Green Tea Protects Rats against Autoimmune Arthritis by Modulating Disease-Related Immune Events$^1,2$

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Abstract

Green tea, a product of the dried leaves of Camellia sinensis, is the most widely consumed beverage in the world. The polyphenolic compounds from green tea (PGT) possess antiinflammatory properties. We investigated whether PGT can afford protection against autoimmune arthritis and also examined the immunological basis of this effect using the rat adjuvant arthritis (AA) model of human rheumatoid arthritis (RA). AA can be induced in Lewis rats (RT.1) by immunization with heat-killed Mycobacterium tuberculosis H37Ra (Mt�), and arthritic rats raise a T cell response to the mycobacterial heat-shock protein 65 (Bhsp65). Rats consumed green tea (2–12 g/L) in drinking water for 1–3 wk and then were injected with Mt� to induce disease. Thereafter, they were observed regularly and graded for signs of arthritis. Subgroups of these rats were killed at defined time points and their draining lymph node cells were harvested and tested for T cell proliferative and cytokine responses. Furthermore, the sera collected from these rats were tested for anti-Bhsp65 antibodies. Feeding 8 g/L PGT to Lewis rats for 9 d significantly reduced the severity of arthritis compared with the water-fed controls. Interestingly, PGT-fed rats had a lower concentration of the proinflammatory cytokine interleukin (IL)-17 but a greater concentration of the immunoregulatory cytokine IL-10 than controls. PGT feeding also suppressed the anti-Bhsp65 antibody response. Thus, green tea induced changes in arthritis-related immune responses. We suggest further systematic exploration of dietary supplementation with PGT as an adjunct nutritional strategy for the management of RA. 


Introduction

Rheumatoid arthritis (RA)$^9$ is a chronic debilitating autoimmune disease affecting over 2.1 million Americans (1,2). This disease is characterized by chronic inflammation of the synovial tissue leading to cartilage and bone damage (3). Nonsteroidal antiinflammatory drugs have formed the mainstay of treatment of RA, but their prolonged used is associated with adverse reactions and discomfort (4,5). Therefore, natural plant products that are beneficial against arthritis are continuously being sought for the management of RA. Although there is some evidence for the antiarthritic activity of certain plant products and other nutraceuticals (6–8), the mechanisms of action of such products are largely unexplored.

Green tea, a product of the dried leaves of Camellia sinensis, is the most widely consumed beverage in the world with no known serious side effects (9–12). The polyphenolic compounds isolated from green tea (PGT) are rich in antioxidants that possess antiinflammatory properties (9–12). The main polyphenolic compounds with a flavonoid structure in PGT include epicatechin (EC), epigallocatechin (EGC), EC-3-O-gallate (ECG), and EGC-3-O-gallate (EGCG) (9–11). In this study based on the rat adjuvant-induced arthritis (AA) model of human RA, we tested whether PGT can afford protection against arthritis and also examined the effect of PGT on antigen-specific immune response involved in the disease process.

AA can be induced in the inbred Lewis rats (RT.1)$^1$ by subcutaneous (s.c.) immunization with heat-killed Mycobacterium tuberculosis H37Ra (Mt�) (13,14), and AA has several clinical and histological similarities with RA. The T cells directed against the 65-kD mycobacterial heat shock protein (Bhsp65) have

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$^4$Abbreviations used: AA, adjuvant arthritis; Bhsp65, mycobacterial heat-shock protein 65; CIA, collagen-induced arthritis; EC, epicatechin; ECG, epicatechin-3-O-gallate; EGC, epigallocatechin; EGC-3-O-gallate; GC, gallocatechin; IFN, interferon; IL, interleukin; LNC, lymph node cell; Mt�, Mycobacterium tuberculosis; PGT, polyphenolic compounds from green tea; RA, rheumatoid arthritis; s.c., subcutaneous.

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been invoked in the pathogenesis of both AA (14–17) and RA (18,19). Antibodies also play a role in the pathogenesis of autoimmune arthritis (20,21). The AA model has been used extensively for evaluation of the antiarthritic activity of new compounds of synthetic or natural origin. In this study, we tested the T cell and antibody response to Bhsp65 in PGT-fed Lewis rats compared with water-fed (control) Lewis rats. For T cell response, we tested 2 proinflammatory cytokines [interleukin (IL)-17 and interferon-γ (IFNγ)] (22–24) and 2 antiinflammatory/ immunosuppressive cytokines (IL-4 and IL-10) (25).

