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Rational Design of Hypoallergenic Vaccines: Blocking IgE-binding to Polcalcin Using Allergen-specific IgG Antibodies

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ABSTRACT

Chenopodium album polcalcin (Che a 3) is characterized as a major cause of cross-reactivity inallergic patients to the Chenopodiaceae family. Therefore, the present study was conducted to develop a hypoallergenic Che a 3 derivatives as the candidate vaccine for type 1 allergy.

Four derivatives were generated from Che a 3. The first was a mosaic peptide derivative computationally identified in Che a 3 which was coupled to keyhole limpet hemocyanin (KLH). The second one was a mutant Che a 3, and the other two derivatives included N- and C-terminal halves of Che a 3 that both coupled to KLH. The IgE-binding capacity of Che a 3 and its derivatives and also their ability to induce there combinant Che a 3 (rChe a 3)-specific IgG antibody, were determined using the enzyme-linked immune sorbent assay (ELISA). Moreover, the lymphopro liferative capacity of rChe a 3 or its derivatives and their pro-inflammatory cytokine response interleukin (IL)-5 and IL-13 were measured in the human peripheral blood mononuclear cells (PBMCs).

Among all derivatives, the N-terminal half peptide and mosaic peptide exhibited the lowest IgE-binding capacity. In addition, in comparison to other antigens, KLH-coupled mosaic peptide induced the highest level of the recombinant Che a 3 (rChe a 3)-specific IgG antibody and ther Che a 3 specific-blocking IgG antibody in mice. Moreover, the mosaic peptide lacked lymphopro liferative capacity and down-regulated expression of pro-allergic IL-5 and IL-13 cytokines.

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Therefore, a peptide-carrier fusion vaccine, composed of the B-cell epitope coupled to the carrier, could be considered as one of the promising hypoallergenic vaccines to treat patients with allergy to low molecular weight allergens such as Che a 3.

Keywords: Chenopodium album; Immunotherapy; Polcalcin; Recombinant fusion proteins; Vaccine

INTRODUCTION

According to the studies, an allergic disease has been considered to be one of the most common health problems in developed countries, which influences more than 25% of the population.[1,2] Weeds pollen has been known as one of the most common aeroallergens in the Middle East area so that a recent meta-analysis showed that weeds are the most common outdoor allergens in Iran.[3] Chenopodium album as one of the allergenic members of the Chenopodiaceae family (a common weed) has been regarded to be the most frequent cause of allergy in desert areas such as some regions in Southern Europe, Iran, and Saudi Arabia.[4-6] In addition, Polcalcins (Che a 3) as the pollen-specific panallergen contain two conserved EF-hand and exhibit potent cross-reactivity.[7,8] In fact, cross-reactivity creates conditions for allergic patients, which make them sensitive to various plant pollens.[2,9-11]

Notably, allergen-specific immunotherapy (SIT) that is the only antigen-specific disease-modifying treatment of allergy involves the administration of the increased doses of the intact allergen to the allergic patients to develop a state of immunologic tolerance.[9,10] However, the presence of whole IgE epitopes on the intact allergen can induce immediate allergic reaction or even life-threatening anaphylactic shocks in SIT.[10,12] In addition, the late-phase reaction induced by allergen T-cell epitope has been considered as one of the concerns about SIT.[13-15] Therefore, to overcome these problematic issues in SIT, researchers have attempted to produce hypoallergenic variants of allergens that can induce IgG, which blocks the binding of IgE to the allergen.[2,10,16,17] For example, Westritschnig et al generated a hypoallergenic polcalcin variant from the timothy grass, phl p7, by disrupting the three-dimensional structure of phl p7 via replacing four calcium-binding sites with alanine.[11] Moreover, the hypoallergenic variant generated by this strategy retained the T-cell epitope to induce high amounts of IgG blocking antibodies, which could compete with the IgE binding activity to the allergen. However, it is well known that T-cell epitopes of the allergen could induce the late-phase reaction.[18,19] Thus, the recently developed strategies resolved concerns about the late-phase reaction by developing the hypoallergenic short peptides lacking T-cell epitope coupled with the carriers to induce a robust allergen-blocking IgG response.[13,14]

Several studies used various immunogenic carriers (i.e., Viral like particles, HBS antigen) to present the hypoallergenic allergen-derived peptides to the immune system for inducing the protective allergen-blocking IgG response, and some of these vaccines passed the early clinical trial phase successfully.[20-22]

The present study designed and immunologically characterized four derivatives of Che a 3 to generate a hypoallergenic vaccine. The first derivative was generated by a mutation in the calcium-binding sites and the second and third derivatives included N-terminal (aa 2–37) and C-terminal (aa 38–78) halves of polcalcin. Moreover, the fourth derivative, mosaic peptide, consisted of the short peptides selected based on five factors, including surface accessibility, antigenicity, flexibility, a high score for linear B-cell epitope, and lack of T-cell epitope.

