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Identification of an Inhibitor for Interleukin 4-Induced ε Germline Transcription and Antigen-Specific IgE Production *in Vivo*

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IgE plays a key role in the pathogenesis of allergic disease. Interleukin (IL) 4 is a potent and critical stimulator of immunoglobulin class switching from IgM to IgE in B cells. IL-4 induces the expression of ε germline transcript (εGT), which is critical to initiate IgE production. While searching for molecules that inhibit εGT expression induced by IL-4, we found that polyphenol strictinin, which was isolated from tea leaves, was able to inhibit the IL-4-induced εGT expression in the human B cell line DND39. Strictinin also acted on human peripheral blood mononuclear cells obtained from healthy donors to inhibit IL-4-induced εGT expression. Strictinin demonstrated similar inhibitory activity in peripheral blood mononuclear cells obtained from atopic donors. Interestingly, strictinin decreased ovalbumin-induced IgE production in mice, whereas the production of IgG and IgM was not affected. Furthermore, we found that the IL-4-induced STAT6 tyrosine phosphorylation, which is essential for IL-4-induced εGT expression, was inhibited in DND39 cells upon treatment with strictinin. Taken together, these results suggest that strictinin can inhibit IgE production through the inhibition of IL-4-mediated signaling in B cells.

Key Words: **allergy; IgE; IL-4; strictinin; germline transcription; STAT6.**

Immunoglobulin (Ig) E is believed to be one of the major mediators of immediate hypersensitivity reactions that underlie atopic conditions such as urticaria and eczema, seasonal allergy, food allergy, asthma, and anaphylaxis (1). In many individuals, total serum IgE level correlates roughly with the severity of the disease (2).

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The Ig heavy chain gene segments encodes constant regions which rearrange by class switch recombination resulting in the production of one of other isotypes (3, 4). Induction of isotype switching to a particular heavy chain constant region correlates with the transcriptional activation of that particular gene in germline configuration (5). Induction of germline transcripts is necessary to target a switch region for recombination and switching. In the case of IgE class switching, Interleukin (IL) 4 activates the ε germline promoter, which leads to the expression of ε germline transcription (εGT) through the tyrosine phosphorylation pathway to activate the signal transducers and activators of transcription (STAT) 6 molecule (6, 7). Transgenic mice deficient in STAT6 fail to produce IgE in response to extracellular pathogens (8). These findings strongly suggest that the expression of εGT is essential for the IgE production, and the inhibitors of εGT expression can suppress the initiation of IgE synthesis by inhibiting IgE class-switch recombination (9).

Tea (*Camellia sinensis*), one of today's most popular beverages, contains various substances. A number of studies have shown that tea has a wide range of biological effects such as anticarcinogenesis and antioxidation (10, 11). Recently, novel catechin derivatives with potent antiallergic activity have been found in tea leaves (12, 13). Therefore we focused on tea leaves as a promising source for effective antiallergic agents. To search for molecules that are able to suppress IgE synthesis, we examined various substances purified from tea for their effect on IL-4-mediated εGT expression. In this paper, we report the identification of an IgE production-inhibitory molecule found in tea leaves. Strictinin (14, 15), a polyphenol in tea, effectively inhibited IL-4-mediated εGT expression and antigen-specific IgE production *in vivo.*

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MATERIALS AND METHODS

Preparation of tea extract. Tea leaves were obtained from the National Research Institute of Vegetables, Ornamental Plants, and Tea in Shizuoka, Japan. Freshly picked tea leaves were dried and stored in a refrigerator before analysis. The extraction and isolation of tea substances were performed according to the method previously reported (16).

Cells and cell culture. The human EBV-negative Burkitt's lymphoma cell line DND39 cells (17) were maintained at 37°C with 5% CO_2 in RPMI 1640 medium supplemented with 5% fetal calf serum (Intergen, Purchase, NY), 100 U/ml penicillin G, 100 mg/ml streptomycin, and 10 mM Hepes buffer. The peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral blood of healthy donors or allergic (atopic dermatitis) patients. After centrifugation over Ficoll-Hypaque, the cells were suspended in RPMI 1640 medium containing 10% fetal calf serum. To assess εGT expression, DND39 cells or PBMC were cultured at a final density of 1×10^6 cells/ml in RPMI 1640 medium containing 5 or 10% fetal calf serum for 48 h with the following reagents: IL-4 (R&D Systems Inc.) at 25 U/ml, TGF-β (R&D Systems Inc.) at 2 ng/ml. For the phosphorylation assay, DND39 cells were treated with strictinin (0, 25, 50, and 100 μM) in the presence of IL-4 (250 U/ml) for 30 min.

