

Green Tea (*Camelia sinensis*) Suppresses B Cell Production of IgE Without Inducing Apoptosis

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Abstract. Green tea (*Camelia sinensis*) is known to possess biological properties that are antioxidative and antimutagenic. Recent studies demonstrated beneficial effects of green tea in inflammatory allergy. However, the effect of green tea on anti-allergic activity/IgE responses in vitro has not been studied. U266 myeloma cells (2×10^6 /ml), which secrete IgE, were cultured for 0-72 hr with or without green tea extract (1-300 ng/ml), and IgE levels in the supernatants were determined (24-72 hr) by ELISA. The effects of green tea extract on U266 cell numbers, viability, and apoptosis were studied by flow cytometry. High levels of IgE produced by U266 cells were observed at 24, 48, and 72 hr ($1.3 \pm 0.3 \times 10^3$, $1.7 \pm 0.3 \times 10^3$, $2.8 \pm 0.4 \times 10^3$ IU/ml, respectively). Addition of green tea extract either as (a) a single dose, or (b) repeated daily doses, suppressed IgE production with increasing suppression over time (up to 90%; $p < 0.05$); the suppression was dose-dependent with the highest concentrations resulting in the greatest suppression. The suppression of IgE production by green tea extract was not mediated by apoptosis or cell death. This study demonstrates that green tea extract has immunoregulatory effects on human IgE responses in vitro.

Keywords: IgE, U266 myeloma cells, B cell, polyphenols, flow cytometry, annexin, propidium iodide

Introduction

Green tea (*Camelia sinensis*) contains a number of bioactive ingredients, including polyphenols such as epigallocatechin gallate (EGCG), caffeine, and vitamins [1-2], which have antioxidative and free-radical scavenging properties [3-6], cancer-preventing actions [7-10], and cause induction of apoptosis of cancer cells [11]. EGCG, the major catechin in green tea, is believed to be the primary source of green tea's beneficial effects [1]. However,

the O-methylated derivative of EGCG, (-)-epigallocatechin-3-O-(3-O-methyl)-gallate (EGCG''3Me), which was isolated from oolong tea, is reported to have more inhibitory effects on type I and IV allergies in mice than does EGCG [1]. EGCG''3Me can inhibit histamine release in a human basophilic cell line KU812 [12], as well as suppress the expression of FcεRI α and γ chain genes [1]. Shiozaki et al [13] found that tea catechins and caffeine have important inhibitory roles in type I allergic reactions in rats.

Gallic acid (3,4,5-trihydroxybenzoic acid), a polyphenyl natural product from green tea, modulates the inflammatory allergic reaction and decreases IgE-induced histamine release from mast cells [14]. Gallic acid also has an anti-allergic effect in allergy models in vivo and in vitro [14], and

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might be useful in the treatment of allergic skin reactions [14]. Kim et al [14] reported that gallic acid inhibits mast cell-derived inflammatory allergic reactions by blocking histamine release and pro-inflammatory cytokine expression.

IgE plays a major role in asthma and allergic reactions through its ability to bind to Fc-epsilon RI on mast cells [15]. The effect of green tea on the production of IgE by B cells has yet to be established. In the present study, we assessed the effect of unseparated green tea extract (GTE) on IgE response in vitro. We used unseparated GTE because this likely closely mimics the advantageous effects of green tea, since it includes all of the potentially bioactive ingredients. We also investigated whether the effect of GTE on IgE production in U266 cells was due to apoptosis, or was independent of cell death. The present study demonstrates that green tea extract suppresses in vitro IgE production, suggesting a potential therapeutic application of GTE in IgE mediated inflammatory diseases such as allergies, asthma, and atopic dermatitis.

