

Plant chitinase III Ziz m 1 stimulates multiple cytokines, most predominantly interleukin-13, from peripheral blood mononuclear cells of latex-fruit allergic patients

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ABSTRACT

Background: Indian jujube is a fruit abundantly cultivated in Taiwan. Its major allergen in latex-fruit syndrome is Ziz m 1 of the chitinase III family. The Ziz m 1 *Pichia* (rZiz m 1-P) has chitinase activity but not Ziz m 1 *E. coli* (rZiz m 1-E).

Objective: This study examined whether plant chitinase III, using rZiz m 1-P and rZiz m 1-E, can stimulate allergic inflammation similar to that of mammalian chitinases.

Methods: Five patients allergic to latex-Indian jujube and five nonallergic controls were evaluated. Their peripheral blood mononuclear cells (PBMC) were cultured with rZiz m 1-E or rZiz m 1-P and pulsed with phorbol 12-myristate 13-acetate. Eleven cytokines were measured by FlowCytomix human Th1/Th2 plex kit and interleukin (IL)-13 by sandwich enzyme-linked immunosorbent assay (ELISA).

Results: Interleukin-13 significantly increased in rZiz m 1-P stimulated PBMC of allergic subjects but was undetectable when stimulated with rZiz m 1-E. The stimulation index significantly increased in IL-13 (380.6 ± 77.33 vs 13.70 ± 6.92), IL-5 (6.70 ± 0.59 vs 0.70 ± 0.37), IL-1 β (32.70 ± 0.83 vs 2.10 ± 1.29), and tumor necrosis factor beta (TNF- β) (17.10 ± 2.66 vs 1.50 ± 0.66) between allergic and nonallergic subjects after rZiz m 1-P stimulation. There was no difference in terms of IL-2, IFN- γ , IL-8, and TNF- α production.

Conclusions: The biological function of chitinase activity is required for Ziz m 1 to induce a Th2-specific immune response. This is the first report on PBMC responses of latex-fruit syndrome subjects toward an active exogenous plant class III chitinase that can stimulate multiple cytokines, especially IL-13, from allergic subjects. This implies the role of cross-reactive food allergens in propagating allergic inflammation among allergic subjects.

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Introduction

Indian jujube (*Zizyphus mauritiana*), also known as Chinese apple, is a sweet fruit commonly found in tropical and subtropical regions and widely grown in Southern China, Africa, India, Malaysia, Australia, and some Pacific countries. It is a very popular fruit in Taiwan. Ziz m 1 is the major Indian jujube allergen involved in latex-fruit syndrome.¹ Its complementary DNA (cDNA) has been cloned, sequenced, and expressed in yeast (rZiz m 1-P),² whereas the immunoglobulin E (IgE)-binding epitope of recombinant Ziz m

1 has been identified using the *Escherichia coli* expression system (rZiz m 1-E).³ The rZiz m 1-P possesses chitinase activity but rZiz m 1-E does not.³

Chitin is the second most abundant polysaccharide in nature after cellulose. It makes up the exoskeleton of lower organisms such as fungi, crustaceans, and insects. Recently, exogenous chitin has been shown to activate macrophages and other innate immune cells. It regulates innate and adaptive immune responses and modulates adaptive type 2 allergic inflammation.⁴⁻⁹ Chitinases, enzymes for digesting chitin, are found in a large variety of organisms, including bacteria, plants, insects, and fungi, as one of their defense proteins.¹⁰

More recently, mammals have been shown to contain such enzymes.^{11,12} Acidic mammalian chitinase is an important mediator of interleukin-13 (IL-13)-induced responses in T helper type 2 (Th2)-dominated asthma.^{13,14} Interleukin-13 is a cytokine secreted

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predominantly by activated Th2 cells that has been implicated in the pathogenesis of asthma in human and animal studies.¹⁵ However, whether plant chitinase is able to induce inflammation in humans has never been investigated.

This study aimed to examine whether plant chitinase III, using Ziz m1 as a model, can stimulate allergic inflammation like mammalian chitinases. To substantiate whether the biological function of chitinase activity is required, rZiz m 1-E was used as a control.

Methods

Subjects

Five subjects with allergies to latex and Indian jujube (P1–P5) and 5 healthy individuals without allergies (NA1–NA5) were recruited from the Allergy and Asthma Clinic of Taichung Veterans General Hospital. The Institutional Review Board of Taichung Veterans General Hospital approved the study, and all of the subjects provided written informed consent. All of them had been exposed occupationally to latex gloves. All subjects allergic to latex and Indian jujube had moderate to severe reactions to latex and Indian jujube whereas the controls had no symptoms and no skin test reactions to latex and Indian jujube extract. No statistical difference was found regarding age and sex between control and study groups.

