expressing $\gamma\delta$ type of TCR and/or CD8 $\alpha\alpha$ homodimer that are very few in other systemic lymphoid tissues, such as lymph nodes and the spleen. IEL are reported to play a role in maintenance of intestinal epithelial homeostasis, infectious prevention, and regulation of immune responses³. However, the physiological functions of IEL are not yet clearly elucidated.

2. Enhanced gene expression of interleukin-10 in IEL derived from ovalbumin-specific TCR-transgenic mice fed ovalbumin

Oral ingestion of a protein antigen usually induces oral

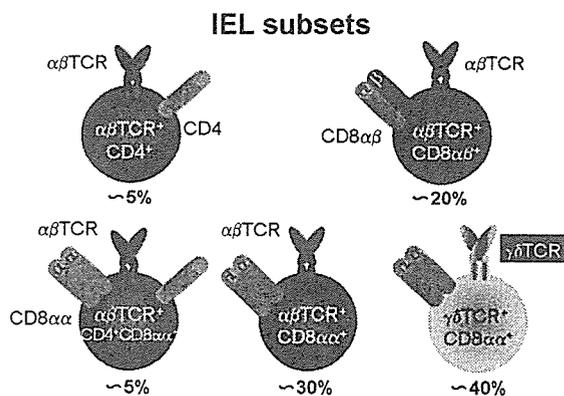


Fig. 2. Subsets of intestinal epithelial lymphocytes. Approximate percentage of each IEL subsets among whole IEL in mice was shown.

tolerance and antigen-specific IgA secretion to prevent harmful immune responses against food antigens. When these systems do not function well, food allergy may develop. However, it is not yet known whether IEL are involved in immune responses to oral antigens. Thus, we investigated what happens to IEL when a protein antigen is orally ingested.

In order to see the response of IEL when an antigen is orally ingested, we used ovalbumin (OVA)-specific TCR transgenic DO11.10 mice⁴. Almost all of the T cells in these mice express the OVA-specific TCR. The mice were fed either the egg white diet containing OVA (EW diet) or the control diet containing casein (CN diet) as a unique source of proteins. After feeding *ad libitum* for 3 days, whole IEL expressing $\alpha\beta$ TCR or $\gamma\delta$ TCR were isolated and purified by flow cytometry. Then we analyzed gene expression profile of IEL by a DNA microarray analysis using GeneChip system (Affymetrix, Santa Clara, CA, USA). The expression of some genes was confirmed by quantitative real-time RT-PCR method.

For DNA microarray analysis, we performed the experiments twice independently. When the EW diet was fed, expression of 98 genes were up-regulated and 24 genes were down-regulated. Among the up-regulated genes, 44 genes are those related to cell cycle regulation

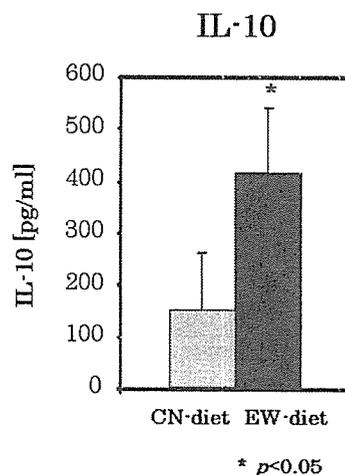


Fig. 3. Increased IL-10 secretion by whole IEL derived from OVA-specific TCR-transgenic mice fed the EW-diet. Whole IEL (2.5×10^6 cells/well) derived from DO11.10 mice fed the EW-diet or the CN-diet for 3 days were stimulated with anti-CD3 mAb and anti-CD28 mAb. After a 72-h culture, IL-10 levels in the supernatants were determined by ELISA.

and 14 genes are related to immune responses. We focused on interleukin-10 (IL-10), an inhibitory cytokine for many aspects of immune responses. The enhanced IL-10 expression by whole IEL was also confirmed at the protein secretion level (Fig. 3). IL-10 secretion from whole IEL stimulated *in vitro* with anti-CD3 and anti-CD28 antibodies was much higher for IEL from the EW-diet group than those from the control diet group.

Next we examined which IEL subset is the source of IL-10. Among IEL subsets derived from DO11.10 mice, a CD4⁺ IEL subset showed the highest gene expression of IL-10. The much higher gene expression of IL-10 in CD4⁺ IEL than in other IEL subsets and CD4⁺ or CD4⁻ cells from other tissues, such as Peyer's patches, mesenteric lymph nodes and spleen, was also observed in normal BALB/c mice. However, the IL-10 expression in CD4⁺ IEL was not significantly enhanced by feeding the EW diet. Therefore, the enhancement of IL-10 gene expression in whole IEL from the EW-fed mice is likely due to the increased number of CD4⁺ IEL in the total IEL compartment. Considering that some kind of regulatory T cells (Tr1) express IL-10⁹, our results suggested that CD4⁺ IEL play a role in suppressing inflammatory responses and/or immune responses induced by the orally fed antigens in the gut.