Materials and Methods

U266 cell culture. Cultures of human B cell myeloma line U266 (ATCC, Rockville, MD) (2×10^6 /ml), which secretes IgE, were cultured for 0-72 hr in complete medium (RPMI containing heat-inactivated fetal calf serum (FCS) (10%) (Gibco, Grand Island, NY), L-glutamine (2 mM) (Gibco), 2 mercaptoethanol (50 μ M) (Sigma, St. Louis, MO), and streptomycin (20 ng/ml) (Lilly, Indianapolis, IN), with or without various concentrations of green tea (1-300 ng/ml). Cells were cultured for 0-72 hr at 37°C in a humidified atmosphere of 5% CO₂ in air. Cell viability was >90%, as judged by trypan blue exclusion. Cell culture supernatants were collected (0, 12, 24, 48, 72 hr) and frozen (-70°C) until assayed.

Fresh green tea extract. Whole green tea (*Camelia sinensis* L) extract was purchased from Topix Pharmaceuticals (West Babylon, NY). As per manufacturer, the composition of green tea extract is 90% polyphenol isolate from whole leaf, containing 80% catechins, of which 70% of the catechin content is EGCG, with no significant lot-to-lot variation. The green tea extract powder was freshly prepared for each experiment by suspension in RPMI media (Gibco) (1 g/100 ml) prewarmed to room temperature and diluted (1-300 ng/ml). Three hundred ng/ml was selected as the maximum concentration of GTE in this study, based on previous studies that found maximum serum levels of 300 ng/ml in human volunteers after multi-gram daily supplements [16-17].

Quantification of IgE production. In vitro quantitative determination of IgE content in cell culture supernatants was performed using a solid-phase sandwich enzyme-linked immunoadsorbent assay (IgE ELISA Test Kit, Bioquant, San Diego, CA). All ELISAs were performed according to the manufacturer's recommended procedure. Specimens were analyzed in triplicate and a standard curve was derived from known concentrations of IgE. Plates were read at 450 nm using an automated microplate reader (Model Elx800; Bio-Tek Instruments, Winooski, VT). Optical densities were converted to IU/ml based on the standard curve.

Flow cytometry. Flow cytometry was performed with an Epics XL/MCL flow cytometer with system II software (Beckman Coulter, Fullerton, CA). The fluidics system and optical alignment were verified daily using flow-check fluorospheres (Beckman Coulter). The gain on the photo-multiplier tube detecting fluorescence intensity was adjusted so that 99% of cells with background fluorescence staining were scored between 10^0 - 10^1 on a 4-decade log scale. Color compensation was performed between channels FL1 to FL2 to exclude the effects of overlap of the fluorescence spectra. Briefly, overlap of FL1 and FL2 was excluded by adjusting the compensation value so that cells that are singly labeled with a FL1 fluorescent antibody will result in positive and negative cell populations that have the same median fluorescence intensity, which is distributed symmetrically around the mean FL2 channel autofluorescence value. Specific fluorescence was reported as the % of cells with relative fluorescence intensity scored above background (isotype control); 50,000 events were counted for all samples.

Apoptosis. Apoptotic cell death was determined using a combination of annexin V (AV) and propidium iodide (PI) labeling and analyzed by flow cytometry. In apoptotic cells, membrane phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the extracellular environment. Annexin V is a 36 kDa Ca²⁺-dependent phospholipid-binding protein that has a high affinity for PS and binds to cells when PS is exposed to the external cellular environment, which occurs when membrane integrity is affected in the early phase of apoptosis [18-20]. PI is a vital dye that binds to DNA, a process implying disrupted cellular membrane and exposed DNA, compatible with late, irreversible cell necrosis, either primary or secondary to late apoptosis.