MATERIALS AND METHODS

Allergic Patients

Sera from 7 male and 9 female (mean age=40) with clinical histories and positive prick test with common weed pollen commercial extract (Greer Laboratory, USA) were collected in February–May 2019 at Shohaday Khalij Fars hospital (Bushehr, Bandar Bushehr, Bushehr: Iran) under supervision of asthma and allergy specialists. In addition, the enzyme allegrosorbenttest (RIDASCREEN Spec. IgE, R-Biopharm, Germany) was used to select the patient's sera containing a specific IgE antibody to common

weed pollen extract. Results showed the mean (±SD) level of common weed pollen-specific IgE as equal to7.68 (±1.27) IU/mL. Then, the sera were analyzed for the presence of the Che a 3-specific IgE antibody. Table 1 presents the demographic data of allergic patients.

Ethics Statement

All human and animal experiments used in the present study were under and following the protocol approved by the Ethics Committee of the University of Medical Sciences–Iran in Bushehr province (Permit number: IR.BPUMS.REC.1397.011).

Table 1. Demographic and serological data of Chenopodium album polcalcin (Che a 3)-allergic patients

Patients	Age (years)	Gender	Common weed pollen extract-Specific IgE (IU/mL)	Symptoms
1	42	M	8.4	As, Rc
2	34	F	9.8	Rc
3	27	F	7.4	R
4	36	M	8.3	RC
5	50	F	9.4	R
6	59	M	6.2	Rc. As
7	35	F	8.2	RC
8	33	M	4.5	R
9	51	F	7.8	R
10	32	F	6.6	Rc. As
11	41	F	8.4	Rc
12	57	M	7.3	Rc
13	50	F	8.5	Rc
14	25	M	7.9	R
15	47	M	7.4	R
16	28	F	6.8	R
	Mean: 40.43		Mean:7.68	

Rc, rhinoconjunctivitis; r, rhinitis; As, Asthma

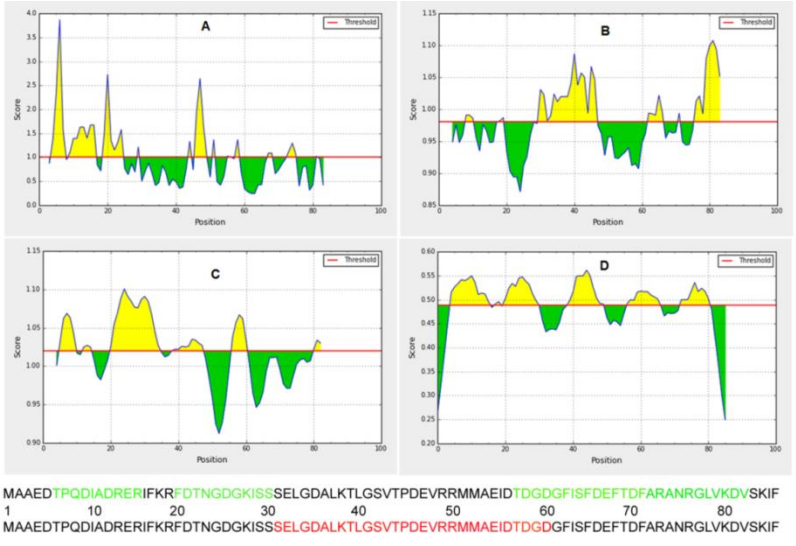

Figure 1. Determination of candidate peptides on Chenopodium album polcalcin (Che a 3) using Immune Epitope Database (IEDB). Peptides were selected according to surface accessibility, antigenicity, flexibility, and B-cell epitope score; using IEDB. The selected peptides have been shownby green color, while MHCII-binding regions have been represented by the red color on the amino acid sequence of Che a 3.

Expression and Purification of Mutant Che a 3

In this section, the mutational form of Che a 3 was generated by substitution of four calcium-binding residues by alanine as described by Westritschnig to design a hypoallergenic form of phl p7, polcalcin from the timothy grass pollen.[11] Then, the coding gene was chemically synthesized, cloned into pET-28a (+), and expressed in E.coli Bl21 (DE3) as mentioned elsewhere.[23,24] To sum up, the expression was induced with 0.5 mM isopropyl--β-D-thiogalactopyranoside (IPTG) at 20˚C for 18 h. In the next step, the bacteria pellet was re-suspended in lysis buffer (0.5 mg/mL lysozyme, 50 mM KH2PO4, pH 7.4, 150 mmNaCl) and ultra-sonicated five times with 80% amplitude for 5 min. The lysate was loaded on a column with Ni-NTA His Bind resin, and the recombinant mutated Che a 3 was eluted with elution buffer (50 mM KH2PO4, pH 7.4, 150 mm NaCl, 250 mm imidazole). Finally, concentration and homogeneity of the purified recombinant protein were evaluated using Bradford and SDS-PAGE, respectively.

Determination of the Che a 3-derived Short Peptides to Generate the Mosaic Peptide

The amino acid sequence of Che a 3, (accession no: AAL92871.1) was retrieved from the protein database of National Center for Biotechnology Center (http://www.ncbi.nlm.nih.gov/protein) and submitted at IEDB database (http://tools.iedb.org/mhcii) to predict the short peptides from Che a 3 according to four criteria of accessibility, flexibility, antigenicity, and score of the linear epitope. Moreover, the human dominant T-cell epitope-binding regions restricted to MHC II, DRB1*11:04, 11:03, 15:01, 04:01 DQA1*05:01, DQB1*03:01 (as dominant haplotype in the Iranian population)[25] were determined using the IEDB(https://www.iedb.org/) and excluded from the selected peptides as much as possible (Figure 2). Additionally, the potential peptides were fused by the GGS linker (Figure 1), and the antigenicity of the peptide was checked using Vaxin Jen (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) (Table 2).