Purification of recombinant Ziz m 1 from *Pichia pastoris* and *Escherichia coli* expression system

The cDNA coding Ziz m 1 was cloned into the pPIC9K vector (Invitrogen, Carlsbad, CA, USA) in frame with the α -factor secretion signal sequence. The transformation of the *P. pastoris* strain GS115 and extracellular expression were performed according to the instruction manual. The rZiz m 1-P was purified from culture supernatant by Resource Q anion-exchange column (Pharmacia, Uppsala, Sweden) as described previously.² The expression of Ziz m 1 in *E. coli* BL21(DE3) (rZiz m 1-E) was performed as described previously.³

Isolation and culture of peripheral blood mononuclear cells

Peripheral blood (30 mL) was obtained by venous puncture on sodium-heparin vacuum tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient (Pharmacia, Sweden), and interphase cells were washed three times with phosphate-buffered saline. The cells were resuspended at a density of 1×10^6 cells/mL in complete medium consisting of RPMI 1640 (Sigma Biochemical Co., St. Louis, Missouri) supplemented with 2 g/mL sodium bicarbonate, 2 mM L-glutamine, 5 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) buffer, 200 U/mL penicillin, 200 μ g/mL streptomycin, and 10% heat-inactivated human autologous serum. The PBMCs were cultured in a 96-well plate (Nunc, Roskilde, Denmark) with or without various concentration of rZiz m 1 (5–50 μ g/mL) for different lengths of time (2–6 days). Before harvesting at each time point, the cultures were pulsed with 50 ng/mL of phorbol 12-myristate 13-acetate for an additional 24 hours.

Cytokine measurement by ELISA

After stimulation, cell-free supernatants were collected by centrifugation at 800g for 5 minutes and assayed for IL-5, IL-13, and interferon-gamma (IFN- γ) with enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol (Bender MedSystems, Vienna, Austria). Interleukin-5, IL-13, and IFN- γ concentrations were determined by interpolation from a standard curve performed with purified human IL-5, IL-13, and IFN- γ . The lowest detection limit of IL-5, IL-13, and IFN- γ were 8, 3, and 16 pg/mL, respectively.

Cytokine measurements by fluorescent bead immunoassay

Culture supernatant was analyzed simultaneously for 11 different cytokines, namely IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IFN- γ , tumor necrosis factor (TNF)- α , and TNF- β , using the bead-based FlowCytomix (Bender MedSystems) as described previously.¹⁶ Briefly, 25 μ L of each analyte and various concentrations of each cytokine standard were added to 25 μ L of antibody-conjugated beads in a 96-well plate. After the final wash, the contents of each well were resuspended in 200 μ L assay buffer and placed in 12×75 -mm polystyrene tubes, and then read on a FACScan flow cytometer (BD Immunocytometry Systems, San Jose, California). The flow cytometer was equipped with a 488-nm laser capable of detecting and distinguishing fluorescence emissions at 575 nm and 685 nm. Cytokine concentrations were calculated by comparison with a standard curve for each cytokine derived from the cytokine standards assayed in the same manner.

RNA extraction and cDNA synthesis

Peripheral blood mononuclear cells were homogenized in Trizol reagent (Invitrogen) according to the manufacturer's instructions. The RNA concentration and purity was determined by measuring the absorbance at 260 and 280 nm in a NanoDrop spectrophotometer (Thermo Scientific, Delaware). Complementary DNA was synthesized from 1 μ g of total RNA in a 20- μ L reaction mixture containing 2.5 μ M oligo-dT primer, 1 mM deoxyribonucleoside triphosphates, 5 μ M dithiothreitol, 40 U RNaseOUT RNase inhibitor, and 15 U ThermoScript reverse transcriptase (Invitrogen, Carlsbad, California). The reaction was performed at 50°C for 60 minutes followed by a heat denaturation step at 85°C for 5 minutes.

Reverse transcription polymerase chain reaction

Expression of IL-5, IL-13, and IFN- γ messenger RNA (mRNA) were analyzed by conventional reverse transcription polymerase chain reaction (PCR). The reaction mixture contained 2 mM of $MgCl_2$, 200 μ M of deoxyribonucleoside triphosphates, 1.25 units of hot-start SuperTherm Gold DNA polymerase (Hoffman-La-Roche, Basel, Switzerland), 10 pmole of specific sense and anti-sense primers for each cytokine gene, and 100 ng of first-strand cDNA in a total volume of 50 μ L. After beginning by a single 95°C for 10 minutes preincubation step, PCRs were performed under the following conditions: denaturation, 94°C for 1 minute; annealing, 60°C for 1 minute; and extension for 1 minute at 72°C in a Perkin Elmer thermocycler. The number of cycles used was dependent on the transcript amplified. Beta-actin cDNA was amplified as an internal control.