U266 cells were gently separated from culture tubes with a rubber policeman, washed twice with cold phosphate buffer solution (PBS) (Gibco), and resuspended in binding buffer (0.1 M Hepes, 1.4 M NaCl, 25 mM CaCl₂) (BD Biosciences Pharmingen, San Diego, CA). Annexin V-FITC and PI were then added with a volume of 1:20 the volume of cells (typically 5 μ l in 100 μ l of cells in solution), followed by gentle vortexing, and incubation for 15 min at room temperature (25°C) in the dark. Finally, 400 μ l of 1X binding buffer was added to each tube, and cells were analyzed by flow cytometry within 30 min. Apoptosis and necrosis were distinguished by the combination of labeling of annexin V (AV) and propidium iodide (PI) (BD Biosciences Pharm-

ingen), and analyzed using WinMDI software (Vers. 2.8, Scripps Research Institute, San Diego, CA). AV-PI- was defined as viable cells; AV+PI- was defined as early apoptosis; AV-PI+ was defined as early necrosis; AV+PI+ was defined as late stage cell death, either by apoptosis or necrosis.

Absolute cell counts. Absolute cell counts after treatment with GTE were determined by single platform flow cytometric immunophenotyping analysis using flow-count fluorospheres (Beckman Coulter) [21-22]. Briefly, the fluorospheres were transferred to a test tube with an equal volume of cells in solution (100 μ l each), followed by vigorous vortexing. After analysis by flow cytometry, the absolute cell count was calculated by the following formula: Absolute Count = [(cells counted) $\times$ (fluorospheres added)] / (fluorospheres counted).

Statistics. Data are reported as mean $\pm$ SD. Non-parametric statistical techniques were employed; the Kruskal-Wallis one way ANOVA and pair-wise Mann-Whitney U tests were used to compare medians between treatment and control groups. Values (two-tailed) of $p \leq 0.05$ were considered significant.

Results

Effect of green tea on IgE responses induced in vitro. With no GTE treatment, high levels of IgE were first detected in vitro at 12 hr ($6.4 \pm 5.6 \times 10^2$ IU/ml) and continued to increase at 24, 48, and 72 hr ($1.3 \pm 0.3 \times 10^3$, $1.7 \pm 0.3 \times 10^3$, $2.8 \pm 0.4 \times 10^3$ IU/ml, respectively) (Fig. 1A). We focused on 24, 48, and 72 hr for studies of the effects of in vitro treatment with green tea extract (GTE) (1-300 ng/ml) on suppression of IgE production.

At 24 hr after treatment with 300 ng/ml GTE, there was moderate suppression of IgE production ($15.2 \pm 1.3\%$), but no or mild suppression with 1,

