Anti-allergic *Psidium guajava* Extracts Exert an Antitumor Effect by Inhibition of T Regulatory Cells and Resultant Augmentation of Th1 Cells

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**Abstract.** Th1 polarization is one of the mechanisms underlying the therapeutic effects of herbal medicine. The action of anti-allergic agents from *Psidium guajava* (P. guajava) on T cell immunity in mice was investigated. The addition of P. guajava extracts blocked IL-10-mediated, in vitro induction of T regulatory (Tr) cells from CD4⁺ splenocytes of C57BL/6 mice, whereas the extracts exerted only a weak or no effect on the development of Th1 and Th2 cells. Accordingly, Tr cells were not induced from splenocytes of mice administered orally with the extracts. Furthermore, P. guajava extracts shifted the Th1/Th2 balance to a Th1 dominant status by directly attenuating Tr cell activity. In a study of tumor immunity, mice pretreated with the extracts exhibited retarded growth of s.c. inoculated B16 melanoma cells. These findings suggest that P. guajava extracts are efficacious for the prevention of tumor development by depressing Tr cells and subsequently shifting to Th1 cells.

In atopic dermatitis (1), bronchial asthma (2) and allergic rheumatism (3), chemical mediators, such as histamine, serotonin and arachidonic metabolites, are secreted from mast cells or basophils upon stimulation with IgE (4, 5), and nitric oxide (NO) is produced by macrophages during inflammation (6, 7) in a Th2 cell-dependent manner. These inflammatory mediators ultimately cause various allergic symptoms. Since the number of patients with these allergic diseases is increasing worldwide, effective anti-allergic agents are necessary. Empirically, anti-allergic agents from natural sources, such as *Bidens parviflora* Willd (*B. parviflora*), *Mallotus japonicus* and *Psidium guajava*, have traditionally been used as mild remedies for pollinosis and atopic dermatitis (8-11) to some effect. For example, polyacetylene glucosides from *B. parviflora* (10), phloroglucinol derivatives from *M. japonicus* (11) and gallic acid derivatives from *P. guajava* (8, 9, 12) have been identified as inhibitors of NO production by macrophages and histamine release from rat mast cells upon stimulation with antigen-antibody reactions.

It has been reported that extracts from the leaves of *P. guajava* polarize the Th1/Th2 balance to Th1 cells and result in the suppression of allergenic reactions (8, 12), although there is little scientific data to clarify whether *P. guajava* extracts directly or indirectly augment Th1 cell activity. Only one research group has demonstrated that the gallic acid derivative, methyl gallate, from *P. guajava* directly attenuates cytokine production by Th2 cells, though the study was incomplete (12).

It has recently been reported that Tr cells act as down-regulators of tissue-specific autoimmune diseases mediated by autoreactive Th1 cells (13, 14) and of tumoricidal immune reactions constructed by tumor-specific cytotoxic T
lymphocytes (CTLs) and Th1 cells (15-17). Tr cells are distinguished from other conventional CD4+ lymphocyte populations, such as Th1 and Th2 cells, by the high cell surface expression of CD25 and CTLA-4, and by the cytokine profile of IL-10 and TGF-β. In tumor immunity, IL-10, expressed early at local tumor sites, is necessary for the systemic maturation of Tr cells (15, 18). Indeed, Tr cells are induced by cultivation of splenocytes from normal mice in the presence of recombinant (r)IL-10 (19). In murine systems, the development and function of Th1 cells are dominantly suppressed by Tr cells in a cell-cell contact or TGF-β-dependent fashion (20, 21). Tr cells have been reported to participate in the tolerance of both Th1 and Th2 cells in a human system (22). Thus, Tr cells play a substantial role in the control of the Th1/Th2 balance, although there have been few studies on the relationship between Tr cells and the Th1/Th2 balance (23).

In this study, we investigated whether B. parviflora and P. guajava extracts modulate Tr cell activities, whether modification of the Th1/Th2 balance by P. guajava extracts leads to a Th1 dominant condition via Tr cells, and whether the growth of inoculated melanoma cells is beneficially influenced in mice administered with P. guajava extracts.