Methods

Rats. Inbred male Lewis (RT1<sup>n</sup>) rats, 5–6 wk old, were purchased from Harlan-Sprague Dawley and maintained in the Central Animal Facility of the University of Maryland School of Medicine, Baltimore, MD. These rats were treated ethically in accordance with the guidelines of the Institutional Animal Care and Use Committee. Rats were killed by carbon dioxide asphyxiation and death was confirmed by thoracotomy.

Extraction, purification, and characterization of PGT. The method used in this work was optimized and modified from previous reports (26,27). Whole dried leaves (100 g) of Korean green tea (Camellia sinensis) were extracted twice with 700 mL of hot water (80°C) for 10 min and then 3 times with 700 mL of 80% ethanol under nitrogen gas. Then, the ethanol extractions were concentrated over a rotary evaporator and combined with the water extractions to a final volume of 1800 mL. The resulting extract was treated with an equal volume of chloroform to remove pigments and caffeine and then made acidic with acetic acid to pH 4, followed by reextraction thrice with 1500 mL of nitrogen-saturated ethyl acetate. The resulting soluble organic fraction was collected, lyophilized, and then dissolved in distilled water.

Antigens/mitogens. Native Bhsp65 (28) was obtained by overexpressing the recombinant protein from pET23b-GroEL2 vector (Colorado State University, Fort Collins, CO) transformed into Escherichia coli strain BL21 (DE3) pLysS (Novagen). The removal of endotoxin and further characterization of the recombinant protein by Western blot analysis using the appropriate antibody (SPA-807, Stressgen) to rule out contamination by hsp65 of plant or other origin. Finally, the PGT extract was subjected to HPLC/UV/MS for determination of specific catechins.

Induction and evaluation of AA. Lewis rats were immunized s.c. at the base of the tail with 200 µL (1 mg/rat) of heat-killed Mtb (Difco Laboratories) suspended in mineral oil (Sigma) (14). Thereafter, these rats were observed regularly for signs of arthritis in their paws. The severity of arthritis in each paw was evaluated on the basis of erythema and swelling and graded on a scale of 0 to 4. The highest score for each paw was 4 and the total maximum score for each rat was 16 (14,29). The relative total arthritic score (%) was derived by comparing the total arthritic score over the entire course of AA of an experimental rat with that of a control rat. To examine the effect of PGT on arthritis, groups of Lewis rats (n = 3–4 each) were fed either PGT (8 or 12 g/L) (experimental group) or water (control group) for 1–3 wk before injecting (s.c.) them with Mtb. The daily PGT feeding continued only until the Mtb injection day. Thereafter, all rats were observed regularly for signs of arthritis. To examine the effect of PGT on the T cell response to the disease-related antigen, Bhsp65, Lewis rats were fed 8 g/L PGT (experimental group) or water (control group) for 2 wk before s.c. injection of Mtb. After 9 d, the draining lymph nodes (LNC) of these rats were tested for T cell proliferation and cytokine production in response to Bhsp65 as the recall antigen. We performed tests for 2 proinflammatory cytokines (IL-17 and IFNγ) and 2 antiinflammatory/immunosuppressive cytokines (IL-10 and IL-4). For antibody testing, blood was collected from water-fed and PGT-fed Lewis rats at defined time points before and after Mtb challenge. The collected blood was allowed to clot and was then centrifuged at 2700 × g; 15 min at 4°C. The separated sera were tested in ELISA.

LNC proliferation assay. The rats were killed 9 d following immunization s.c. with Mtb. Thereafter, the draining lymph nodes (superficial inguinal, iliac, and popliteal) of these rats were harvested and a single cell suspension was prepared. These LNC were then cultured with antigen for 4 d before pulsing with [3H]-thymidine for another 16–18 h as described (29). Concanavalin A (mitogen) or mycobacteria-derived purified protein derivative (Mycos Research) was used as a positive control antigen, whereas hen eggwhite lysozyme was used as a negative control antigen. The results based on the incorporation of radioactive [3H]-thymidine were expressed either as Bq or as a stimulation index (disintegrations per minute (dpm) with recall antigen/dpm with cells in medium alone).