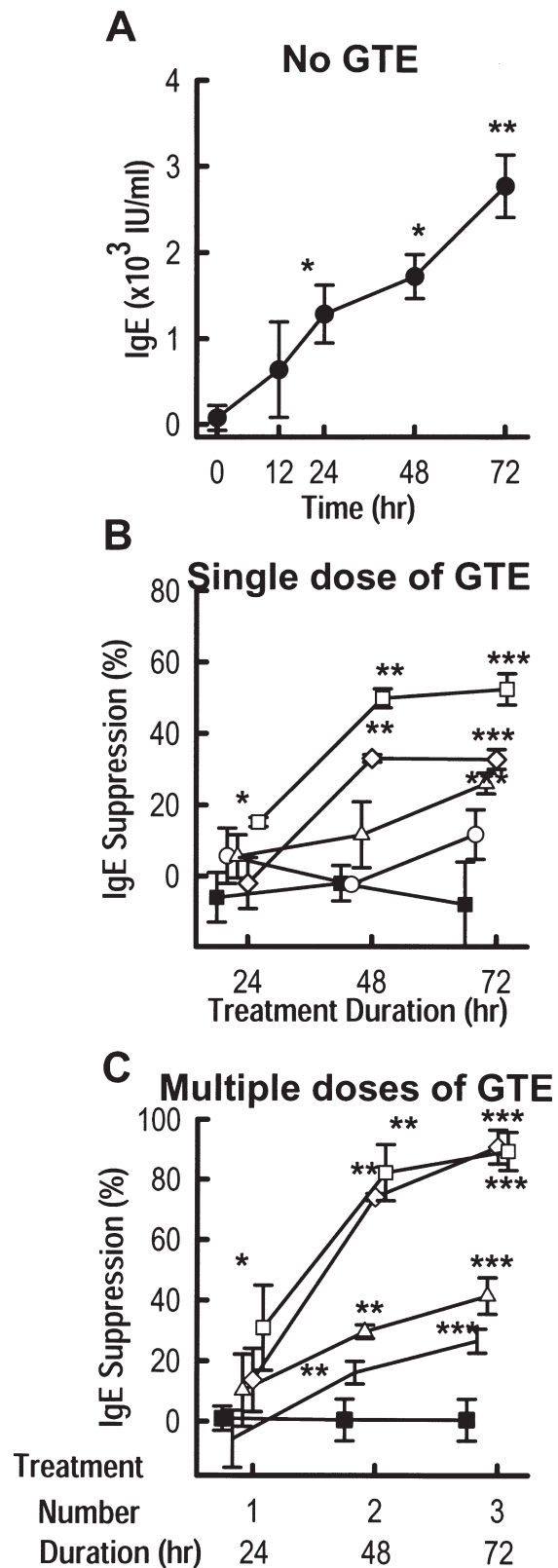


Fig. 1. Green tea extract (GTE) suppresses IgE production by U266 cells. U266 cells were grown in complete RPMI medium to logarithmic growth phase. Cells received either: (A) no treatment (solid square), (B) a single treatment at day 0, or (C) multiple treatments at days 0, 1, and/or 2 of 1 (open circle), 10 (open triangle), 100 (open diamond), or 300 (open square) ng/ml GTE added in vitro. Cell culture supernatants were collected at 0, 12, 24, 48, or 72 hr. Sandwich ELISA was performed (triplicate). Data are reported as IU of IgE per ml (mean $\pm$ SD; $n = 4$ experiments). Panel A: *, ** = significant difference from 0 hr ($p = 0.04$) and 24 hr ($p = 0.03$), respectively. Panel B: *, **, *** = significant difference from other treatment groups at 24 hr ($p = 0.05$) and 48 hr, and from similar treatment at 24 hr ($p < 0.04$) and 72 hr, and from similar treatment at 24 hr ($p < 0.0001$), respectively. Panel C: *, **, *** = significant difference from other single treatment groups at 48 hr ($p < 0.03$) and 72 hr, and from similar treatment at 48 hr ($p < 0.05$).