Modulation of ovalbumin-induced IgE production in mice treated with strictinin. Female 6-week-old C57BL/6J mice (Seiwa Experimental Animals, Japan) were injected intraperitoneally with 10 μg ovalbumin (Sigma; grade IV) and 1 mg aluminum hydroxide (LSL Inc., Japan) to favor specific IgE response (18). Mice were administered 200 μl of water containing (+) or not containing (−) 0.5 mg strictinin every 2 days for 8 days. At 2 months after immunization, levels of ovalbumin-specific IgE, IgM, and IgG were measured in sera by ELISA. Specific IgE, IgM, and IgG antibody were quantified using the horseradish peroxidase-conjugated antibody. Sera samples were diluted at 1:18 for specific IgE, 1:4500 for specific IgM, and IgG.

Primers. Primer sequences used in this experiment are as follows: PεGT-F, 5′-AGGCTCCACTGCCCGGCACAGAAAT-3′; PεGT-R, 5′-ACGGAGGTGGCATTGGAGGGAATGT-3′; PGAPD-F, 5′-GCTCAGACACCATGGGGAAGGT-3′; PGAPD-R, 5′-GTGGTGCAGGAGGCATTGCTGA-3′.

Analysis of εGT expression. Total RNA was isolated from the cultured cells using Trizol reagent (GIBCO/BRL) according to the manufacturer's instructions. Ten micrograms of the total RNA was reverse transcribed to cDNA using 20 units of MMLV-reverse transcriptase (Amersham, UK) and 0.5 μg of $(dT)_{20}$ primer in a reaction volume of 20 μl. When synthesizing cDNA for detection of εGT, 1 μM of the PεGT-R which is an anti-sense direction primer for εGT, was used for reverse transcription. RT-PCR was performed in a 10 μl reaction volume using 0.5 unit of AmpliTaq Gold DNA polymerase (Perkin-Elmer Cetus), 1 mM concentration of each deoxynucleotides (Amersham, UK), 1 μM of the appropriate primer (PεGT-F and PεGT-R for εGT, or PGAPD-F and PGAPD-R for GAPDH), and 0.5 or 0.05 μg of total RNA-equivalent cDNA. Samples were predenatured at 95°C for 9 min followed by amplification at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s for 13 cycles (εGT) or 10 cycles (GAPDH), followed by a final 7 min extension step at 72°C. The amplified PCR products were subjected to electrophoresis on a 1.5% agarose gel, transferred to a Hybond-N^+ membrane (Amersham) followed by hybridization using Gene Images (Amersham) according to the manufacturer's instructions. Specific probes for εGT and GAPDH genes were made by labeling the following DNA: εGT, 5′-AGCTGTCCAGGAACCCGACAGGGAG-3′; GAPDH, PCR-amplified GAPDH genes (19).

Immunoprecipitation and Western blotting. Cells were lysed in lysis buffer (2×10^7 cells/ml) containing 10 mM Tris–HCl (pH 7.6), 50 mM NaCl, 200 mM sodium orthovanadate, 0.5% Triton-X 100, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml aprotinin. The cell extracts were passed once through a protein A-Sepharose (Pharmacia Biotech Inc.). After the addition of anti STAT6 antibodies (Santa Cruz), the reactions were then allowed to continue for 4 h at 4°C. To remove the antibody-antigen complexes, prewashed protein A-Sepharose was added to the reaction. After incubation for 1 h, the complexes were removed by centrifugation and washed extensively with buffer containing 0.5% NP-40, 25 mM Tris–HCl (pH 7.6), and 150 mM NaCl. Samples were prepared by the addition of an equal volume of 1 × SDS–PAGE buffer containing 2% 2-mercaptoethanol to the washed resin and boiling for 5 min before electrophoresis through 8% SDS–PAGE. For Western blot analysis, the immunoprecipitated proteins were subjected to SDS–PAGE and electrotransferred onto nitrocellulose membranes (Schleicher & Schuell). Antibody to phosphotyrosine (PY20) (Santa Cruz) or STAT6 was added at optimal concentrations and the blots were incubated for 1 h at room temperature. The immunoreactive proteins were visualized using horseradish peroxidase-conjugated goat anti-mouse IgG (Zymed) and the chemiluminescence reagent (ECL) (Amersham). Stripping of nitrocellulose membranes was performed as described by the manufacturer (Amersham).