Primer sequences were: IL-5, 5'-GCTTCTGCATTGAGTTT-GCTAGCT-3' (forward) and 5'-TGGCCGTCATGTATTCTTTAT-TAAG-3' (reverse); IL-13, 5'-CTCCTCAATCCTCTCCTGTT-3' (forward) and 5'-GTTGAACCGTCCCTCGCGAAA-3' (reverse); IFN- γ , 5'-TCCAGATGCAATCAATGCC-3' (forward) and 5'-CATGGGA-GCTAGAGGAGGAACG-3' (reverse); and β -actin, 5'-AAAGACCTG-TACGCCAACACAGTGC-3' (forward) and 5'-CCGGACTCGTCAT-ACTCTGCTTGC-3' (reverse). The PCR products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining followed by Kodak molecular imaging system (NY).

Statistical analysis

Data were presented as mean $\pm$ standard error of mean unless otherwise specified. Differences between means were analyzed using nonparametric Mann-Whitney *U* test, using SPSS 12.0 software (SPSS Inc., Chicago, Illinois). A *P* < .05 was considered statistically significant.

Table 1
Demographic, clinical and laboratory characteristics of the study subjects

No.	Age/ Sex	Occupation	Latex-Specific IgE CAP (kU/l)/SPT	rZiz m 1-P ELISA (OD)/SPT	Symptoms
P1	34/F	Nurse	27.4/4+	1.3/4+	AS, OAS
P2	31/F	Nurse	2.3/2+	3.8/2+	AS, AR
P3	39/F	Medical technician	20.2/4+	0.5/4+	AS, OAS
P4	45/M	Physician	12.9/3+	2.1/4+	AR, AC, A
P5	57/M	Physician	0.7/2+	3.1/3+	AR, AC, OAS
NA1	42/F	Physician	—/—	—	None
NA2	28/M	Lab technician	—/—	—	None
NA3	26/F	Lab technician	—/—	—	None
NA4	34/M	Lab technician	—/—	—	None
NA5	23/F	Lab technician	—/—	—	None

Abbreviations: SPT, skin prick test; ELISA, enzyme-linked immunosorbent assay; OD, optic density; AS, asthma; OAS, oral allergy syndrome; AR, allergic rhinitis; AC, allergic conjunctivitis; A, angioedema.

Results

Characteristics of the subjects

The demographic, clinical, and laboratory data of the five allergic patients and five nonallergic controls are shown in Table 1. All of the patients showed positive SPT reactions and increased specific IgE antibodies to latex by ImmunoCAP (Phadia, Uppsala, Sweden) and to rZiz m 1-P in-house ELISA, respectively. The five nonallergic controls had negative SPT reaction and no specific IgE antibodies against latex and rZiz m 1.

rZiz m 1-P-induced IL-13 protein secretion but not rZiz m 1-E

In this experiment, rZiz m 1-P and rZiz m 1-E were used to stimulate PBMCs from latex-Indian jujube-allergic patients or normal subjects. Interleukin-13 and IFN- γ protein secretions were then measured. Cytokine production was measured by ELISA in culture supernatants harvested on days 3, 4, 5, 6, and 7. Cytokine secretion in culture supernatants significantly increased and reached its highest level on day 7 for IL-13 after stimulation with rZiz m 1-P (Fig 1A). However, the level of IL-13 in rZiz m 1-E-stimulated cells was undetectable in both groups (Fig 1A).

The rZiz m 1-P also induced more IFN- γ proteins from allergic subjects compared with rZiz m 1-E on all time points (Fig 1B). Interestingly, rZiz m 1-P and rZiz m 1-E induced similar amounts of IFN- γ proteins from nonallergic subjects. Such results suggested that IL-13 production but not IFN- γ required chitinase activity.