Peptide Synthesis

N- and C-Terminal half peptides from the Che a 3 (molecular weight: 4967.35 and 4600.16 dalton, respectively) and the mosaic peptide (molecular weight: 5348.70 daltons) were synthesized using the solid-phase synthesis method with N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry (Pepmic, Suzhou, China), and some peptides were modified at C-terminal with one cysteine to facilitate conjugation with KLH. Moreover, the synthesized peptides were purified using the RP-HPLC equipped with SHIMADZU Inertsil ODS-SP (4.6*250 mm* 5µm) column, and eluted by 0–100% H2O/acetonitrile gradient containing 0.1% trifluoroacetic acid (TFA). Table 1 summarizes the characteristics of Che a 3-derived peptides.

Evaluation of IgE Reactivity by Dot Blot

rChe a 3, which has been kindly gifted by Dr. A. Varasteh (Bu-Ali research center, Mashhad, Iran), and its derivatives were investigated by dot blot as previously described (26-28). For dot blot, one microgram of rChe a 3 or its derivatives was blotted onto the nitrocellulose membrane and blocked with 1% bovine serum albumin (BSA). The membrane was then incubated with sera of C. album-allergic patients at 37˚C under continuous shaking for 2 h. After the washing steps with phosphate-buffered saline with 0.5% Tween-20 (PBS-T), biotin-labeled anti-human IgE (Abcam, USA) was added and incubated at 37˚C for 1 h. Afterward, the horseradish peroxidase (HRP)-conjugated streptavidin (Sigma, USA) was added, and finally, the reactive proteins were detected using the chemiluminescent substrate (Thermo Scientific Pierce ECL) and visualized by G: BOX Chemi XX9 gel doc system (Syngene, UK).

Table 2. Properties of the investigated peptides and proteins

Proteins & Peptides	Borders & Amino acid exchanges	Molecular weight(Da)	Antigenicity score
Mutated Che a 3	EF-1 :(D25A, E32A) EF-2: (D60A, E67A)	9347.47	0.07
N-Terminal half	2-45	4967.35	0.50
C-terminal half	45-86	4600.16	-0.25
Fused peptides	aa:5-15 (GGS) aa:20-30 (GGS) aa: 57-82	5348.70	0.74

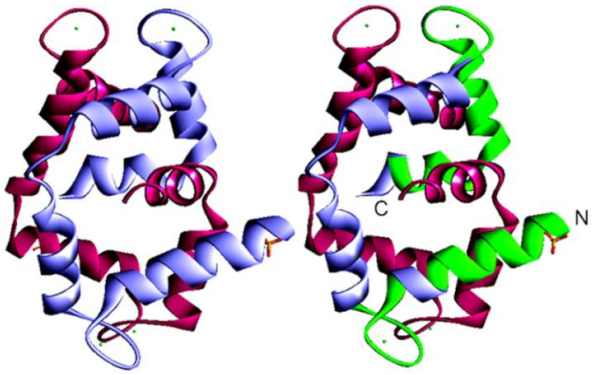

Figure 2.The representation of selected short peptides on the ribbon structure of polcalcin. The figure represents a dimeric form of Chenopodium album polcalcin (Che a 3) that retrieved from PDB (Pdb cod: 2opo). Monomers formed dimeric Che a 3 showed via dark red and dark blue color. Potential short peptides comprising mosaic peptide have been shown with green color.

Evaluation of IgE Reactivity by ELISA

According to the research design, to perform ELISA, the wells of 96-well microplate (NuncMaxiSorpTM, Denmark) coated with two micrograms of peptides and blocked with 1% BSA were exposed to 1:5 diluted sera of C. album-allergic patients. The wells were incubated with biotin-labeled anti-human IgE Abs and HRP-conjugated streptavidin as described above. Upon the addition of 3, 3′, 5, 5′-Tetramethylbenzidine/H2O2 chromogen/substrate, the plates were incubated in the dark for 15 min. Afterward, the reaction was stopped by adding 100 µL of 2 N H2SO4 solutions, and optical density (OD) of the wells was measured at 450/630 nm using the microplate reader(BioTek Synergy 4, USA)

Evaluation of IgE-binding Capacity of Peptides and the Che a 3 Protein with Competitive ELISA

To determine the IgE cross-reactivity between the Che a 3 protein and its derivatives, 100 µL of allergic patients' 1:3 diluted sera separately preincubated with an equal volume of peptides (5, 10, 20 or 40 µg/mL), rChe a 3 (40 µg/mL) or BSA (as the negative control) were examined for IgE reactivity with the rChe a 3 protein coated on the microtiter plate wells. Then, the inhibition percentage was calculated as follows:

$$\left(\frac{\mathrm{ODwi} - \mathrm{ODi}}{\mathrm{ODwi}}\right) \times 100$$

OD_{wi} and OD_i represent the optical density of a sample without inhibitor and with inhibitor, respectively

Immunization of the Mice with KLH-coupled Peptides or Che a 3 Protein

According to the research design, six groups of female Balb/c mice (6-8 weeks old) were subcutaneously immunized with 25 µg of KLH-coupled peptides three times on days 0, 14, and 28 or Che a 3 protein formulated with adjuvants, including CFA/IFA, as classical strong adjutant, or Al $(OH)_3$ (1 mg/mice), as the most widely adjutant in human. Therefore, one week after the last immunization, blood was taken from the lateral vein of the tail, and the isolated sera were evaluated for Che a 3-specific IgG level.