RESULTS

Identification of an Inhibitor for IL-4-Induced εGT Expression in Tea

Selective inhibition of IgE by B cells would have great therapeutic potential. Therefore, we tried to identify inhibitory molecules for IL-4-induced εGT expression, which is critical for initiation of IgE production. For screening, we used the human B cell line DND39, which has been shown to express εGT upon IL-4 stimulation (17). DND39 cells were treated with IL-4 (25 U/ml) and/or tea samples (10 μg/ml) for 48 h, and then assessed for levels of εGT by quantitative polymerase chain reaction after first being reversed transcribed. The extract from green tea was fractionated into five separate fractions by successive extraction with the following organic solvents: methanol (fraction I), chloroform (fraction II), ethylacetate (fraction III), *n*-butanol (fraction IV), and water (fraction V) (Fig. 1A). These fractions were then assessed for the inhibitory effect on IL-4-mediated εGT expression in the DND39 cells (Fig. 1B). The ethylacetate fraction (polyphenol-rich) was found to have a significant suppressive effect (Fig. 1B, lane 7). We therefore proceeded to purify the ethylacetate fraction. Various tea substances (10 μg/ml) obtained from the ethylacetate fraction were tested, and at least two substances were found to be able to reduce the IL-4-induced εGT expression. A representative result is shown in Fig. 2A. One of the substances (Fig. 2A, lane 4) was determined to be strictinin (1-*O*-galloyl-4,6-(−)-hexahydroxydiphenoyl-β-D-glucose) (14, 15) by proton nuclear magnetic spectrometry and carbon-13 nuclear magnetic spectrometry analysis and the structure of strictinin is shown in Fig. 2B.

Strictinin Inhibits IL-4-Induced εGT Expression in DND39 Cells

DND39 cells were treated with IL-4 (25 U/ml) with or without strictinin (1, 10, and 25 μM) for 48 h, and