LNC cytokine concentration. The draining LNC of PGT-fed and water-fed (n = 4–6 per group) Lewis rats immunized with Mtb were tested for cytokine response.

Real-time PCR. The LNC (1 × 10<sup>6</sup> cells/L) were restimulated with antigen for 48 h as in a LNC proliferation assay. Thereafter, total RNA was extracted from these LNC, reverse-transcribed to cDNA, and amplified using specific primers for the genes encoding the rat IFNγ, IL-17, and IL-4, as described elsewhere (30,31). The levels of cytokine mRNA transcripts were normalized to that of the hypoxanthine-guanine phosphoribosyltransferase gene and the relative gene expression levels were determined.

ELISA. The LNC were restimulated with antigen in vitro for 72 h. Thereafter, the culture supernates were assayed by ELISA for IFNγ and IL-10 using commercially available kits (Biosource) (30,31). The results were expressed as ng/L (ng/L of cytokine in the supernatant of antigen-treated cells – ng/L of cytokine in supernate of cells in medium alone) after subtracting the background cytokine secretion by cells cultured in the absence of antigen.

Measurement of the level and isotype of serum antibodies. Sera of the test and control group of rats (n = 3 each) were pooled separately and then added at different dilutions to antigen-coated wells (100 ng/well) of a high-binding ELISA plate (Greiner Bio-One). The plate was incubated for 1 h at room temperature (32). Following thorough washings, the plate-bound total Ig and isotypes IgG1 and IgG2a were detected by using the appropriate horseradish peroxidase-conjugated goat anti-rat antibodies. The color intensity was read at 450 nm and Δ OD was calculated by subtracting the background OD from OD value with antigen.

Statistical analysis. The data were analyzed using the repeated-measures model in SAS and when appropriate using GraphPad Prism 4.0 program (GraphPad Software). In Figure 1, the comparisons of the control group with each of the PGT-fed groups are within each panel. Although rats were scored repeatedly for arthritic scores, the comparisons between the control group with each of the PGT-fed group were conducted such that data for a particular day were compared among groups utilizing the repeated-measures model to account for within-subject variation. In addition, the severity of arthritis over the entire course of the disease in PGT-fed rats was compared with that of the respective controls. Dunnett’s test was used as the post hoc test in this analysis. A Student’s t test was employed to assess the effect of PGT on cytokine levels (Fig. 2). A P-value of <0.05 was considered significant.

Results

HPLC/UV/MS analysis of PGT extract. Prior to using the PGT extract for experiments in Lewis rats, it was chemically...
characterized using HPLC/UV/MS. Based on analysis of the molecular ions and their specific fragment ions achieved by MS detection, catechins were successfully identified as gallocatechin (GC), EGC, catechin (C), EC, EGCG, GC-3-O-gallate, and ECG. The concentrations of the individual and total catechins in the PGT extract were quantified using the UV detector at 280 nm. The PGT extract contained 3.3% (of total weight) GC, 10.2% EGC, 0.7% C, 4.6% EC, 30.5% EGCG, 2.9% GC-3-O-gallate, and 5.3% ECG, with a total of 57.5%.

**PGT feeding protects against AA in Lewis rats.** Lewis rats fed 8 g/L PGT for 2 wk before Mtb injection had less (P < 0.05) severity of AA compared with the respective control group (Fig. 1A). Similarly, those fed PGT (8 g/L or 12 g/L) for either 2 wk (Fig. 1B) or 3 wk (Fig. 1C) before Mtb injection also had lower arthritic scores (P < 0.05) than the corresponding control groups. In the groups fed for 2 wk, rats fed 8 g/L PGT had lower arthritic scores than those fed 12 g/L PGT (Fig. 1B). However, in the rats fed for 3 wk, the scores of those fed 8 g/L and 12 g/L PGT did not differ (Fig. 1C). Taken together, the optimal feeding regimen of 8 g/L PGT for 2 wk led to a marked (P < 0.05) reduction in disease severity from d 12 to 24 compared with the respective control group (Fig. 1A). The same PGT concentration (8 g/L), but fed for 3 wk, led to reduced arthritic scores from d 13 to 22 (P < 0.05) (Fig. 1C). For each of the 3 sets (Fig. 1A–C), the overall severity of arthritis throughout the course of the disease in 8 g/L PGT-fed rats was lower than that of the respective controls (P < 0.003–0.012). Furthermore, the total arthritic scores over the entire course of AA of the rats fed 2, 4, 8, and 12 g/L PGT were 94.5, 90.3, 47.4, and 69.4%, respectively, of the score of the controls (data not shown). These results demonstrate that the oral administration of PGT successfully suppressed the development of AA in Lewis rats and that feeding 8 g/L PGT for 2 wk prior to Mtb injection had the optimal antiarthritic effect. Therefore, we used this optimal 8 g/L PGT, 2 wk-feeding regimen for the rest of our experiments in this study.