Evaluation of the Polcalcin-specific IgG Response

Similar to the step "evaluation of IgE reactivity by ELISA", the wells of 96-well microplate were coated with rChe a 3 (2 µg/well) and probed with 1:1000 diluted sera of the immunized mice. Upon the washing steps, 1/2000 diluted goat anti-mouse IgG-HRP (Abcam, USA) was added to the wells and incubated at 37˚C for 2 h. Then, rChe a 3-specific IgG Abs in the sera of the immunized mice was assessed by addition of TMB/H2O2 chromogen/substrate into the wells and measurement of OD at 450/630 nm using the microplate reader (BioTek Synergy 4, USA)

Evaluation of Blocking IgG Antibody in Sera of the Immunized Mice by Inhibition ELISA (iELISA)

In this stage, ELISA and blot inhibition were used to measure the blocking IgG antibody. Then, the wells coated with rChe a 3 were incubated with the increased dilutions of sera from the immunized mouse or sera of the non-immunized mice (1:500, 1:1000, 1:2000,

1:400, 1:8000 or 1:16000) as competitors or without a competitor, respectively. Afterward, 1:5 diluted sera of allergic patients (n:10) were added to the wells and IgE reactivity measured as described above. The inhibition percentage was calculated as follows:

$$\frac{\text{ODwc} - \text{ODc}}{\text{ODwc}} \times 100$$

ODwc and ODc represented OD of the samples without competitor (sera of non-immunized mice) and OD of the samples with a competitor (sera of the immunized mice), respectively.

Evaluation of Blocking IgG Antibody in Sera of the Immunized Mice by Inhibition Dot Blot

For the dot blot experiment, similar to iELISA, the nitrocellulose membrane coated with rChe a 3 (1 µg) were pre-incubated with the increased dilutions of the sera from the immunized mice as described for inhibition ELISA. Then, the disks were incubated with 1:5 diluted sera of the allergic patients (n:10) at 37°Ċ for 2 h, and IgE reactivity was evaluated as described above for dot blot experiment

Cell Proliferation Assay using Tetrazolium-based Colorimetric (MTT) Assay and Cytokine Responses of PBMCs

The heparinized blood samples were obtained from the rChe a 3-allergic patients (n=5; mean age=38), and PBMCs were isolated by the Ficoll density gradient centrifugation and cultured with RPMI 1640 supplemented with FBS serum, penicillin (100 U/mL), and streptomycin (100 µg/mL) (SIGMA). Then, the cells were added to the well (1×106 cells/well) and treated with rChe a 3 or its derivatives (1 µg/mL). In addition, treatment with IL-2 (5 U/well) (Roche Diagnostics, Rotkreuz: Switzerland) was performed as the positive control. After 7 days, the lymphocyte proliferation induced by rChe a 3 or its derivatives were assessed using Vybrant MTT cell proliferation assay kit (Thermo Scientific) and expressed as the proliferation index (OD570 of the stimulated cells/the mean OD570 of control cells). Moreover, the level of the pro-allergic cytokines IL-5 and IL-13 in the supernatant of PBMC cultures stimulated with rChe a 3 or its derivatives were determined; using the RayBiotech ELISA kit (RayBiotech, Inc.).

Statistical Analysis

The one-way analysis of variance (ANOVA) was used to compare the mice group and other results with each other using the GraphPadPrism software version 8. The p-value of less than 0.05 was considered statistically significant.

RESULTS

Mutated Che a 3 was Expressed and Purified

The mutated Che a 3 was successfully expressed in E.coli and purified using the Ni-NTA Purification System. Then, a single-protein band corresponding to the expected molecular weight for the mutated Che a 3 protein was observed in SDS-PAGE, which confirmed the homogeneity of the purified protein (Figure 3). Finally, the concentration of the purified protein was estimated to be 2.5 mg/mL.

The Derivatives of Che a 3 Showed Highly Reduced IgE-binding Capacity

Evaluation of the IgE reactivity with sera of allergic patients using ELISA demonstrated a highly reduced IgE reactivity for the peptide-based derivatives of Che a 3 compared to the parent protein (rChe a 3). However, among all derivatives, the N-terminal half

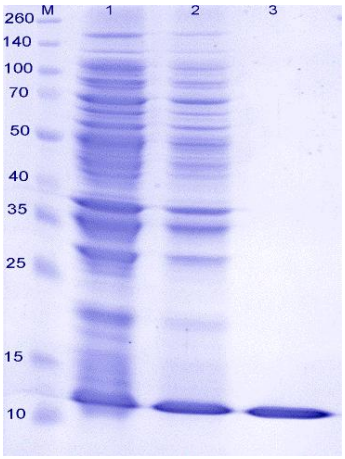

Figure 3. Evaluation of expression and purification of the mutated Chenopodium album polcalcin (Che a 3) using SDS-PAGE. Line 1: lysate of bacteria transformed with plasmid pET-28a; Line 2: lysate of bacteria transformed by a plasmid containing mutated Che a 3 -coding sequence and line 3: recombinant mutated Che a 3 purified by Ni-NTA purification system; Line M is a low molecular weight protein marker (10, 15, 25, 35, 40, 50, 70, 100, 140 and 260 kDa).