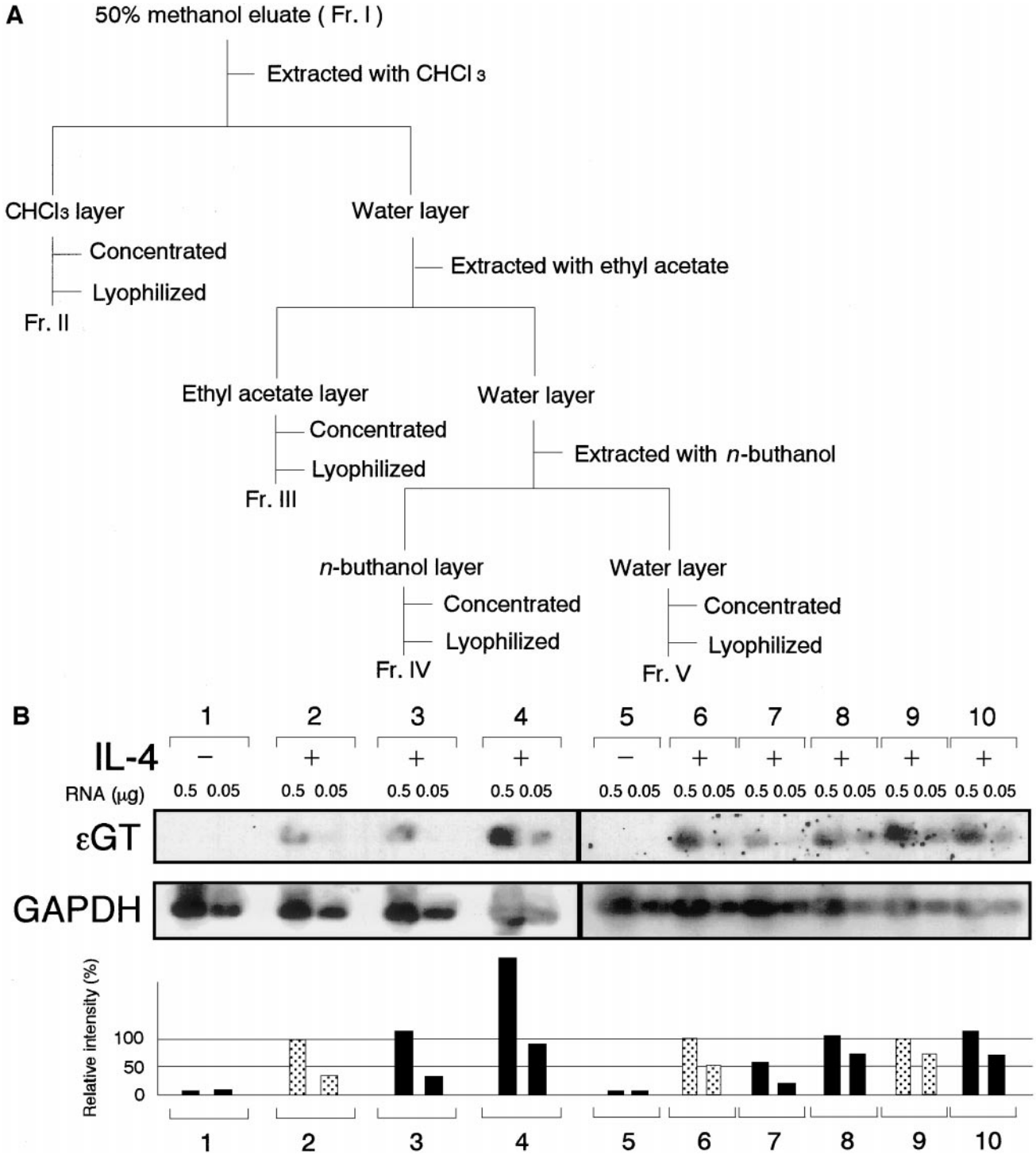

FIG. 1. Screening a tea extract fraction to detect inhibitory activity for IL-4-induced εGT expression. (A) Flow chart describing how the extraction of tea leaves was performed. (B) The human B cell line DND39 was treated with the each extract fraction (10 μg/ml) in the presence of IL-4 (25 U/ml) for 48 h, and then assessed for levels of εGT by quantitative reverse transcriptase (RT)-PCR followed by Southern hybridization. Dilutions of 0.5 or 0.05 μg of total RNA-equivalent specific primer-primed cDNA were used as templates for the PCR amplification. GAPDH gene amplification followed by Southern hybridization was simultaneously performed as a gel loading control. The amount of the εGT from DND39 cells treated with IL-4 only (columns 2, 6, and 9; 0.5 μg RNA) was used as the 100% standard for columns 3 and 4 (Fr. I and II), columns 7 and 8 (Fr. III and IV), and column 10 (Fr. V), respectively.