3. The effect of food ingredient on IEL: nucleotides and apple condensed tannin

Nucleotides are known to exist abundantly in breast milk, which implies that dietary nucleotides have some physiological functions⁶. Dietary nucleotides have been reported to have immune stimulatory activities, such as enhancement of antibody production, and activation of natural killer cells and macrophages^{7, 8}. For intestinal epithelial cells, dietary nucleotides induce promotion of proliferation and differentiation, and enhancement of digestive enzyme activity⁹. Thus, we investigated the effect of dietary nucleotides on the gut immune system¹⁰.

Young BALB/c mice at 3 weeks of age were fed either a nucleotide-free diet (NT(-) diet) or a diet supplemented with nucleotide mixture to the NT(-) diet (NT(+) diet). After feeding *ad libitum* for 2 weeks, IEL were isolated, and the composition of IEL subsets was examined by flow cytometry. As shown in Fig. 4, we found that, in the NT(+) diet group, the proportion of $\gamma\delta$ TCR⁺ IEL was increased, whereas the proportion of $\alpha\beta$ TCR⁺ IEL was decreased. This shows that dietary nucleotides increased the number of $\gamma\delta$ TCR⁺ IEL population.

IEC is known to secrete many kinds of cytokines and chemokines¹¹. To consider the mechanism underlying the increase in the number of $\gamma\delta$ TCR⁺ IEL, we focused on IL-7 secretion from IEC, because it was reported that in IL-7

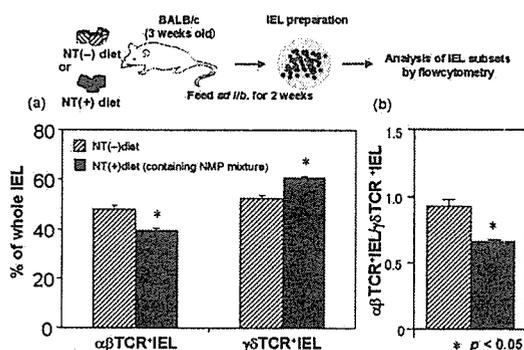


Fig. 4. Dietary nucleotides increased the number of $\gamma\delta$ TCR⁺ IEL. (a) BALB/c mice (3 weeks old) were fed *ad libitum* the NT(-) or NT(+) diet for 2 weeks. IEL were prepared and analyzed by flow cytometry to determine the percentages of $\alpha\beta$ TCR⁺ IEL and $\gamma\delta$ TCR⁺ IEL among whole IEL. (b) The ratio of $\alpha\beta$ TCR⁺ T cells and $\gamma\delta$ TCR⁺ T cells among IEL were calculated based on the data shown in (a).

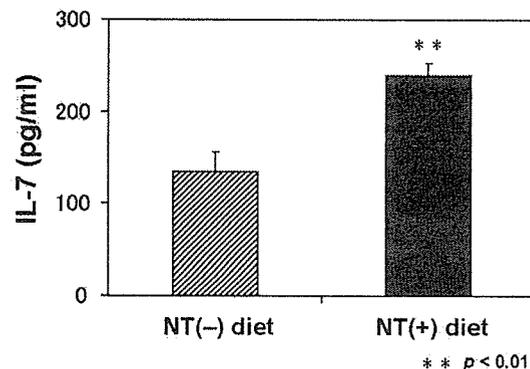


Fig. 5. Dietary nucleotides enhanced IL-7 secretion from intestinal epithelial cells. BALB/c mice (3 weeks old) were fed *ad libitum* the NT(-) or NT(+) diet for 2 weeks. IL-7 levels in the culture supernatants of IEC (2×10^5 cells/well) derived from each group of mice were measured by ELISA.

deficient mice as well as in IL-7 receptor deficient mice, there is no $\gamma\delta$ TCR⁺ IEL^{11,12}. Moreover, when the IL-7 gene was expressed under an IEC-specific promoter in IEC of IL-7 knockout mice, the emergence of $\gamma\delta$ TCR⁺ IEL was observed¹³. Therefore, it was proved that IL-7 secreted by IEC is necessary for the development of $\gamma\delta$ TCR⁺ IEL. Therefore, we next examined whether dietary nucleotides enhanced IL-7 secretion from IEC.