peptide and mosaic peptide exhibited the lowest IgE-binding capacity, whereas the mutated Che a 3 showed the highest level of IgE reactivity. In addition, the mean ODs±SD for IgE reactivity against rChe a 3, N-terminal half, C-terminal half, mosaic peptide, and the mutated Che a 3 were 0.99±0.32, 0.07±0.01, 0.21±0.09, 0.08±0.050, and 0.46±0.03, respectively (Figure 4.a). The mean ODs±SD for IgE reactivity of rChe a 3, N-terminal half, C-terminal half, mosaic peptide, and the mutated rChe a 3 incubated with the healthy individuals' sera were 0.030±0.01, 0.026±0.01, 0.024±0.01, 0.024±0.01 and 0.04±0.02, respectively.

It should be mentioned that inconsistent with the results obtained from ELISA, the results of the dot blot demonstrated a strongly reduced IgE-binding capacity for the N-terminal half of Che a 3 and mosaic peptide as much as no detectable IgE-reactivity was observed, whereas the mutated rChe a 3 exhibited a considerable reactivity with the Che a 3-specific IgE antibody in sera of the Che a 3-sensitized patients (Figure 4b).

In the next stage, competitive ELISA was used to investigate which derivatives of Che a 3 could inhibit the IgE reactivity of rChe a 3 due to the possession of the same IgE epitopes. According to the results, among the mentioned derivatives, the mutated Che a 3 had the highest inhibitory activity against the binding of IgE to rChe a 3. In addition, the IgE-binding capacity of rChe a 3 reduced by 50-93%, 3-7%, 8- 22%, 3- 6 %, and 15-51% when the sera of the Che a 3-sensitized patients were preincubated with the increased concentration of rChe a 3, N-terminus half, C-terminus half, mosaic peptide or mutated rChe a 3, respectively (Figure 5).

KLH-coupled Peptides Induced a Robust Che a 3-Specific IgG Antibody in Mice

The results of the mice immunization by Che a 3-derivatives showed that the KLH-coupled mosaic peptide possessed the highest ability to induce the Chea 3- specific IgG response in comparison with other peptides coupled to KLH, including the mutated rChe a 3 and N- or C- halves. In addition, it was known that the mice immunized with the peptides coupled to KLH remarkably elicited a higher level of rChe a 3-specific IgG Ab response in comparison with those of the non-coupled antigens, including rChe a 3 and the mutated Che a 3 (Table 3).

KLH-coupled Mosaic Peptide Had Extremely High Ability to Induce the IgG Abs Blocking the IgE-Binding to rChe a 3.

Following the examination of peptides for their capacity to induce the Che a 3-specific IgG in the immunized mice, the ability of rChe a 3-specific Abs was evaluated to inhibit the binding of patient's IgE to rChe a 3. The obtained results demonstrated that IgG Abs induced by the KLH-coupled mosaic peptide significantly possessed a high activity to inhibit the binding of the patient's IgE to Che a 3.

Moreover, at lower dilutions of mice antisera, the mosaic peptide coupled with KLH, rChe a 3 and the mutated Che a 3 exhibited a comparably high activity to inhibit the binding of the patient's IgE to rChe a 3 in comparison to the KLH-coupled N and C terminal halves. At the increased dilutions of antisera, a significantly rapid decline was observed in the inhibitory activity mediated by rChe a 3 and the mutated Che a 3 (approximately from 94% to 38%), but not by KLH-coupled mosaic peptide (from 93% to 57%) (Figure 6b).

Then, the inhibition dot blot was used to compare the inhibitory activity of IgG induced by the mutated Che a 3 and mosaic peptides to block the binding of IgE to rChe a 3. As shown in Figure 6, when the mice antisera dilution increased, the inhibitory activity of the anti-mutated Che a 3 Ab to inhibit the binding of IgE to rChe a 3 reduced, while the anti-mosaic peptide Ab could block the binding of IgE to rChe a 3 even at the increased dilutions of the mice antisera (Figure 6a).

Table 3. Total allergen-specific IgG in the mice immunized by Chenopodium album polcalcin (Che a 3) or its derivatives. Data represent the mean±standard deviation (SD) of three independent experiments

Adjuvant		N.terminus-KLH	C.terminus-KLH	Mosaic peptide-KLH	Mutated-r.Che a 3	r.Che a 3
CFA/IFA	Preimmune(ODs)	0.03 ±0.01	0.04 ±0.03	0.02 ±0.01	0.03 ±0.02	0.03 ±0.02
	Immune(ODs)	1.12 ±0.05	1.28 ±0.10	1.53 ±0.08	0.95 ±0.06	0.98 ±0.10
ALU	Preimmune(ODs)	0.03 ±0.01	0.02 ±0.04	0.31±0.01	0.02 ±0.01	0.02 ±0.02
	Immune(ODs)	0.36 ±0.07	0.75 ±0.03	0.93±0.05	0.51 ±0.10	0.51 ±0.09