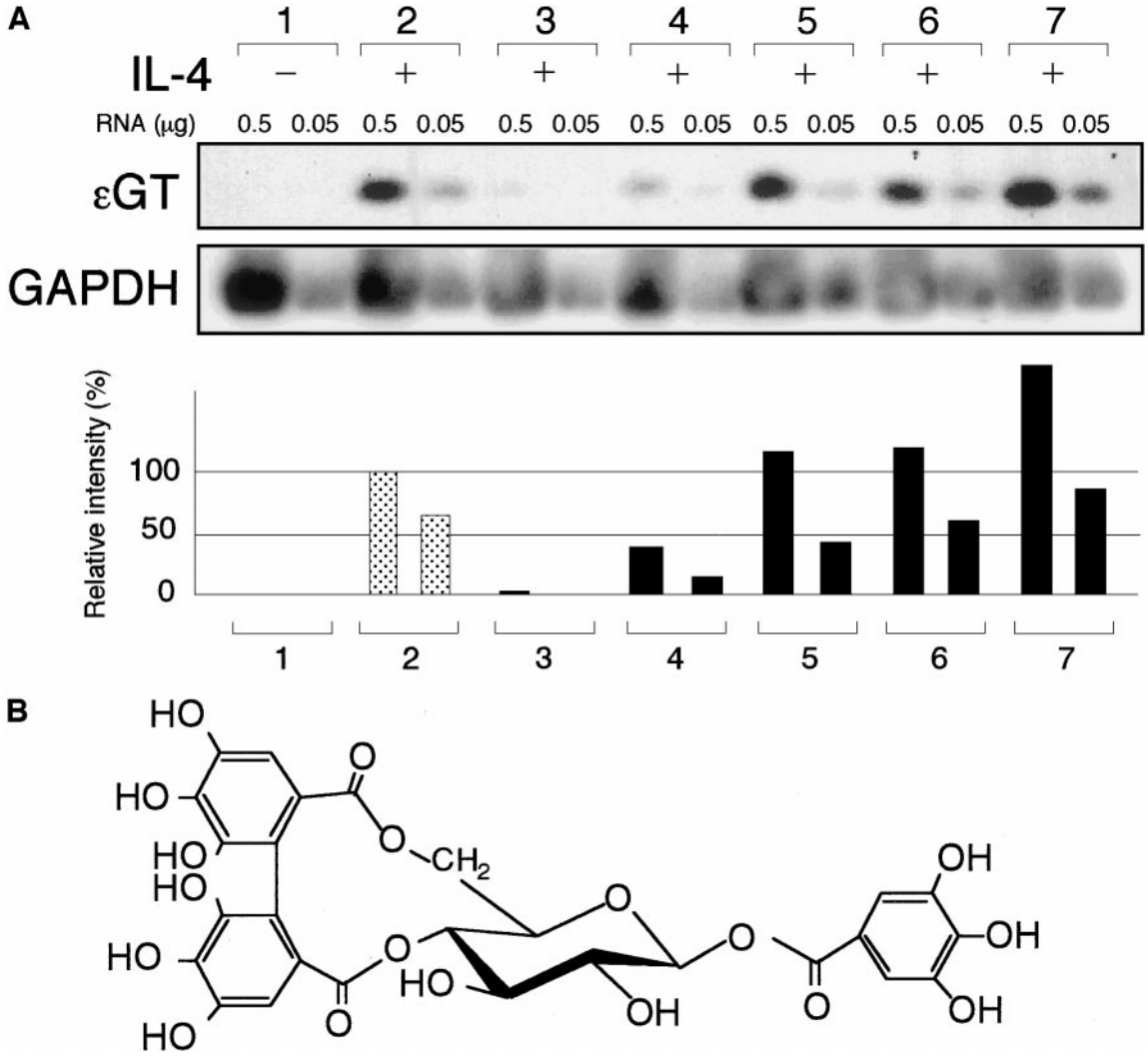

FIG. 2. Identification of an inhibitor for εGT expression. (A) DND39 cells were treated with or without substances (each 10 μg/ml) isolated from the ethylacetate fraction (fraction III) shown in Fig. 1B in the presence of IL-4 (25 U/ml) for 48 h. Evaluation of the production levels of εGT by quantitative RT-PCR was done as described in Fig. 1. GAPDH gene amplification was simultaneously performed as a gel loading control. TGF-β (2 ng/ml), which is known to down-regulate εGT expression, was used as a positive suppressor control (lane 3). The amount of εGT produced in DND39 cells stimulated with IL-4 only (column 2, 0.5 μg RNA) was used as the 100% standard. (B) The structure of strictinin.

then assessed for εGT expression (Fig. 3). TGF-β, which is known to down-regulate εGT expression in the DND39 cells (17), was used as the positive suppressor control. TGF-β (2 ng/ml) completely inhibited the IL-4-induced εGT expression as previously shown (17). Strictinin dose-dependently inhibited IL-4-induced εGT expression. No cytotoxicity of strictinin was seen at any of the concentrations tested.

The Effect of Strictinin on εGT Expression in Human Peripheral Blood Mononuclear Cells

Based on the observation that strictinin was able to suppress IL-4-induced εGT expression in DND39 cells, we tested the effect of strictinin on the expression of εGT in human peripheral blood mononuclear cells obtained from either healthy or atopic donors. A representative result for 25 μM of strictinin is shown in Fig. 4. We found that strictinin inhibited IL-4-induced εGT expression in the cells from healthy donors. Interestingly, cells isolated from atopic donors were able to express εGT without additional IL-4 stimulation. In the presence of IL-4, strictinin was also able to inhibit εGT expression. Thus, one can speculate that strictinin may have therapeutic applications on the inhibition of IgE production *in vivo*.