After feeding BALB/c mice (3 weeks old) with the NT(+) diet or the NT(-) diet *ad libitum* for 2 weeks, IEC were prepared. IEC from the NT(+) diet-fed mice produced more IL-7 than those from the NT(-) diet-fed mice (Fig. 5). The expression levels of IL-7 receptor and IL-2 receptor on IEL were not different between the two dietary groups. Our findings suggest that the increased population of a $\gamma\delta$ TCR⁺ IEL subset by feeding nucleotides may be caused by the enhanced production of IL-7 by IEC.

In order to examine whether nucleotides directly influence IEC to enhance IL-7 secretion, we cultured IEC derived from non-treated BALB/c mice in the presence of nucleotide mixture. IL-7 secretion was significantly increased in the presence of the nucleotide mixture in the culture (Fig. 6). This indicates that nucleotides are recognized directly by IEC and enhance IL-7 secretion. These results do not exclude the possibility that dietary nucleotides influence IEC indirectly with the help of

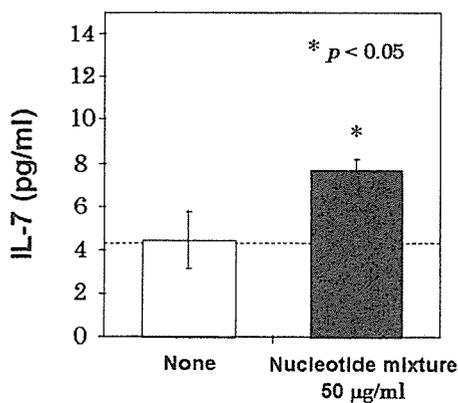


Fig. 6. Nucleotides added to the culture enhanced IL-7 secretion by murine intestinal epithelial cells. IECs freshly isolated from normal BALB/c mice were cultured at 2×10^5 cells/well for 24 h with or without 50 µg/ml nucleotide mixture. The culture supernatants were concentrated by ultrafiltration and the concentrations of IL-7 were determined by means of ELISA. Broken lines indicate the detection limit of IL-7.

intestinal microflora or other cells. This is a typical example of the modulation of interaction between IEC and IEL by a food ingredient.

Dietary nucleotides affect not only GALT but also the systemic immune responses. Feeding nucleotides resulted in the skewed induction of Th1-type immune responses (Fig. 7), and also suppressed antigen-specific IgE production (Fig. 8)¹⁰. It has been reported that adoptive transfer of small numbers of $\gamma\delta$ TCR⁺ T cells from mice tolerant to OVA selectively suppressed IgE antibody production without affecting the parallel IgG response¹⁹.

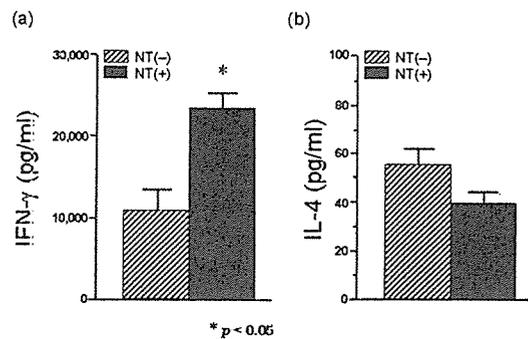


Fig. 7. Interferon-γ and Interleukin-4 production *in vitro* by spleen cells from OVA-specific TCR-transgenic mice fed OVA with or without dietary nucleotides. OVA-TCR Tg mice (3 weeks old) were fed *ad libitum* the NT(-) or NT(+) diet, plus water supplemented with 2% OVA for 4 weeks. The spleen cells were cultured in the presence of OVA at 100mg/ml for 2 days. Interferon-γ (a), and Interleukin-4 (b) levels were determined by ELISA.

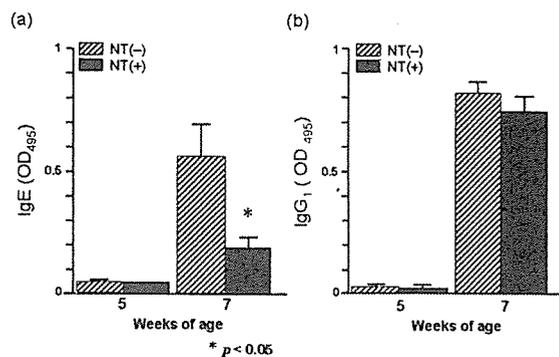


Fig. 8. Effects of dietary nucleotides on serum levels of OVA-specific immunoglobulin in OVA-immunized BALB/c mice. BALB/c mice were immunized intraperitoneally with OVA together with alum at 3 and 5 weeks of age, and then the mice in one group were fed the NT(-) diet and the others were fed the NT(+) diet. The mice were bled at 5 and 7 weeks of age for determination of antibody levels. The serum levels of OVA-specific IgE (a), and IgG₁ (b) were determined by ELISA.