IL-5, IL-13, and IFN- γ mRNA expression by rZiz m 1-P-stimulated PBMCs

RNA was isolated from PBMC cells stimulated with rZiz m 1-P for 5 days in the presence of phorbol 12-myristate 13-acetate and analyzed by reverse transcription PCR. Interleukin-5 and IL-13

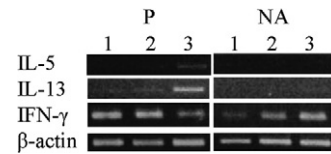


Figure 2. Reverse transcription PCR analysis of IL-5, IL-13, IFN- γ , and β -actin mRNA expression in PBMCs from a latex-Ziz m 1-allergic subject (P) and a nonallergic control (NA). PBMCs were cultured with medium (lane 1), Con A (lane 2), or rZiz m 1-P (lane 3) for 5 days and restimulated with PMA for 24 h.

mRNA expression were up-regulated by rZiz m 1-P stimulation in PBMC from patients compared with medium control (Fig 2). In contrast, no significant IL-5 and IL-13 mRNA expression was seen in PBMCs from nonallergic subject. The PBMCs from allergic patients expressed significantly lower amounts of the Th1 cytokine IFN- γ after rZiz m 1-P stimulation compared with medium control, whereas IFN- γ mRNA expression in nonallergic subjects was up-regulated.

Cytokine Profiles of rZiz m 1-P-stimulated PBMCs

Eleven cytokines were measured by FlowCytomix human Th1/Th2 plex kit (Bender) and IL-13 by sandwich ELISA. The results were represented as stimulation index to correct baseline differences between individuals. The stimulation index of cytokines was calculated as a ratio of the cytokine levels after rZiz m 1-P stimulation divided by the basal levels from unstimulated cells. The rZiz m 1-P stimulation significantly increased the production of the Th2-associated cytokines IL-5 ($P < .0001$) and IL-13 ($P = .004$), as well as IL-1 β ($P = .024$) and TNF- β ($P = .002$) in allergic individuals compared with nonallergic subjects (Fig 3). No difference was seen between groups in the production of Th1 cytokines IL-2 and IFN- γ and IL-8 and TNF- α . Production of IL-6 and IL-10 by rZiz m 1-P-stimulated PBMCs from the allergic group were higher than those of control subjects, but without statistical significance. Interleukin-4 and IL-12p70 were not detected in this study.

Discussion

Ziz m 1 is the major Indian jujube allergen involved in latex-fruit syndrome. A previous study showed that Ziz m 1 is a plant class III chitinase.² The current study is the first to report on PBMC responses of latex-fruit syndrome patients toward an active exogenous chitinase, Ziz m 1. The data show a distinct pattern of cytokine production from PBMCs of latex-fruit-allergic patients after stimulation with rZiz m 1-P. Compared with controls, latex-fruit-allergic patients have significantly increased production of Th2 cytokines, IL-13, IL-5, and IL-10, and pro-inflammatory cytokines, IL-1 β , IL-6, and TNF- β , on stimulation with rZiz m 1-P, the recombinant allergen that possesses chitinase activity. Nonetheless, rZiz m 1-E,

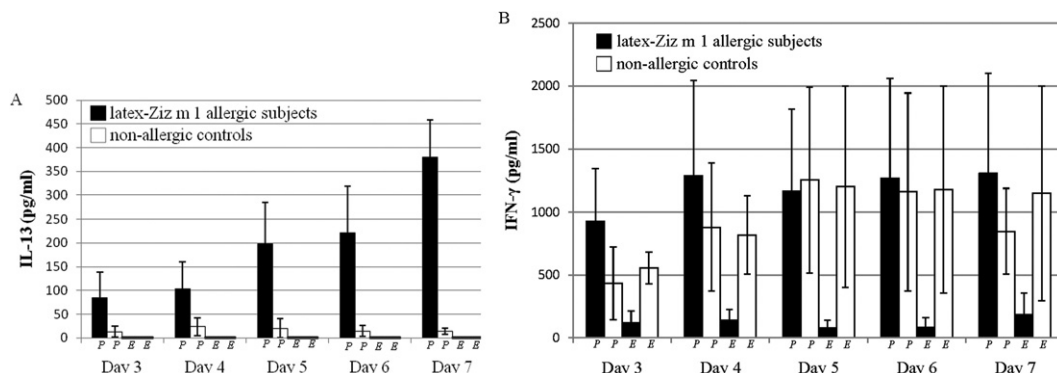


Figure 1. Kinetics of (A) IL-13 and (B) IFN- γ production by rZiz m 1-P (P) and rZiz m 1-E (E) stimulated PBMC from five allergic subjects and five nonallergic controls. Cytokine levels were assessed by ELISA in culture supernatants harvested on days 3, 4, 5, 6, and 7. Data were expressed as mean $\pm$ SEM of 5 independent experiments.