Strictinin Inhibits Antigen-Specific IgE Production in Vivo

The *in vitro* findings were extended to the *in vivo* mouse model (Fig. 5). Mice were first treated with

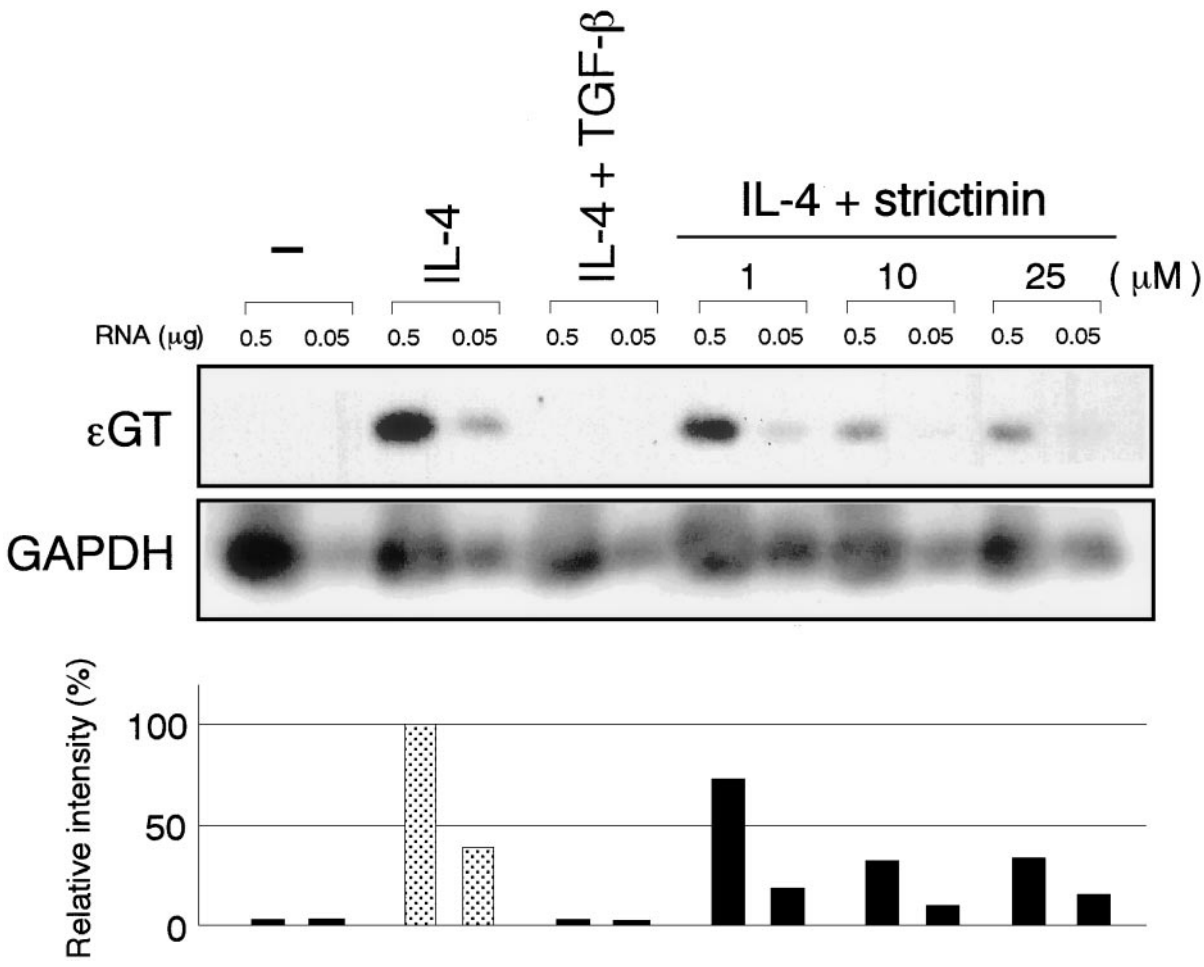

FIG. 3. Inhibition of IL-4-induced εGT expression in the human B cell line DND39 by strictinin. DND39 cells were treated with or without strictinin (1, 10, and 25 μM) in the presence of IL-4 (25 U/ml) for 48 h, and then assessed for levels of εGT as described in Fig. 1. GAPDH gene amplification was simultaneously performed as a gel loading control. TGF-β (2 ng/ml) was used as a positive suppressor control. The amount of the εGT produced in DND39 cells stimulated with IL-4 only (0.5 μg RNA) was used as a 100% standard.

ovalbumin to induce an IgE response and were then administrated 200 μl of water containing or not containing 0.5 mg strictinin every 2 days for 8 days. The amount of ovalbumin-specific IgE was shown to be reduced for the strictinin administered mice as compared with the water only control mice. Moreover, the amount of other allergen-specific Ig isotypes (IgG and IgM) was not significantly affected. These results suggest that *in vivo* strictinin selectively down-regulates the production of antigen-specific IgE antibody, which is the isotype regulated by IL-4.