Therefore, one of the physiological roles of $\gamma\delta\text{TCR}^+$ T cells may be in the regulation of IgE responses. Therefore, the increased population of $\gamma\delta\text{TCR}^+$ IEL induced by dietary nucleotides may have a relation with the anti-allergic effects of nucleotide feeding.

Another example of a food ingredient influencing both gut and systemic immune responses was seen for a kind of apple polyphenol¹⁶. Feeding apple condensed tannin could increase the number of $\gamma\delta\text{TCR}^+$ IEL in mice. In addition, feeding apple condensed tannin also decreased the serum level of the antigen-specific IgE induced by oral sensitization in mice. In both of the cases with nucleotides and apple condensed tannin, the number of $\gamma\delta\text{TCR}^+$ IEL was increased, and, at the same time, the change in systemic immune response was observed. Taken together, to explore the mechanisms underlying immune regulatory effects of food ingredients, it seems important to clarify the physiological function of IEL in terms of the relationship with systemic immune responses.

4. Modification of IEC response to yeasts by butyric acid

Caco-2 cells, a human IEC-like cell line, secrete IL-8 in response to various stimulation. IL-8 is a chemokine which has been shown to recruit neutrophils and trigger the firm adhesion of monocytes to vascular endothelial cells. It has been reported that Caco-2 cells are much less reactive to gram-positive bacteria and yeasts than to gram-negative bacteria¹⁷. However, we found that Caco-2 cells secrete significant amount of IL-8 in response to yeast cells, *Candida albicans* and *Saccharomyces cerevisiae*, only in the presence of butyric acid, which is one of bacterial metabolites of non-digestible carbohydrate¹⁶.

In the medium without butyric acid, *C. albicans* as well as *S. cerevisiae* had no significant effect on IL-8 secretion from Caco-2 cells after co-culture for 7 or 24 h (data not shown). In the presence of 10 mM butyric acid, however, *C. albicans* and *S. cerevisiae* induced secretion of IL-8 in a dose-dependent manner (Fig. 9). The *S. cerevisiae* cell wall components, zymosan and glucan, but not mannan, also

induced secretion of IL-8 only in the presence of butyric acid.

Mammalian cells recognize pathogens through pattern-recognition receptors including Toll-like receptor (TLR) family¹⁹. There are currently 11 known TLR family members, and each recognizes a specific molecule derived from microbes. Some of TLRs are known to be expressed on IEC. Peptide glycan and zymosan, which are components of gram-positive bacteria and yeasts, respectively, are recognized by TLR2 in combination with

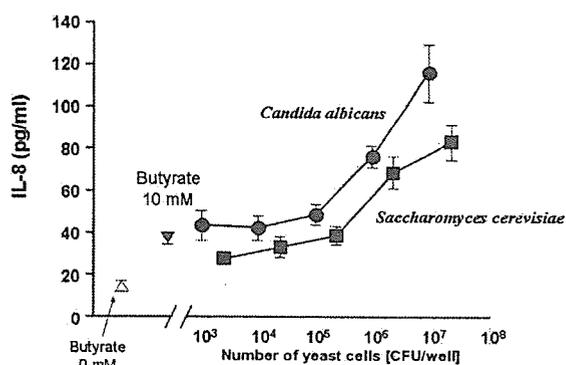


Fig. 9. IL-8 secretion from Caco-2 cells in response to intact *C. albicans* and *S. cerevisiae* in the presence of butyric acid. Caco-2 cells were pre-cultured on a 12-mm Millicell-HA filter for 4 days in a medium containing 10 mM butyric acid. The cells were cultured for 7 h in the presence or absence of *C. albicans* or *S. cerevisiae*, which was added to the apical side of filter. IL-8 secreted into the basolateral supernatants was assayed by ELISA.