Materials and Methods

**Mice and tumor cells.** Eight- to 12-week-old female B6 and BALB/c mice were obtained from Japan SL(C (Hamamatsu, Japan). OVA323-339-specific 1-A4-restricted T cell receptor (TCR) gene-transgenic DO11.10 mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA), and maintained under the same conditions in our laboratory. B16 melanoma cells were cultured in RPMI-1640 medium supplemented with 10 % fetal calf serum (FCS, Nissui Pharmacy Co. Ltd., Tokyo, Japan).

**Cytokines, monoclonal antibodies (mAbs), peptide.** Recombinant murine IFN-γ, IL-4, IL-10 and IL-12 were purchased from B.D. PharMingen (San Diego, CA, USA). Purified mAbs specific for murine IFN-γ (R4-6A2), IL-4 (11B11), IL-10 (JES5-2A5), IL-12 (C15.6) and TGF-β1 (A75-2.1), and biotin-conjugated mAbs specific for murine IFN-γ (XMG1.2), IL-4 (BVD6-24G2), IL-10 (JES516E3), IL-12 (C17.8) and TGF-β1 (A75-3.1) were obtained from B.D. PharMingen. The OVA323-339 peptide was obtained from the American Peptide Company (Sunnyvale, CA, USA).

**Preparation of P. guajava and B. parviflora.** Extracts were prepared from the air-dried bark, roots and leaves (50 g) of P. guajava with 250 ml of 80% ethanol (Wako Pure Chem. Industries Ltd., Osaka, Japan) at 20°C for 24 h. The extracts were evaporated at 40°C under reduced pressure, designated PG-Et, and used in *in vitro* and *in vivo* studies at varying concentrations. Alternatively, extracts from the leaves (50 g) of P. guajava were prepared with boiling water (250 ml) for 20 min, named PG-boil, and used after freeze-drying.

Five compounds (P3Dc-1, P3Dc-2, P3Dc-3, P3Dc-4 and P3Dc-5), possessing anti-allergic properties, were extracted from air-dried whole plants of B. parviflora WILLD twice with 60 % ethanol (Wako) and purified as described previously (10). After evaporation of the solvent under reduced pressure, the combined extracts were suspended in water and partitioned with hexane, ethyl acetate and n-butanol, respectively. Evaporation of the solvent yielded the hexane fraction (P1), the ethyl acetate fraction (P2), the n-butanol fraction (P3) and the aqueous fraction (P4). The P3 fraction was subjected to Sephadex LH-20 column chromatography (Pharmacia Fine Chemicals AB, Uppsala, Sweden) with methanol (Wako)-H2O (0-100) to give fractions, P3-A, P3-B, P3-C, P3-D, P3-E, P3-F, P3-G, P3-H, P3-1 and P3-J. Fraction P3-D was separated by normal-phase silica gel column chromatography (SiO2, 400 g, eluted with CHCl3 and methanol in increasing polarity; Pharmacia) to obtain nine fractions (P3-Da, P3-Db, P3-Dc, P3-DD, P3-Dc, P3-Dd, P3-Dg, P3-Dh, P3-Di). The fraction P3-De was then purified by Sephadex LH-20 column chromatography (Pharmacia), eluted with methanol-H2O (1:1), and the eluates were further separated by preparative high-performance liquid chromatography (INW 125 Fluorix, 10 mm i.d. 3250 mm; Pharmacia) and eluted with 18% CH3CN (Wako) to give compounds P3Dc-1, P3Dc-2, P3Dc-3, P3Dc-4 and P3Dc-5.