Strictinin Inhibits IL-4-Induced STAT6 Tyrosine Phosphorylation

To examine the mechanism responsible for the suppression of IL-4-induced εGT expression by strictinin, DND39 cells cultured in the presence of IL-4 were treated with strictinin in a range of 25–100 μM. STAT6 was immunoprecipitated, and assayed by Western blot analysis for tyrosine phosphorylation (Fig. 6). No tyrosine phosphorylated STAT6 was observed in the unstimulated control, and phosphorylation occurred when exogenous IL-4 was added. However, STAT6 tyrosine phosphorylation was shown to be reduced in strictinin-treated cells in a dose-dependent manner. Reblotting of the same blot membrane for STAT6 showed that changes in tyrosine phosphorylation were not due to a loss of protein expression. This observation suggest that strictinin inhibits STAT6 tyrosine phosphorylation, which in turn blocks εGT expression and the subsequent IgE class switching necessary for driving IgE production.

DISCUSSION

IgE plays a key role in the pathogenesis of allergic disease. IL-4 is critical for the synthesis of IgE in B cells. Inhibition of IL-4 may therefore be effective in

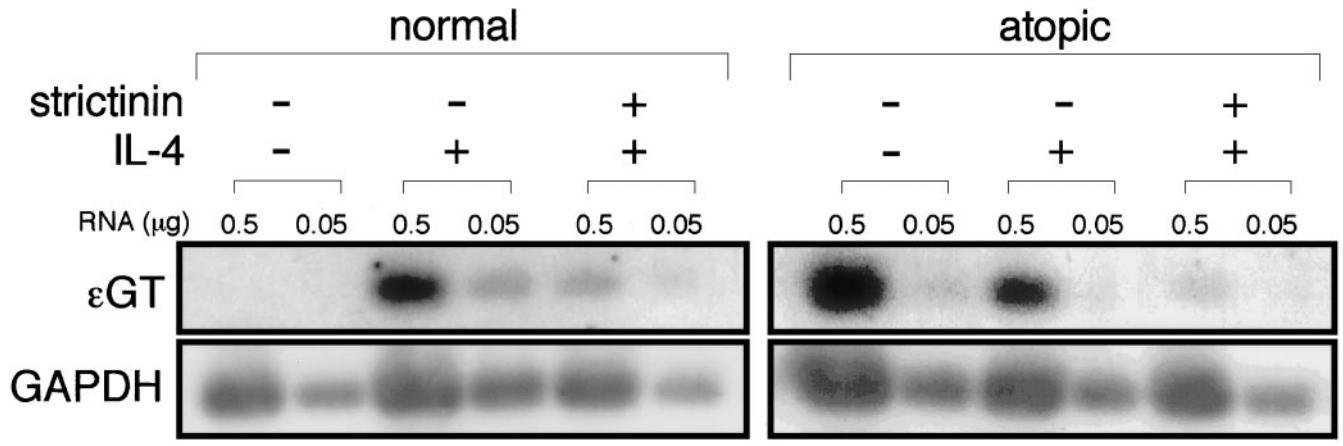

FIG. 4. Strictinin inhibits IL-4-induced εGT expression in human peripheral blood mononuclear cells. The peripheral blood mononuclear cells from either healthy or atopic donors were treated with IL-4 (25 U/ml) with or without strictinin (25 μM) for 48 h, and then assessed for levels of εGT as described in Fig. 1. GAPDH gene amplification was simultaneously performed as a gel loading control.

inhibiting allergic diseases. In the current study, we identified strictinin as a inhibitory molecule that inhibits IL-4-induced εGT expression and IgE production.

Strictinin is a member of the broad class of hydrolyzable vegetable extracts known as ellagitannins (20). The ellagitannins, the structures of which bear at least one axially chiral diphenic acid unit, are found in plants and are reported to have important biological activities such as anti-HIV, antitumor, anti-topoisomerase activity (21–24). However, no information in currently available about the effects of not only strictinin but also other ellagitannins on immunoglobulin synthesis as well as the immune system *in vivo*. To our knowledge, this is a first report that demonstrates the effects of ellagitannins on IL-4-mediated signaling and IgE production.