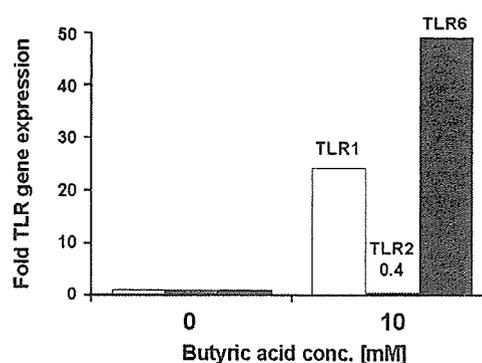


Fig. 10. Butyric acid enhanced the gene expression of TLR1 and TLR6 in Caco-2 cells. Caco-2 cells were cultured on a 12-mm Millicell-HA filter for 4 days in medium with or without 10 mM butyric acid which was added to the apical side of the filters. Data are shown as expression of each specific mRNA relative to that in Caco-2 cells pre-cultured for 4 days in medium without butyric acid. The level of each transcript was normalized to that of GAPDH in the same sample.

either TLR1 or TLR6. Therefore, we next examined whether the gene expression of these TLRs was enhanced in the presence of butyric acid.

In the absence of butyric acid and yeast stimulation, Caco-2 cells expressed TLR1, TLR2, and TLR6 mRNAs as shown by RT-PCR. A quantitative RT-PCR analysis showed that the expression of each message was not affected by stimulation with yeast cells (data not shown). In contrast, culture of Caco-2 cells for 4 days in a medium containing 10 mM butyric acid without yeast stimulation increased expression of TLR1 and TLR6 mRNAs, but had no effect on TLR2 message (Fig. 10). Thus, the increased expression of TLR1 and TLR6 mRNAs may be responsible for Caco-2 cells to acquire the responsiveness to yeast cells in the presence of butyric acid.

Although IL-8 secretion is often detected in proinflammatory responses, the ability of yeasts to enhance IL-8 secretion from IEC does not necessarily mean that yeasts in the intestine are promoters of inflammation. Triggering of the immune system by nonpathogenic microorganisms in the absence of inflammation may serve to enhance host defenses against occasional pathogenic microorganisms. In conclusion, these results clearly show that butyric acid, and therefore non-digestible carbohydrate, can modify the IEC response to microbial stimulation.

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〔日本語訳 (要旨)〕

腸管免疫系：アレルギーあるいは免疫寛容の選択的誘導部位としての役割

戸塚 護

東京大学大学院農学生命科学研究科応用生命化学専攻
 東京都文京区弥生1-1-1

1. 腸管関連リンパ組織 (GALT)

腸管は常に様々な抗原や病原菌に曝されており、栄養成分の吸収と同時に、共生細菌との共存、病原菌の排除などの複雑な役割を担っている。そのため、腸管にはパイエル板、腸間膜リンパ節などからなる腸管関連リンパ組織 (GALT) という特異な免疫系が備わっている。我々は、GALTを構成する細胞のうち、特に腸管上皮層に存在する2つの細胞、すなわち小腸上皮

細胞 (IEC) と小腸上皮内リンパ球 (IEL) に注目し、その免疫学的機能と食品成分がこれらの細胞に及ぼす影響について検討した。IECは栄養素の消化吸収に重要な役割を果たすほか、様々なサイトカインやケモカインを分泌し、T細胞の活性化に必須のMHCクラスII分子を発現することから、免疫応答の調節機能も有することが明らかにされつつある。一方、IELはT細胞レセプター (TCR) を発現しておりT細胞の一種ではあるが、

脾臓などの全身免疫系のT細胞とは性質や機能が異なる細胞群である。IELの生理機能や特性は、これまで明確に明らかにされてはいない。

2. 食品抗原摂取による小腸上皮内リンパ球の遺伝子発現変化

食品抗原に対する免疫応答においてIELが果たす役割を検討するため、我々は抗原経口摂取時におけるIELの遺伝子発現プロファイルの変化をDNAマイクロアレイを用いて解析した。卵白アルブミン (OVA) 特異的TCR遺伝子を導入したトランスジェニックマウス (DO11.10マウス) にOVAを含む卵白食を摂取させた場合にIELに生じる変化を解析したところ、98遺伝子の発現上昇、24遺伝子の発現低下が観察された。発現が上昇したのものとしては細胞周期、免疫応答に関連するものが多数認められた。そのうち、我々は免疫応答において抑制的に作用するインターロイキン10 (IL-10) に着目した。IL-10はCD4⁺ IELサブセットで発現していることが明らかとなった。卵白食摂取によりCD4⁺ IELの細胞あたりのIL-10発現は上昇せず、全IELにおけるCD4⁺ IELの割合が増加したことから、卵白食摂取によるIL-10遺伝子発現の増加はCD4⁺ IEL細胞数の増加によるものであることが示唆された。IL-10は免疫応答を抑制的に制御する制御性T細胞の一部が発現するサイトカインであり、食品抗原摂取によって制御性T細胞機能を有するIELが増加することにより、腸管での免疫応答が調節されている可能性が考えられた。