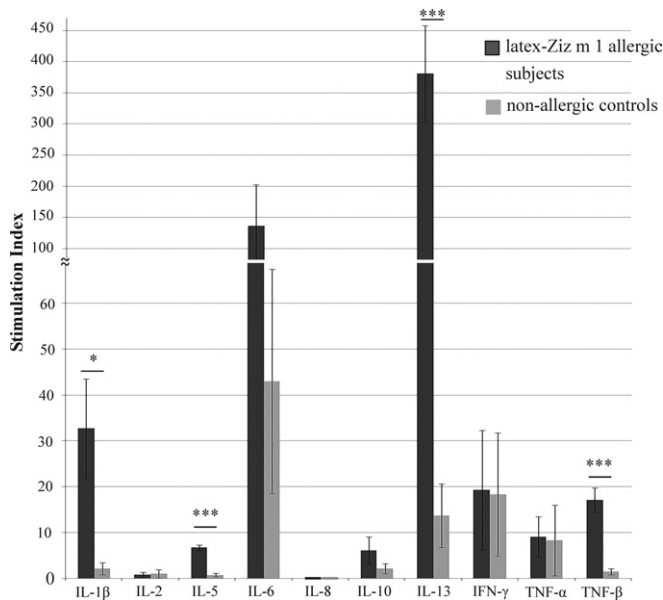


Figure 3. Stimulation index of cytokines from rZiz m 1-*P*-stimulated PBMCs. The PBMCs from nonallergic ($n = 5$) or latex-Ziz m 1 allergic ($n = 5$) subjects were cultured with rZiz m 1 (5 $\mu\text{g/mL}$) for 7 days. Cytokines levels in the supernatant were measured by FlowCytomix assay except IL-13, which was measured by sandwich ELISA. Data were shown as mean of stimulation index $\pm$ SEM and analyzed by Mann-Whitney *U* test. * $P < .05$; *** $P < .001$.

which has no chitinase activity, is unable to induce IL-13 from these subjects. Thus, the biological function of chitinase activity is required for Ziz m 1 to induce Th2-specific cytokine response in PBMC from latex-fruit-allergic patients.

T-helper type 2 cells propagate allergic inflammation by secreting various cytokines, including IL-5, IL-13, and IL-4. Interleukin-5 induces the differentiation of eosinophils and may be responsible for the tissue damage observed in chronic asthma. Interleukin-13 is thought to be one of the central mediators of allergic asthma.¹⁷ It shares many of the properties of IL-4^{18,19} and reportedly directs naïve human B cells to switch to IgG4 and IgE production²⁰ and induces the pathophysiologic features of asthma independent of classical IgE and eosinophil mechanisms.¹⁷

Acidic mammalian chitinase is induced during Th2 inflammation through an IL-13-dependent mechanism in mice and human subjects.^{13,14} The presence of chitinase-like proteins in the lungs and in circulation correlates with the severity of asthma.²¹ Of particular interest, the cytokine most robustly stimulated by rZiz m 1-*P* is IL-13. By inducing IL-13, Ziz m 1, a fruit allergen possessing chitinase activity, may aggravate allergic inflammation in latex-fruit-allergic patients in addition to IgE cross-reactivity.

Interleukin-10 is an anti-inflammatory cytokine produced by regulatory T cells. It inhibits the activation and cytokine production of both Th1 and Th2 cells.²² Latex-fruit-allergic subjects in this study secreted more IL-10 than nonallergic subjects, although the difference did not reach significance. Allergen extracts reportedly induce IL-10 from activated human B cells²³ to modulate IgE production. However, the ability of an allergen to produce IL-10 differs among allergens.²³ In the current study, Ziz m 1 may not be a good IL-10 stimulator compared with what has been reported from dog allergen.²³

Interleukin-1 β is a pro-inflammatory cytokine thought to be involved in the pathogenesis of allergic asthma²⁴ and cutaneous allergic reactions.²⁵ The expression of IL-1 β is closely controlled in healthy tissue, and it has been postulated to represent an important central cytokine in the inflammatory diseases.²⁶ In the current study, the protein level of IL-1 β also shows significant difference between the patient and control groups after rZiz m 1 stimulation ($P < .05$).

In conclusion, this is the first report on PBMC responses of latex-fruit-allergic subjects toward an active exogenous plant class III chitinase, Ziz m 1. The Th2 predominant cytokine production from PBMCs of allergic patients occurs after rZiz m 1 stimulation. Of particular interest, the cytokine most robustly stimulated is IL-13, which is similar to reports on mammalian chitinase.^{13,14} Plant chitinase III can stimulate multiple cytokines, especially IL-13, from latex-Indian jujube allergic subjects. This implies the role of cross-reactive food allergens in propagating allergic inflammation among allergic subjects.

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