Tea leaves contain many polyphenols known as catechins, including epicatechin gallate and epigallocatechin gallate (25). Strictinin, like the two catechins, have one galloyl group. Surface plasmon resonance analysis revealed that strictinin and the two catechins can bind to the cell surface of DND39 cells (unpublished result). However, these catechins were shown to not be able to suppress the IL-4-induced εGT expression (data not shown). The defining structural feature of strictinin that is different from the two catechins is a hexahydroxydiphenoyl moiety. Whether or not other substances possessing the hexahydroxydiphenoyl moiety similar to strictinin can suppress IgE production through inhibiting IL-4-induced εGT expression is an interesting possibility that needs to be explored in future studies.

We have shown that strictinin is able to inhibit IL-4-induced STAT6 tyrosine phosphorylation. When STAT6 is phosphorylated upon IL-4 treatment, the phosphorylated form of STAT6 translocates to the nucleus where it can activate the transcription of IL-4-responsive genes (26, 27). Therefore, the inhibition of IL-4-induced εGT expression by strictinin may be due to the inhibition of STAT6 tyrosine phosphorylation. Several mechanisms by where STAT6 tyrosine phosphorylation is affected have been previously shown (28). Further experiments are necessary to determine the exact target point of strictinin in the STAT6 tyrosine phosphorylation pathway which leads to IgE production in B cells.

In summary, our data show that the strong suppression of IL-4-induced εGT expression as well as allergen-specific IgE production is caused by the addition of strictinin, and that the inhibition of εGT expression may occur through inhibition of STAT6 tyrosine phosphorylation. These findings also highlight the therapeutic potential of strictinin and its analogues as

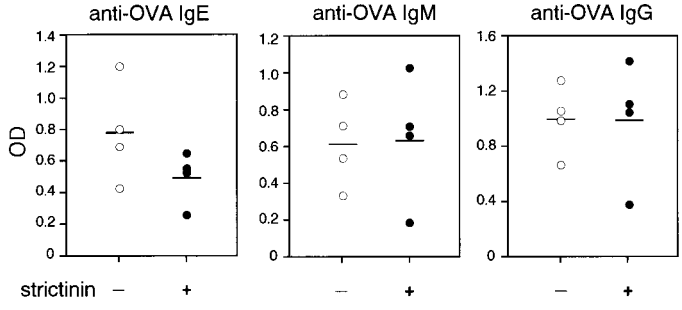

FIG. 5. Oral administration of strictinin decreases specific IgE antibody response in ovalbumin-immunized mice. C57BL/6J mice injected intraperitoneally with ovalbumin were administrated 200 μl of water containing (+) or not containing (−) 0.5 mg strictinin every 2 days for 8 days. Two months after immunization, the amount of ovalbumin-specific IgE, IgM, and IgG in the sera was measured. Results are expressed in OD values. Results from one set of experiments with four mice per group are presented.

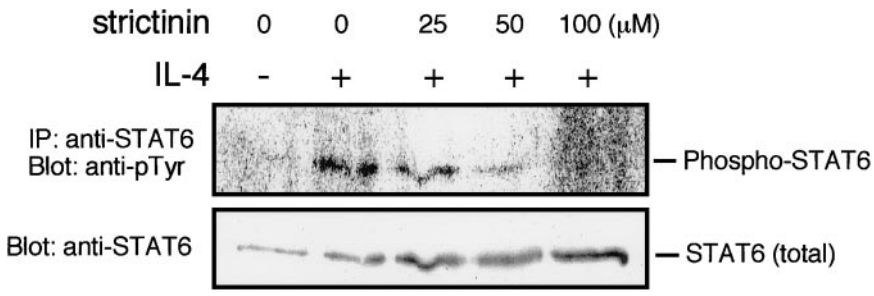

FIG. 6. Strictinin inhibits tyrosine phosphorylation of STAT6. DND39 cells were treated with strictinin (0, 25, 50, and 100 μM) in the presence of IL-4 (250 U/ml) for 30 min as indicated. STAT6 was immunoprecipitated, separated on a 8% SDS–PAGE, and immunoblotted with the anti-phosphotyrosine antibody. Shown in the lower panel are protein levels from the same filter blotted again with the anti-STAT6 antibody.

potential pharmaceutical agents that may be useful to inhibit the progression of IgE-mediated disease such as food allergy.

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