3. ヌクレオチド摂取が小腸上皮内リンパ球に及ぼす影響

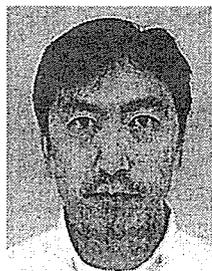
食品抗原以外の食品成分がIELに変化をもたらす可能性について検討した。マウスにヌクレオチドを摂取させた場合のIELのサブセット構成を調べたところ、 $\gamma\delta$ 型のTCRを発現するIELサブセットの割合が有意に上昇することを見いだした。また、ヌクレオチドの摂取によって $\gamma\delta$ TCR⁺ IELの分化に重要なIL7の産生がIECで増強しており、これが $\gamma\delta$ TCR⁺ IELの細胞数の増加の原因であることが示唆された。ヌクレオチドをマウスIEC培養系に添加したところ、IL-7産生の増強が認められたことから、ヌクレオチドはIECに直接作用し、IL-7産生を増強する働きがあることが示された。上記のことは、IECとIELの相互作用に食品成分が影響を及ぼす例として重要な知見であろう。

ヌクレオチドの経口摂取はGALTにのみ作用するのではなく、全身免疫系にも作用する。これまでに、アレルギー発症に抑制的に作用するI型ヘルパーT細胞 (Th1) 応答を誘導し、IgE産生を低下させることを明らかにしている。ヌクレオチドと同様に、リンゴ由来ポリフェノールを摂取させた場合にも $\gamma\delta$ TCR⁺ IELが増加するとともに、IgE産生の低下が認められる現象が示されており、 $\gamma\delta$ TCR⁺ IELの増加と全身免疫系におけるアレルギー応答の抑制との間に何らかの関係がある可能性も示唆された。

4. 小腸上皮細胞の酵母に対する応答の酪酸による修飾

腸内細菌による難消化性糖質の代謝により産生される酪酸がIECに及ぼす影響について検討した。酪酸非存在下でCaco-2細胞は酵母 (*Candida albicans*および*Saccharomyces cerevisiae*) に対して応答性を示さなかったが、10mM酪酸存在下では、*C. albicans*および*S. cerevisiae*の添加によりIL-8産生応答が認められた。同様の効果は酵母の細胞壁成分であるザイモザンやグルカンの添加でも認められた。酵母由来成分の認識に関わるToll様レセプター (TLR) の発現を調べたところ、TLR1およびTLR6のmRNA発現が酪酸添加により上昇していた。またTLR2の発現も認められたが、酪酸の添加では変化しなかった。これらの結果より、酪酸はIECのTLR1およびTLR6の発現を誘導し、これらの分子がTLR2とともに酵母由来成分を認識してIECが活性化されることが示唆された。このことは、難消化性糖質の摂取がIECの微生物認識、さらには腸管免疫系の応答に影響を及ぼす可能性を示している。

PROFILE



戸塚 護

東京大学大学院農学生命科学研究科
応用生命化学専攻
客員助教授
農学博士

1988年東京大学農学部農芸化学科卒業、
1990年東京大学大学院農学系研究科農芸
化学専攻修士課程修了、同年東京大学農
学部助手、1998年東京大学大学院農学生
命科学研究科客員助教授、現在に至る。

Effects of *Ginkgo biloba* extract on blood pressure and vascular endothelial response by acetylcholine in spontaneously hypertensive rats

Yoko Kubota, Naoko Tanaka, Satomi Kagota, Kazuki Nakamura, Masaru Kunitomo, Keizo Umegaki and Kazumasa Shinozuka

Abstract

We previously demonstrated that *Ginkgo biloba* extract (Ginkgo) produced vasodilation via the nitric oxide pathway in aortic segments isolated from Wistar rats. In this study, we have analysed the effects of daily long-term oral Ginkgo treatment on blood pressure, vascular tone, and calcium mobilization to evaluate the clinical availability. Spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY) were fed either a control diet or a diet containing 0.05%–0.5% Ginkgo for 30 days. Administration of Ginkgo did not change systolic blood pressure in WKY, but significantly decreased systolic blood pressure in SHR. In thoracic aortic preparations isolated from SHR, diminished relaxation in response to acetylcholine was improved by a Ginkgo-containing diet. This diet significantly decreased the EC₅₀ value and significantly increased maximum relaxation in response to acetylcholine in SHR. In aortic segments isolated from WKY, acetylcholine-induced relaxation was not affected by a Ginkgo-containing diet. Sodium nitroprusside-induced relaxation was unchanged by a Ginkgo-containing diet in SHR and WKY. We also examined the effects of a Ginkgo-containing diet on the intracellular calcium level of aortic endothelium using a fluorescent confocal microscopic imaging system. Calcium Green 1/AM preloading indicated that acetylcholine significantly increased the endothelial intracellular calcium level. The Ginkgo-containing diet significantly enhanced this increase in the aortic endothelium of SHR, but did not change that of WKY. The results suggested that Ginkgo enhanced endothelium-dependent vasodilation and elevation of the endothelial intracellular Ca²⁺ level in SHR, resulting in hypotension. This accelerative effect of Ginkgo on Ca²⁺ mobilization seemed to be associated with restoration of impaired dilatory function induced by acetylcholine in endothelial cells.