**In vitro induction of Th1, Th2 and Tr cells.** Th1 and Th2 cells were differentiated from splenocytes of OVA323-339-specific TCR gene-transgenic DO11.10 mice, as described previously (24). Splenocytes of the DO11.10 mice were subjected to the magnetic cell sorting (MACS) system (Miltenyi Biotec, Gladbach, Germany) to separate CD4+ CD45RB+ cells. CD4+ splenocytes, separated with anti-mouse CD4 mAb-conjugated beads (Dynal A.S., Oslo, Norway), were further treated with rat mAb specific for mouse CD45RB (B.D. PharMingen). After washing 3 times, anti-CD45RB-treated CD4+ splenocytes were incubated with anti-rat mAb-conjugated beads, and separated by the magnet, Vario MACS (Miltenyi Biotec). CD4+ CD45RB+ splenocytes were cultured with mitomycin C-treated spleen cells of BALB/c mice in the presence of rIFN-γ (1 ng/ml), rIL-2 (5 U/ml), rIL-12 (50 ng/ml), anti-IL-4 mAb (50 μg/ml) and OVA323-339 peptide (10 μg/ml) for Th1 cell induction, and rIL-2 (5 U/ml), rIL-4 (1 ng/ml), anti-IFN-γ mAb (50 μg/ml), anti-IL-12 mAb (50 μg/ml) (all from B.D. PharMingen) and OVA323-339 peptide (10 μg/ml) for Th2 cell induction at a cell density of 1x106 cells/ml. After 48 h, the cells were restimulated with the same concentration of OVA323-339 peptide and cultured for another 5 days. Tr cells were prepared from CD4+ splenocytes of B6 mice (19). CD4+ splenocytes of B6 mice, separated with antimouse CD4 mAb-conjugated beads (Dynal A.S.), were cultured in medium supplemented with rIL-2 (5 U/ml), rIL-10 (5 ng/ml), anti-IFN-γ mAb (50 μg/ml), anti-IL-4 mAb (50 μg/ml) and anti-IL-12 mAb (50 μg/ml) at a cell density of 2x106 cells/ml for 48 h. Half the medium volume was changed with fresh medium supplemented with rIL-2 (5 U/ml) and cultured for another 5 days.

**Cell proliferation assays.** The proliferation of Th1, Th2 and Tr cells was measured by Alamar blue assay, according to the manufacturer’s instructions (25). Th1, Th2 and Tr cells were cultured in 96-well plates at a cell density of 1x106 cells/well. Alamar blue solution (Alamer Biosciences Inc., Sacramento, CA, USA) was added to the cultures at 1:10 volume, and incubated for 5 h in a CO2 incubator. The fluorescence of each well at an excitation wavelength of 560 nm and emission wavelength of 590 nm was measured with a fluorescence analyzer (Synergy HT, Biotek, Instruments Inc., Winooski, VT, USA).
Enzyme-linked immune spot (ELISPOT) assay. Cytokine profiles of CD4+ splenocytes, from B6 mice untreated or inoculated with B16 cells, were examined by ELISPOT assay as described previously (15). Monoclonal Abs (1 μg/ml in 100 μl of 0.1 M carbonate buffer, pH 9.0) to murine IFN-γ (R4-6A2), IL-4 (11B11), IL-10 (JES5-2A5), IL-12 (C15.6) and TGF-β1 (A75-2.1) (all from B.D. PharMingen) were added to each well of 96-well ELISPOT plates (MultiScreen-HA, Millipore, Bedford, MA, USA) and incubated overnight at 4°C. After incubation, the plates were washed twice with phosphate-buffered saline (PBS), the wells were filled up with PBS containing 10% FCS and incubated at 37°C for 1 h to avoid non-specific reactions, and then washed twice with PBS. The cells (5x10^3 cells), suspended in medium, were cultured for 1 day in each well (100 μl) of mAb-coated ELISPOT plates at 37°C. Each well was then washed vigorously 10 times with PBS and incubated with 0.5 μg/ml of biotin-conjugated mAbs (prepared in 100 μl of PBS containing 10% FCS) to murine IFN-γ (XMG1.2), IL-4 (BVD6-24G2), IL-10 (JES516E3), IL-12 (C17.8) and TGF-β1 (A75-3.1) (all from B.D. PharMingen) at 37°C for 2 h. After washing 5 times with PBS, the wells were incubated with 100 μl of PBS containing 10% FCS and 0.1 μl of streptavidin-peroxidase (Boehringer Mannheim, Mannheim, Germany) at 37°C for 30 min, and washed 5 times with PBS. The spots were visualized by incubation with 100 μl of 1 mg/ml substrate (3,3’-diaminobenzidine tetrahydrochloride plus 0.003% H2O2; both from Sigma Chemical Co. Ltd., St. Louis, MO, USA) at 37°C for 15 min, and counted under the microscope.

Administration of PG extracts in mice. To investigate the in vivo effects of PG extracts, B6 mice were administered orally with PG-Et or PG-boil at 20 μg/mouse/day for 3 consecutive days. Five days after the final administration, B16 melanoma cells (2x10^5 cells/mouse) were inoculated subcutaneously (s.c.) into mice, and the subsequent B16 progression was observed by measuring the tumor diameters. Alternatively, B16 tumor-bearing mice were prepared by s.c. inoculation of B16 cells (2x10^5 cells/mouse). One week after B16 inoculation, the mice were administered orally with PG-Et or PG-boil at 20 μg/mouse/day for 3 consecutive days, and subsequent B16 progression was observed.