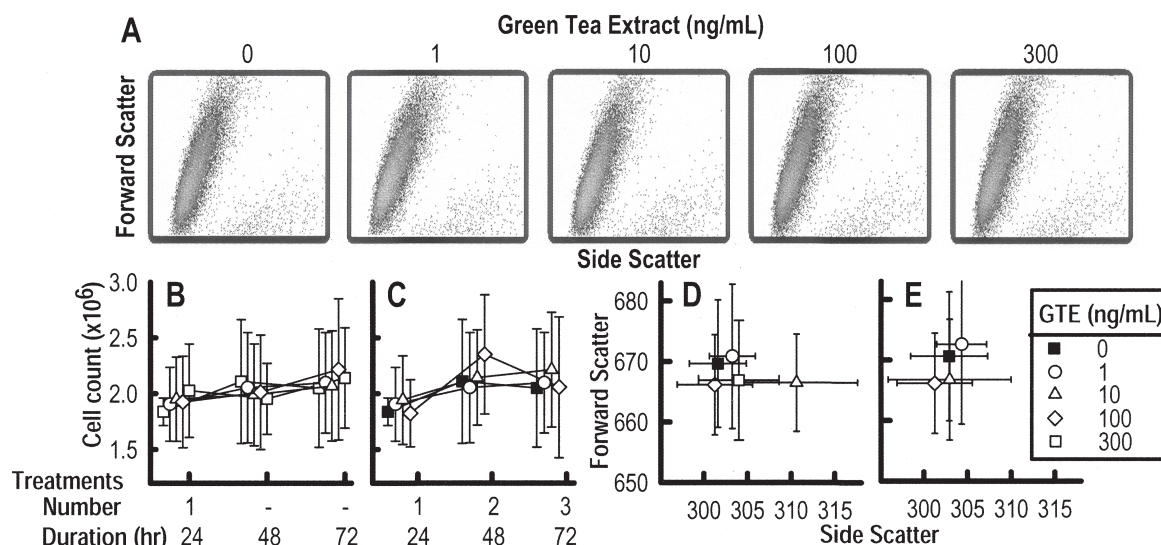


Fig. 2. Green tea extract (GTE) is not toxic to U266 cells. U266 cells were grown in complete RPMI medium to logarithmic growth phase. Cells received either (A, B, D) a single treatment at day 0, or (C, E) multiple treatments at days 0, 1, and/or 2 of 1, 10, 100, or 300 ng/ml GTE. Panels A, D, and E: forward scatter (FS) and side scatter (SS) parameters are relative measures of cell size and surface irregularity, respectively. Panels B and C: absolute cell counts were determined by flow cytometry using flow-count fluorospheres (50,000 cells counted). Data are expressed as means $\pm$ SD ($n = 3$ experiments).