Introduction

Over the last decade, interest by the general public in the use of herbal dietary supplements has risen exponentially. One of the most popular herbal supplements is *Ginkgo biloba* extract (Ginkgo), which is commonly used in the treatment of early-stage Alzheimer's disease, vascular dementia, peripheral claudication, and tinnitus of vascular origin (Sierpina et al 2003). Ginkgo is marketed as a dietary supplement in the United States and Japan. However, in some European countries, it is prescribed clinically and is recognized as being particularly effective in the amelioration of peripheral vascular diseases such as intermittent claudication and cerebral insufficiency (Kleijnen & Knipschild 1992). Ginkgo exerts various pharmacological actions, including the scavenging of free radicals, improvement of the microcirculation, and antagonism of platelet-activating factor (Spinnewyn et al 1987; McKenna et al 2001; De Smet 2002; Ernst 2002). Ginkgo and its constituents, specifically terpenoids (bilobalide and ginkgolides A, B, and C) and flavonoids (quercetin and rutin), are also reported to possess vasorelaxant properties (Duarte et al 2001a; Ibarra et al 2003). This finding has led to the postulation that Ginkgo might have protective effects in cardiovascular disease. Indeed, Sasaki et al (2002) showed that Ginkgo decreased blood pressure in stroke-prone spontaneously hypertensive rats. In this study, we have analysed the effects of daily long-term oral Ginkgo treatment on blood pressure and endothelial function in spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto rats (WKY), to elucidate the mechanism of hypotension by Ginkgo.

Department of Pharmacology,
School of Pharmaceutical
Sciences, Mukogawa Women's
University, 11-68 Koshien
Kyuban-cho, Nishinomiya
663-8179, Japan

Yoko Kubota, Naoko Tanaka,
Satomi Kagota, Kazuki
Nakamura, Masaru Kunitomo,
Kazumasa Shinozuka

National Institute of Health and
Nutrition, 1-23-1 Toyama,
Shinjuku-ku, Tokyo 162-8636,
Japan

Keizo Umegaki

Correspondence: Kazumasa
Shinozuka, Department of
Pharmacology, School of
Pharmaceutical Sciences,
Mukogawa Women's University,
11-68 Koshien-cho, Nishinomiya
663-8179, Japan. E-mail:
kazumasa@mwu.mukogawa-u.
ac.jp

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Materials and Methods

Materials

Ginkgo powder containing 24.2% flavone glycosides, 2.7% ginkgolide A, 1.1% ginkgolide B, 1.7% ginkgolide C, 3.9% bilobalide, and less than 1 ppm ginkgolic acid was supplied by Tama Biochemical Co., Ltd (Tokyo, Japan); this mixture was similar to that of EGb 761, a preparation used in European countries. Calcium Green 1/AM was obtained from Molecular Probes, Inc. (Eugene, OR). Other reagents used were purchased from Wako Pure Chemical Co. Ltd (Osaka, Japan).