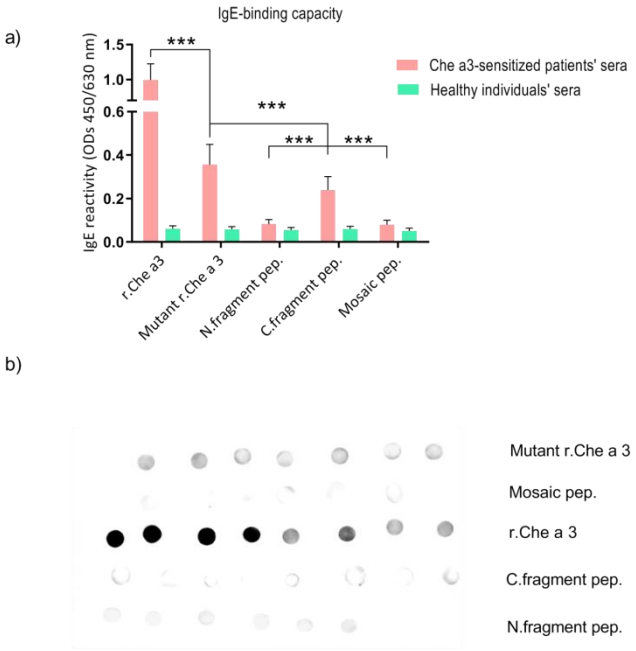

Figure 4. Evaluation of IgE-binding capacity of Chenopodium album polcalcin (Che a 3) and its derivatives. a) IgE reactivity to Che a 3 and its derivatives determined with sera from Che 3-sensitized patients (n:16) and healthy individuals (n:5), the data represented in the graph are mean±SD. b) IgE reactivity of r Che a 3 and its derivatives showed using dot blot and sera from Che 3-sensitized patients. Symbol meaning; * p<0.05, ** p<0.01, ***p<0.001

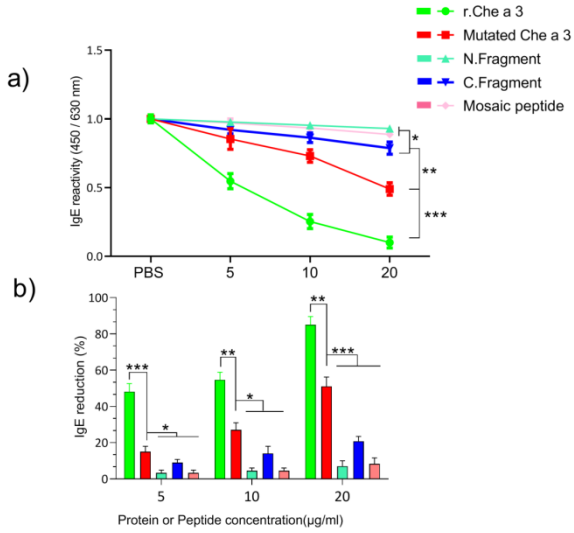

Figure 5. Evaluation IgE cross-reactivity between recombinant Chenopodium album polcalcin (rChe a 3) and its derivatives; using competition ELISA. a) IgE cross-reactivity of rChe a with its derivatives was evaluated using sera from Che a 3-sensitized patients (n:16) preincubated with various concentrations (5, 10, 20 µg/mL) of rChe a 3 or its derivatives. The result represents mean±SD of optical density (OD540/630) obtained from duplicate IgE binding assay. b) The figure shows the percentage of the reduced IgE reactivity against rChe a 3 its derivatives. Symbol meaning; * p<0.05, ** p<0.01, ***p<0.001