Results

Preferential inhibition of in vitro Tr cell induction by P. guajava extracts. We first studied whether P. guajava extracts affect the process of in vitro induction of Th1, Th2 and Tr cells. Th1 cell priming with OVA_{323-339} peptide was not affected by PG-Et, nor did PG-boil alter Th1 induction (Figure 1A). In the induction of OVA_{323-339}-specific Th2 cells, 20-30% growth inhibition was found with a high concentration (10-100 μg/ml) of PG-Et from leaves, although there was a weak or no effect of PG-Et from bark and roots, or PG-boil from leaves (Figure 1B). Of particular interest is the observation that PG-Et strongly suppressed the development process of Tr cells in a concentration-dependent manner (Figure 1C). No inhibitory effect was observed with PG-boil from leaves. On the other hand, 5 compounds purified from B. parviflora WILLD had weak inhibitory effects on the induction of Tr cells (Figure 2), although these substances share with P. guajava extracts the
abilities to suppress the NO production by murine macrophages and the histamine release from rat mast cells (8). These results indicated that *P. guajava* extracts preferentially inhibit Tr cell induction, and that the active ingredients in *P. guajava* extracts were sensitive to heating.

Down-regulation of Tr cell activity and polarization of Th1 cell dominant condition after administration of *P. guajava* extracts. Mice were administered orally with PG-Et or PG-boil from leaves at a concentration of 20 µg/mouse for 3 consecutive days. Five days after the final administration, CD4⁺ splenocytes were subjected to the induction assay for Tr cells and ELISPOT assay for IL-4, IL-10, IFN-γ and TGF-β. As shown in Figure 3A, the induction of Tr cells was dramatically attenuated when mice were administered with PG-Et, with inhibition in the order of leaves, bark and roots. Consistent with the *in vitro* study, PG-boil from leaves did not affect the development of Tr cells. The number of cytokine-producing cells was counted using the ELISPOT assay to further examine the alteration of the Th cell balance in *P. guajava* extract-treated mice. Whereas IL-4-, IL-10- and TGF-β-producing cells were present in spleens from normal mice, *P. guajava* extract-treated mice had decreased numbers of IL-10- or TGF-β-producing cells and an increased number of IFN-γ-producing cells (Figure 3B).

These effects of PG-Et were strongly observed in the order of leaves, bark and roots. The profile of cytokine-producing cells was not changed with PG-boil from leaves. Thus, PG-Et, but not PG-boil, conditioned the Th1 cell dominant status in association with attenuation of Tr cell activity.

**Inhibition of s.c. inoculated B16 tumor growth by oral administration of PG-Et.** The polarization to Th1 dominant immunological status, along with down-regulation of Tr cell activity induced by PG-Et, suggested the hypothesis that this herbal medicine has an antitumor effect. To address this possibility, mice were administered orally with *P. guajava*...
extracts and inoculated s.c. with B16 melanoma cells. PG-Et-pretreatment attenuated the growth of s.c. inoculated B16 tumor cell growth, whereas PG-boil had no protective effect against B16 growth (Figure 4). This prophylactic effect of PG-Et was found strongly in the order of leaves, bark and roots. The B16 tumor diameter 20 days after inoculation in mice pretreated with PG-Et from leaves was approximately one-seventh of the untreated control mice. On the other hand, there was no inhibitory effect of PG-Et extracts in mice with existing tumors (Figure 5). These results indicated that PG-Et exhibited a vaccine effect, but not a therapeutic effect, against tumors.

Down-regulation of Tr cell activity in mice that are pretreated with PG-Et and protected from B16 tumor development. The proliferative capacity of Tr cells from CD4+ splenocytes in mice that were administered with PG-Et, and thus protected from the development of B16 tumors, was compared with that of B16 tumor-bearing mice or normal B6 mice. In confirmation of the previous study (15), Tr cells were markedly expanded from CD4+ splenocytes of B16 tumor-bearing mice in medium with or without rIL-10 (Figure 6). In addition, Tr cells were capable of proliferating when CD4+ splenocytes were cultured even from normal B6 mice in the presence of rIL-10. However, Tr cells were not induced with IL-10 from mice pretreated with PG-Et and inoculated with B16 melanoma cells (B16 protected mice). Therefore, the number and/or activity of Tr cells in PG-Et-
treated mice seemed to be reduced profoundly, compared with those in normal and B16 tumor-bearing mice.