Animals and diets

Male spontaneously hypertensive rats (SHR/Izm) and Wistar Kyoto rats (WKY/Izm) were obtained from SLC (Hamamatsu, Japan). They were cared for in accordance with the procedures outlined in the Guidelines for Animal Experimentation of Mukogawa Women's University, which was compiled from the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science. Rats were housed individually in stainless steel, wire-bottomed cages at a constant temperature (22–24°C) with a 12-h light–dark cycle. After adaptation to these conditions for three to five days, SHR were divided into five groups of six 6-week-old rats each and WKY were divided into two groups of six 6-week-old rats each, as follows. Group 1, control WKY rats; group 2, WKY rats treated with 0.5% Ginkgo (0.5% Ginkgo WKY); group 3, control SHR rats; group 4, SHR rats treated with 0.05% Ginkgo (0.05% Ginkgo SHR); group 5, SHR rats treated with 0.1% Ginkgo (0.1% Ginkgo SHR); group 6, SHR rats treated with 0.5% Ginkgo (0.5% Ginkgo SHR). Ginkgo was added to the commercial rodent diet (CE-2; Clea Japan Inc., Tokyo, Japan) and was given to animals in those groups other than controls. Animals had free access to drinking water. All rats were initially fed a control diet (CE-2 without Ginkgo) for seven days before receiving the experimental diet for 30 days. Blood pressure was measured by the tail-cuff method (Model MK-2000, Muromachi Kikai Co., Ltd, Tokyo, Japan) in unanaesthetized rats between 1300 and 1700 h (light on), at 23–25°C. Rats were placed in plastic restrainers. A cuff with a pneumatic pulse sensor was attached to the tail. Rats were trained to become familiar with this procedure for seven days before the experiment. The first day of blood pressure measurement was the day before the experimental diet was first administered. The final day of blood pressure measurement was the thirtieth day after the experimental diet was first administered.

Relaxation studies

After administration of the experimental diet for 30 days, rats were then anaesthetized with pentobarbital sodium (60 mg kg⁻¹, i.p.) and the thoracic aorta was rapidly removed. A part of the thoracic aorta was immediately placed in a Krebs–Henseleit solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; MgSO₄, 1.2; CaCl₂, 2.5; NaHCO₃, 25.0; KH₂PO₄, 1.2; and glucose, 10.0. After periaortic fat and

connective tissue were removed, the aorta was cut into ring segments of approximately 3 mm in length, and used for a relaxation study and intracellular calcium ion measurement. Each ring preparation was mounted vertically under a resting tension of 1 g in a 5-mL water-jacketed organ bath filled with Krebs–Henseleit solution and attached to a force-displacement transducer (Model T-7, NEC San-ei Instruments, Ltd, Tokyo, Japan). The bath solution was maintained at 37°C and bubbled with a 95% O₂/5% CO₂ gas mixture. Each preparation was allowed to equilibrate for at least 60 min before the initiation of experimental procedures, and during this period the incubation media were changed every 10 min. After this equilibration period, the ring preparation was contracted with noradrenaline (10⁻⁷ M) before the putative relaxing agents were cumulatively added. The relaxation response was expressed as a percentage of the maximal relaxation produced by papaverine (10⁻⁴ M).

Measurements of intracellular Ca²⁺ level

Using microscissors, a window of approximately 1 mm in diameter was opened in the thoracic aorta wall to observe the endothelium. The remaining aortic sections were then immediately immersed in physiological saline solution (PSS) of the following composition (mM): NaCl 140.0; KCl 4.0; MgCl₂ 2.0; CaCl₂ 2.0; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 10.0; and glucose 10.0, adjusted to pH 7.4 with NaOH. The aorta was loaded with 5 μM Calcium Green 1/AM for 30 min at room temperature and then rinsed three times with PSS. The aorta was then allowed to incubate in PSS for an additional 15 min at 37°C in a 95% O₂/5% CO₂ atmosphere to permit complete hydrolysis of any intact ester linkages in the intracellular Calcium Green 1/AM.

A U-shaped stainless steel wire (diam. 0.3 mm) was inserted into the aortic lumen to flatten the surface (endothelial layer). This aortic preparation was placed in a glass-bottomed chamber so that the window faced the objective lens of a microscope. The chamber was immediately filled with PSS and placed on a microscope stage (ECLIPSE TE 300, Nikon, Tokyo, Japan) coupled to a Nipkow disk confocal scanner (CSU10, Yokogawa Electric Corporation, Tokyo, Japan). An excitation wavelength of 488 nm was provided by an argon-krypton laser (Omnichrom Inc., Chino, CA, USA) and the emitted light was collected with a 510-nm long-pass dichroic reflector and a 515-nm long-pass emission filter through the planfluor objective (× 20). The fluorescence images were acquired with an ICCD camera (Hamamatsu Photonics, Hamamatsu, Japan) and captured on a personal computer using the ARGUS-50 program (Hamamatsu Photonics, Hamamatsu, Japan). To quantify the Ca²⁺ responses in endothelial cells, images constructed from 195 × 130 pixels were collected every 5 s. Calcium Green 1 fluorescence intensity was directly indicated from the resulting values.

Statistics

All values are reported as mean ± s.e.m. Statistical analysis of data for the groups was carried out using analysis of variance followed by post-hoc tests. A probability of less than 0.05 was considered significant. Statistical analyses were carried