**FIGURE 1** Arthritic scores in Lewis rats fed 0, 8, or 12 g PGT/L drinking water for 1–3 wk before immunization with Mtb. Values are means ± SEM, n = 3–4. *Different from control, P < 0.05.

**FIGURE 2** LNC cytokine concentrations in control rats and rats fed 8 g/L PGT for 2 wk. LNC IL-10 concentration (A), the ratio of IFNγ:IL-10 (B), and IL-17 mRNA in response to test (Bhsp65) and control (HEL/Ova) recall antigens in rats fed 0 or 8 g/L PGT for 2 wk before Mtb injection. Values are means ± SEM, n = 4–6. *Different from control, P < 0.05. In A, background cytokine levels in the medium were subtracted and in C, cytokine mRNA levels were normalized to that of hypoxanthine phosphoribosyltransferase and then presented as fold of mRNA in medium, which was set to 1.
The influence of PGT on the cytokine response of antigen (Bhsp65)-reactive T cells. PGT-fed and water-fed rats had comparable T cell proliferative responses to Bhsp65 (data not shown). However, PGT feeding suppressed the proinflammatory cytokine, IL-17 \( (P < 0.005; \text{Fig. 2}) \) without affecting IFNγ or IL-4 levels (data not shown). In addition, PGT feeding induced a significant increase in IL-10 secretion in response to Bhsp65 compared with the water-fed controls without altering IFNγ secretion. This resulted in a predominantly antiinflammatory cytokine milieu. These results show that PGT feeding induced marked changes in cytokine secretion that might be involved in mediating the immunomodulatory effect of this natural plant extract on AA in Lewis rats.

Dietary PGT treatment was associated with decreased antibody response to Bhsp65. To gain more insight into the effects of PGT on immune response to Bhsp65, we compared the anti-Bhsp65 antibody response in PGT-fed and control Lewis rats that were immunized with Mtb. PGT feeding induced a significant reduction in the total Ig as well as IgG2a antibody against Bhsp65 (Fig. 3). A similar suppressive effect occurred for IgM, although the levels of IgM (data not shown) were lower than those of IgG2a. The level of IgG1 was only marginally above the background (data not shown). These results further reinforce the antigen-directed immune modulation of AA by PGT.

Discussion

Evidence for the beneficial effects of green tea has been gathered from several disease models. Green tea has been reported to attenuate colitis in IL-2–deficient mice (33); offer protection against autoimmune reactivity in the salivary glands and the skin in Sjögren syndrome and lupus, respectively (34); afford protection against type 2 diabetes (35), hepatic steatosis and injury (36,37), bone loss (38), and transplant-reactive T cell immunity (39); and execute a lipid-lowering and antiatherogenic effect (40,41). In this study, we demonstrate that PGT (9–11) are protective against AA in Lewis rats. A similar beneficial effect of orally administered PGT or equivalent extracts has been observed in another model of arthritis, collagen-induced arthritis (CIA) (27). The disease models (AA and CIA) differ in many aspects, including the genetic susceptibility, disease-related antigens, and relative contribution of cellular compared with humoral effector mechanisms (14,21,27). Therefore, our study using the AA model complements and further enlarges the scope of mechanisms underlying the antiarthritic activity of PGT reported earlier in the CIA model. Taken together, the above-mentioned reports highlight the importance of studying immunological and biochemical changes associated with the use of natural dietary plant products for the management of autoimmune and other disorders.