Discussion

In this study, it was demonstrated that the Th1 polarized immunostatus after administration of P. guajava extracts is caused by attenuation of Tr cell activity. The effective ingredients of P. guajava were eluted with ethanol and sensitive to heating, although the active substances remain to be identified. Since anti-allergic agents from B. parviflora WILLD (10) require a Tr cell-independent mechanism to shift to Th1 dominancy, the immune mechanism underlying the modification of the Th1/Th2 balance by each natural source seems to differ. The most significant finding in our study was that P. guajava extracts have a protective action against tumor development, as well as the widely known anti-allergic action against Th2 cell-mediated allergy. Figure 7 illustrates possible mechanisms of the modification of the Th1/Th2 balance and restoration from Th2-dominant diseases by P. guajava.

The active ingredients of P. guajava extracts were inactivated in the process of boiling. Methyl gallate, a gallic acid derivative isolated from P. guajava extracts, inhibits the production of Th2 cytokines, but is insensitive to heating (12). Therefore, it is likely that substances other than gallic acid derivatives displayed suppressive properties against Tr cells. The purification and identification of these active substances in P. guajava extracts are necessary. P. guajava leaves brewed with hot water have been widely used in the treatment of patients with allergic diseases (26), although our investigations indicate that methods other than brewing are required. The ingestion of powder extracts obtained by ethanol elution is one of the options.

The Th1/Th2 balance is important for the evaluation of susceptibility and prognosis of various diseases (27). The Th2-dominant condition is a feature of patients with atopic dermatitis, pollinosis, asthma and others (1-7, 28, 29). SLE, which occurs with tissue attack by autoantibodies and immune complexes, may be a Th2-dominant disease (30). On the other hand, articular rheumatism (31), contact dermatitis (32), tissue-specific autoimmune diseases (33) and type-II diabetes (34) are classified as CTL- or autoreactive T cell-mediated diseases with Th1 cytokine preponderance. We demonstrated that Tr cells participate in the Th1/Th2 balance, implying the involvement of Tr cells in Th1-imbalanced disorders. Similarly, murine Tr cells, induced in IL-10-containing medium, have been known to down-regulate Th1 cell activities (15, 18, 19). In humans, it has been reported that both Th1 and Th2 cells are suppressed by Tr cells (22). Therefore, the inhibitory effect of P. guajava extracts on Tr cells may be efficacious for Th1-dominant diseases as well as Th2-dominant allergic diseases and malignancies. Our study also showed that the inhibitory effect of P. guajava extracts on Th2 cell activity is weak but significant. This effect may be derived from the gallic acid derivatives, with known Th2 cell suppressive potency (12), obtained during the ethanol extraction.

In addition to the immunoregulatory role of Tr cells in allergic diseases and tumor development, it has recently been reported that they impair the function of CD8+ CTLs in murine persistent Friend virus infection (35). Furthermore, Tr cells are involved in the chronic phase of hepatitis C virus (HCV) infection in humans (36), possibly in association with the impairment of HCV-specific CTLs (37). IL-10-expressing Tr cells seem to increase in number during HIV disease progression (38, 39). Thus, accumulating evidence indicates the down-regulation of CTLs by Tr cells in virus infections, suggesting that P. guajava extracts may be therapeutically effective in the treatment of viral infection.

Although the specific markers that completely distinguish Tr cells from other conventional Th cells have not been identified yet, the immunocompetent molecules of Tr cells have been clarified. Glucocorticoid-induced tumor necrosis factor receptor (GITR) and CTL antigen-4 (CTLA-4), and TGF-β-induced foxp3 have been found as crucial molecules of Tr cells for the maintenance of immune suppression (40). LAGE1 peptide, in the context of HLA-DR13, has recently been identified as a Tr cell-specific tumor antigen (41). Galactin-1, a negative regulator of T cell activation and survival expressed on tumor cells, might be useful for tumor immunotherapy on the basis of control of Tr cell functions, as described in CTLA-4 blocking (42,43). Although further
study is necessary, it is possible that *P. guajava* extracts down-modulate the expression of GITR, CTLA-4 and ifox3 in Tr cells and directly inhibit LAGE1 and galactin-1 expression in tumor cells.

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