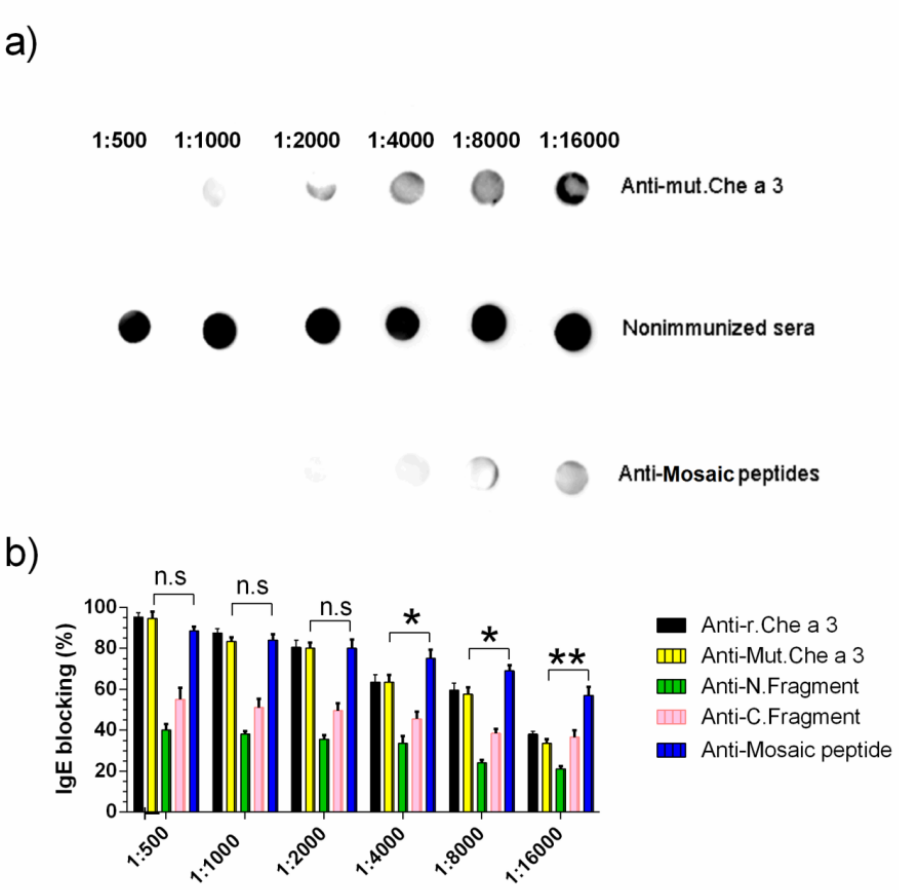

Figure 6. Evaluation of IgG blocking Chenopodium album polcalcin (Che a 3)-IgE binding in sera from the mice immunized by Che a 3 or derivatives. a) Dot blot represents the potency of blocking IgG induced by KLH- mosaic peptide (Anti- mosaic peptides) and mutated Che a 3 (Anti-Mut.Che a 3). Blotted rChe a 3 was incubated with the mixture sera including pool sera of Che 3-sensitized patients (n:10) and the increasing dilution of anti-mutant Che a 3 Ab or anti- mosaic peptides Ab, then IgE reactivity against r.Che a 3 was determined. b) Thepercentage of inhibition of Che a 3-IgE binding by Anti-Che a 3, Anti-Mut.Che a 3, Anti-N.Fragment, Anti.C-fragment, or Anti- mosaic peptide. Pool sera from Che 3-sensitized patients (n:10) used to investigate IgE reactivity of rChe a 3 probed with a different dilution of anti-sera. Symbol signs: *, $p \leq 0.05$; **, $p \leq 0.01$.n.s: *not* significant.

The Mosaic Peptide Could not Induce the PBMC Proliferation Isolated from the C.album-allergic Patients

The late-phase allergic reaction caused by the allergen-specific T-cell has been known as one of the main concerns in SIT with crude extract or intact allergen.[13,14] Therefore, we avoided incorporation of the computationally determined Che a 3-T cell epitope in the mosaic peptide. Then, the proliferation induced by the Che a 3 derivatives using the colorimetric MTT assay as described previously.[29] According to the MTT proliferation, the mutated Che a 3 and rChe a 3 possessed a comparable potency to induce the proliferation of the PBMC obtained from the C.album-allergic patients. In addition, the proliferation assay revealed that the mosaic peptide had the least potency for inducing the lymphocyte proliferation (Figure 7), confirming the lack of the dominant MHC binding region in the mosaic peptide. Furthermore, quantification of the pro-allergenic cytokines secreted from the stimulated PBMC revealed that rChe a 3 and the mutated rChe a 3 induced a considerable level of IL-5 and IL-13 in comparison with other peptide-based derivatives (Figure 7).