We observed that feeding PGT to Lewis rats influenced the immune response to the disease-related antigen (Bhsp65) following challenge of these rats with the arthritogenic stimulus, Mtb. Arthritis as well as other autoimmune diseases such as multiple sclerosis and its animal model are associated with T helper 17- and T helper 1-dependent (inflammatory and pathogenic) responses against the disease-related antigens (22–24). There is evidence from several experimental studies (22–25) that the suppression of IL-17 as well as the deliberate induction of the antiinflammatory (IL-4 or IL-10) cytokines can prevent or alleviate these conditions. In this regard, the PGT-induced suppression of IL-17 and the increased production of IL-10 represent 2 of the major mechanisms that could afford protection against AA. We suggest that PGT influences the T cell and cytokine response in vivo by conditioning the immune system components before as well as early after antigenic (Mtb) challenge. Subsequently, when LNC are harvested and tested in vitro, the in vivo primed T cells tend to secrete the same cytokines. Considering the limited in vitro test conditions used, our conclusion regarding the PGT-induced antiinflammatory milieu is based on 2–3 d of antigenic restimulation of LNC in vitro. Nevertheless, our results of cytokine changes in rats fed PGT in the AA model are supported by those of other investigators studying CIA (27), inflammation in IL-2–deficient mice (33), and transplant rejection (39). However, in our study, PGT induced an increase in IL-10 secretion but without much change in IFNγ production, whereas in other studies (27,33,39), decreased IFNγ secretion was observed. This difference could be due to the properties of different T cell subsets directed against specific target antigens involved in the experimental models under study. Because Bhsp65 has multiple codominant/subdominant epitopes (14–17), PGT can differentially influence the T cells of diverse specificities. As for PGT, we have shown that another plant extract, Boswellia carterii, also can induce protection against AA (42). Furthermore, considering the role of antibodies in the pathogenesis of autoimmune arthritis in the murine CIA and K/BxN models (20,21), our results showing an association between reduced disease severity and PGT-induced suppression of anti-Bhsp65 antibody response point to the possibility that antibodies produced soon after Mtb challenge might be either directly or indirectly pathogenic. Our results also suggest additional but not yet fully explored immune mechanisms that might contribute to the antiarthritic activity of green tea.

On the basis of the results of studies performed by other investigators, we suggest that the beneficial effect of PGT on the
severity of AA might involve additional mechanisms besides cytokine changes and suppression of the antibody response. For example, EGCG, one of the active ingredients of green tea, has been shown to inhibit the IL-1β-induced expression of matrix metalloproteinases (43,44) as well as the chemokine Regulated on Activation, Normal T Expressed and Secreted (44), and to decrease the expression of tumor necrosis factor-α gene through the inhibition of nuclear factor-κB/activator protein-1 pathway (45). Furthermore, EGCG has been reported to inhibit IL-18–induced neutrophil chemotaxis in vitro (46), to block neutrophil-mediated angiogenesis in vivo (46), to reduce the activity of cyclooxygenase-2 and neutral endopeptidase (27), to inhibit angiogenesis (47), and to inhibit degradation of cartilage proteoglycan and type II collagen (48).

In our study, the optimal antiarthritic activity of PGT was manifest at 8 g/L concentration (w/w) compared with the higher (12 g/L) or lower (2 or 4 g/L) concentrations. This reverse bell-shaped dose curve is reminiscent of that frequently observed with T cell response to protein or peptide antigens. A similar beneficial effect of the oral administration of PGT (average dose range of 1–5 g/L) has been observed by other investigators using a variety of experimental model systems (41,49). The bioavailability (9,12,38,50) and biotransformation (51–53) of green tea polyphenols after dietary intake of tea either as a beverage or as a dietary therapeutic product is an active area of research. Orally administrated polyphenols appear in blood and (−)-EC and other flavones absorbed from the intestinal tract are metabolized to a conjugate that is glucuronidated, methylated, and sulfated in plasma (53–57). Furthermore, polyphenols are thought to form various soluble complexes with tannin as a functional unit (58). Therefore, it is likely that besides the native compounds within PGT, the metabolites of these polyphenols produced in vivo may also contribute to the protective effect of PGT against AA.

In summary, PGT, a dietary product, mediates its antiarthritic activity via suppression both of the proinflammatory cytokine IL-17 and of the antibodies to Bhsps65 combined with an increase in the antiinflammatory cytokine IL-10. Our results suggest that this promising antiarthritic activity of green tea should be further explored as a dietary therapy for the management of RA in conjunction with conventionally used drugs.

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