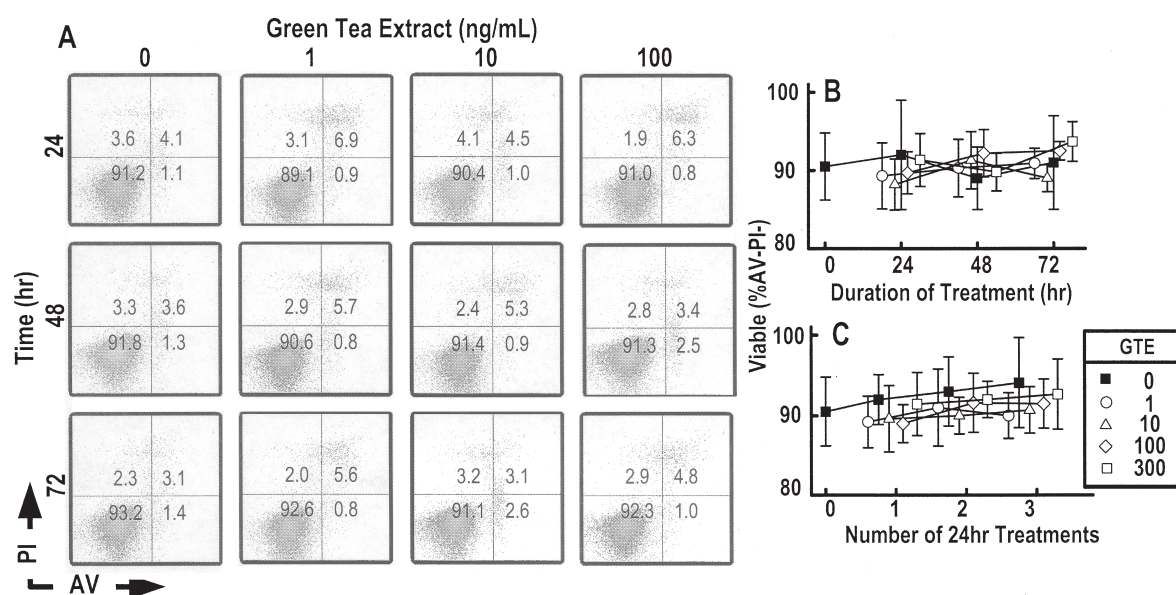


Fig. 3. Green tea extract (GTE) does not induce apoptosis or necrosis of U266 cells. U266 cells were grown in complete RPMI medium to logarithmic growth phase. Panel A, B: At day 0, a single treatment of 1, 10, 100, or 300 ng/ml GTE was added. Panel C: At days 0, 1, and 2, a treatment of 1, 10, 100, or 300 ng/ml GTE was added. Cells were collected at 24, 48, or 72 hr, washed, and stained with annexin V (AV) and propidium iodide (PI). Apoptosis and necrosis were distinguished by flow cytometry (50,000 cells counted) using the combined labeling of AV and PI. AV-PI- was defined as viable cells; AV+PI- was defined as early apoptosis; AV+PI+ was defined as early necrosis; AV+PI+ was defined as late stage cell death, either by apoptosis or necrosis. Panel A: Representative data for a single treatment with 1, 10, and 100 ng/ml. Panels B and C: Data are expressed as % of cells that were AV- and PI- (means $\pm$ SD, $n = 3$ experiments).

10, or 100 ng/ml ($5.7 \pm 7.8\%$, $5.6 \pm 6.1\%$, and $-2.0 \pm 7.2\%$, respectively) (Fig. 1B). At 48 hr after treatment with 100 and 300 ng/ml GTE, there was strong suppression of IgE production ($33.1 \pm 1.1\%$ and $49.9 \pm 2.7\%$, respectively), but no suppression with 1 and 10 ng/ml GTE ($-2.3 \pm 0.07\%$ and $11.6 \pm 9.2\%$, respectively) (Fig. 1B). At 72 hr after treatment with 10, 100, and 300 ng/ml GTE, there was strong suppression of IgE production ($26.0 \pm 2.9\%$, $32.8 \pm 2.8\%$, and $52.4 \pm 4.4\%$, respectively), but no significant suppression with 1 ng/ml GTE ($11.7 \pm 6.9\%$) (Fig. 1B).

To determine the effects of sustained GTE exposure on IgE production by U266 cells, repeated treatments of GTE were added in vitro every 24 hr. As shown in Fig. 1C, repeated GTE dosing caused additional suppression of IgE production by U266 cells compared with single dosing. After 2 doses (48 hr treatment duration), there was even greater suppression of IgE production with 1, 10, 100, and 300 ng/ml GTE than a single dose at 48 hr (1: $16.3 \pm 3.7\%$ vs $-2.3 \pm 0.07\%$; 10: $29.9 \pm 2.2\%$ vs $11.6 \pm 9.2\%$; 100: $74.3 \pm 1.2\%$ vs $33.1 \pm 1.1\%$; 300: $82.4 \pm 9.3\%$ vs $49.9 \pm 2.7\%$) (Fig. 1C). Similarly, after 3 doses (72 hr treatment duration), there was even greater suppression of IgE production with 1, 10, 100, and 300 ng/ml GTE than with a single dose at 72 hr (1: $26.8 \pm 4.1\%$ vs $11.7 \pm 6.9\%$; 10: $41.8 \pm 5.9\%$ vs $26.0 \pm 2.9\%$; 100: $91.0 \pm 5.6\%$ vs $32.9 \pm 2.8\%$; 300: $89.6 \pm 6.3\%$ vs $52.4 \pm 4.4\%$) (Fig. 1C). Further, after 3 doses, there was significantly greater suppression of IgE production with 1, 10, and 100 ng/ml GTE than after 2 doses ($p < 0.04$), but not with 300 ng/ml.

GTE is non-toxic to U266 cells. Since GTE was previously shown to kill cancer cells, including multiple myeloma cells in vitro [23], we investigated whether GTE suppression of IgE was related to cell death. U266 cell morphology was analyzed via forward scatter (FS) and side scatter (SS) parameters of flow cytometry, which are relative measures of cell size and surface irregularity, respectively (representative data, Fig. 2A). A single treatment with any concentration of GTE did not change U266 cell morphology (Figs. 2A, 2D). Similarly, multiple treatments at 24 hr intervals (1, 2, or 3) with any concentration of GTE (1, 10, 100, or 300 ng/ml) did not change cell morphology (Fig. 2E).

In particular, there were no increases in numbers of small dense cells (decreased FS and increased SS, respectively) that would be consistent with the morphological changes of apoptosis [24].

The absolute numbers of U266 cells in culture after 24, 48, and 72 hr without GTE treatment ranged from $1.7 - 2.5 \times 10^6$ cells. A single treatment with any concentration of GTE (1, 10, 100, or 300 ng/ml) did not affect the absolute numbers of cells at 24, 48, or 72 hr (Fig. 2B). Moreover, multiple treatments at 24 hr intervals (1, 2, or 3) with any concentration of GTE (1, 10, 100, or 300 ng/ml) did not have any effect on the absolute numbers of cells (Fig. 2C).

GTE does not induce apoptosis of U266 cells. To determine whether concentrations of GTE sufficient to suppress IgE production by U266 cells induced apoptosis, we studied U266 apoptosis using co-labeling with annexin V (AV) and propidium iodide (PI) via flow cytometry. AV-PI- cells represent viable cells; AV+PI- cells represent early apoptosis; AV+PI+ cells represent early necrosis; AV+PI+ cells represent either primary necrosis, or secondary necrosis in apoptotic cells (representative data, Fig. 3A). The majority of U266 cells in the untreated group were viable at all time points tested (90.5–92.0%) (Fig. 3A).

A single treatment with any concentration of GTE did not increase the numbers of apoptotic or necrotic cells in vitro at 24, 48, or 72 hr (Fig. 3B). Similarly, multiple treatments at 24 hr intervals (1, 2, or 3) with any concentration of GTE (1, 10, 100, or 300 ng/ml) did not increase the numbers of apoptotic or necrotic cells (Fig. 3C).

Discussion

The present study demonstrates that green tea extract suppresses human IgE production in vitro in a dose-dependent fashion, where the highest concentration of GTE (300 ng/ml) results in the strongest suppression of IgE production. A single treatment with GTE suppresses IgE production as early as 24 hr, with strongest suppression at 72 hr. The continued suppression of IgE production at 72 hr suggests that GTE modifies certain transcription pathways with a sustained effect.

Previous studies showed EGCG, the principal green tea catechin, to be oxidized in cell culture medium with a half-life of only 130 min for HT-29 human colon adenocarcinoma cells [25] and 30 min for esophageal squamous cell carcinoma KYSE 150 cells and epidermoid squamous cell carcinoma A431 cells [26]. These studies suggest that a single dose of EGCG in cell cultures would be almost entirely consumed within 12 hr. Therefore, it seems likely that a single dose of GTE suppresses U266 cell production of IgE, with sustained effect at 48 and 72 hr by propagation of other mediators. In the present study, repeated daily GTE treatments resulted in stronger suppression than a single equivalent dosage. These data suggest that there may be a cumulative stimulatory effect with repeated and dose-dependent efficacy.

The results of this study demonstrate a novel role for GTE in suppressing B cell production of IgE. Previous reports established that green tea polyphenols (eg, epicatechin-3-gallate (ECG) and epigallocatechin-3-gallate (EGCG)), exhibit antimutagenic and anticarcinogenic activity in microbial systems (*Salmonella typhimurium* and *Escherichia coli*), mammalian cell systems, and in vivo [27]. However, the role of GTE in suppression of allergic/IgE responses in vitro has not been studied previously and the mechanism(s) are undefined. The present study in U266 B cells suggests that GTE acts directly on B cells, resulting in suppression of IgE production.

Several studies have tested the effects of natural plant derivatives in suppression of IgE production and treatment of atopic disease, because the plant derivatives are generally safe, affordable, and easily accessible. Kim et al [34] demonstrated suppression of IgE production by *Siegesbeckia glabrescens* whole plants in U266 cells in vitro. Preparations from the kiwifruit, *Actinidia arguta*, induced suppression of in vitro IgE production by U266 cells and in vivo IgE suppression in human subjects [35]. Two catechins isolated from Taiwanese oolong tea, (-)-epigallocatechin 3-O-(3-O-methyl)gallate and (-)-epigallocatechin 3-O-(4-O-methyl)gallate, inhibited type I allergic reactions in mice sensitized with ovalbumin and Freund's incomplete adjuvant [36]. The efficacy of various plant extracts in inhibiting IgE production suggests a general suppressive effect of plant-derived catechins.

In contrast, other investigators have reported that green tea or its components can be deleterious or induce clinical asthma [37-39]. In those studies, the asthma was reported in individuals who worked in green tea factories. In such cases, occupational exposure to components involved in the processing of green tea might conceivably cause hyperresponsiveness to green tea or its components, which would not be found in the general population. Future studies will examine whether individual catechins and other plant extracts cause suppression of IgE production in vivo.

The present study of GTE effects in an IgE producing cell line may have limited physiological relevance and further studies are necessary to test GTE suppression of IgE production in vivo in animals and humans. Nevertheless, the present study demonstrates suppression of IgE responses in vitro by single and repeated treatments with GTE; the suppression appears to be independent of green tea's anti-cancer properties and does not reflect altered cell viability and proliferation.

In this study, GTE did not cause increased apoptosis, necrosis, or changes of cell morphology, suggesting that suppression of IgE production by GTE is not mediated by apoptosis or cell death. In contrast, previous studies demonstrated specific killing of multiple myeloma cells by high doses of EGCG extracted from green tea [23,40]. These differences may be explained by (a) a lower concentration of specific catechins, or (b) the presence of multiple other catechins and bioactive ingredients that may interact with the effects of EGCG. Mechanisms by which tea polyphenols may possibly act include the inactivation of mutagens and carcinogens, modulation of DNA replication or repair, and inhibition of invasion and metastasis of tumor cells [27]. Ahmad et al [41,42] suggested that EGCG-caused cell cycle deregulation and apoptosis of cancer cells may be mediated through NF-kappaB inhibition. Most of these reports require further investigation [27]. Further, the effects of bioactive ingredients of green tea other than EGCG are not entirely known. The health effects associated with consumption of green tea reflect the biologic activities of all its polyphenols, rather than of a single component [43-45].

The mechanism of GTE suppression of IgE production is unknown. It may be that green tea

modulates U266 IgE production at the transcriptional or translational level by downregulation of key IgE potentiating genes, such as IL-6, STAT3, TLR-2, or BSAP/Pax5, as we have shown with other anti-inflammatory agents [46,47].

The present study used unseparated green tea extract, whereas other studies examined the cellular effects of separated ingredients from green tea, such as EGCG and ECG [1,6,10,23,40-42,48]. We elected not to use such products as there are numerous bioactive ingredients in green tea that may contribute to its potential therapeutic effects. Moreover, there may be additive or multiplicative interactions between various catechins and other molecules present in green tea. Thus, an unseparated extract is more analogous to the green tea beverages that are ubiquitously consumed in Asia and other parts of the world.

The concentrations of green tea extract used in this study merit discussion. Green tea polyphenols have low bioavailability in serum, with maximum serum levels of approximately 300 ng/ml even after multi-gram daily supplements [16,17]. In the present in vitro study, we selected a range of 1-300 ng/ml concentrations, which is comparable to achievable serum concentrations in vivo. We therefore believe that green tea suppression of IgE production may be achievable in the clinical setting. However, it seems likely that metabolism and binding proteins could account for differential effects of green tea and its bioactive components when ingested in vivo.

In conclusion, GTE suppression of IgE production in vitro is not associated with cell apoptosis or necrosis. The GTE concentrations used in this study are likely to be physiologically achievable, suggesting that GTE suppression of IgE production may also be demonstrated clinically. However, in vivo confirmation of these findings in animals and humans is needed, and the mechanism by which GTE suppresses IgE responses remains to be elucidated. The model system used in the present study may be used to identify the mechanism of green tea suppression of IgE production. Clinical studies of green tea supplements are warranted to test their therapeutic efficacy in allergies, asthma, and atopic dermatitis.

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