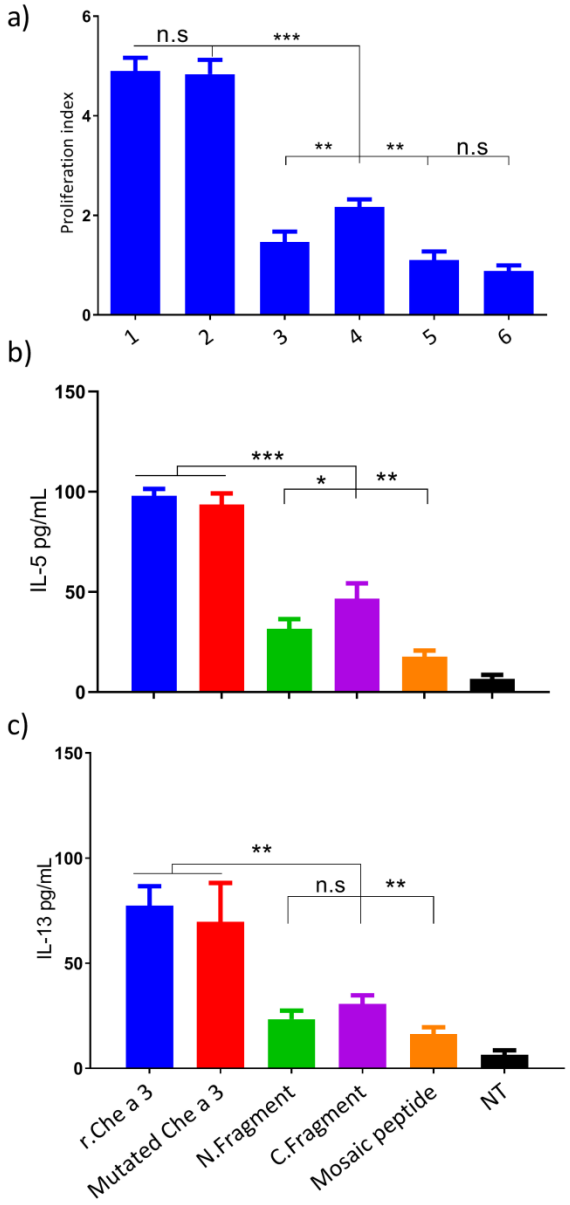

Figure 7. The proliferation of peripheral blood mononuclear cells (PBMCs) and pro-allergenic cytokine response. a) The lymphocytes obtained from Che 3-allergic patients were induced by Chenopodium album polcalcin (Che a 3) or its derivatives and lymphocyte proliferation was determined. b, c) Quantifying of pro-allergenic cytokine (IL-5, IL-13) secreted in the supernatant of PBMCs (from Che 3-sensitized patients) induced by Che a 3 or its derivatives. Symbol meaning; * *p*<0.05, ** *p*<0.01, ****p*<0.001, NT, no treatment

DISCUSSION

The current study characterized a mosaic peptide comprising the Che a 3-derived peptides, lacking the ability to induce the lymphocyte proliferation and bind to IgE. It was also capable of inducing ahigh level of the allergen-specific IgG coupled to KLH. Evaluation of the IgE-binding capacity by various methods, including indirect ELISA, dot blot, and competitive ELISA provided strong evidence that peptide-based derivatives significantly had lower IgE-reactivity compared to the one generated by the mutational strategy. Therefore, this result could be related to the complete disruption of the three-dimensional structure of the protein allergen by peptide-based strategy. These results were consistent with the findings of the previous study, which demonstrated the significantly lower IgE-binding capacity of the Phl p7-derived fragments relative to the parent protein, Phl p7.[11] In addition, another study showed similar observations about Phl p1 that is the major allergen of timothy.[30] Furthermore, inconsistent with the result of the IgE-binging assay, the PBMCs from the allergic patients induced the highest level of proliferation and pro-allergenic cytokines secretion from the T helper type 2 (e.g., IL5, IL13) was incubated with rChe a 3 or the mutated Che a 3. This finding could be due to the high IgE-binging capacity of these proteins leading to the improvement in the IgE-mediated allergen uptake, allergen presentation, and consequently stimulation of the allergen-specific T-cells.[31] High lymphocyte proliferation and secretion of pro-allergenic cytokines induced by rChe a 3 and the mutant Che a 3 were related to the IgE-binding capacity and existence of the T-cell epitope, resulting in the IgE-mediated allergen presentation and stimulation of the allergen-specific lymphocytes, respectively.

We also found that the KLH-coupled derivatives induced more level of Che a 3-specific IgG Ab compared to the non-coupled protein or even the parent protein, providing evidence that KLH enhanced the immunogenicity of the peptides; however, sera of the mice immunized with the KLH-coupled N- and C-terminal halves possessed lower IgE-blocking activity in comparison to the sera from those immunized with the KLH-mosaic peptide, mutated, or wild type of Che a 3. In this regard, the most likely explanation would be that the IgG Abs in sera of the mice immunized with N- or C-terminal fragments could not create the steric

hindrance for all the IgE-reactive epitopes on Che a 3. In addition, when the increased dilutions of the mice immunized sera were examined for their inhibitory activity, sera of the mice immunized with the KLH-mosaic peptides significantly had more inhibitory activity against the binding of patients' IgE to rChe a 3 relative sera of the mice immunized with the non-coupled KLH proteins, including mutant or wild type Che a 3. Therefore, this finding could be related to the higher immunogenicity of the KLH-bound mosaic peptide and so higher ability to induce a stronger IgG Ab response in comparison to the mutant and parent proteins. Another explanation was that the IgG Ab induced by the KLH-coupled mosaic peptide could recognize a large number of the IgE-reactive epitopes on Che a 3 relative to the IgG Ab induced by Che a 3 fragments.

In contrast to our results, the titer of the blocking IgG induced by hypoallergenic Phl p 7 derivatives in the study reported by Westritschnig et al was very lower than the ones induced by the parent protein.[11] Since the late-phase reaction was considered to be one of the considerable side effects of SIT induced by the development of allergen-specific Th2, the present study avoided incorporation of the predicted relevant T-cell epitopes of Che a 3 into the mosaic peptide. However, lack of the T-cell epitopes in the mosaic peptide was experimentally confirmed by the lymphocyte proliferation assay, whereas the mutated Che a 3 and rChe a 3 comparably induced the proliferation of the lymphocytes obtained from the allergic patients.

In general, the mosaic peptide designed and characterized in the present study could not induce the lymphocyte proliferation and bind to IgE. The design of the mosaic peptides could be considered as one of the solutions to overcome these problematic issues, which existed for the hypoallergenic mutated variants.[11,32] Therefore, the current study successfully added the bioinformatics knowledge and immunochemical experiments to determine the allergen-derived mosaic peptides, which could be a candidate for Che 3-specific immunotherapy. However, this mosaic peptide ability to induce the therapeutic response and alleviate the allergic symptoms in the mice model of the allergy should be evaluated by the experts in the field. Furthermore, the most common shortcoming in the current study was the measurement of the IgG1/IgG 2a antibody response as well as the IFN-γ cytokine to determine the profile of the T lymphocyte response induced by the mosaic peptides.

In conclusion, the mosaic peptides were suggested as the candidates so that when they were coupled to an acceptable carrier for human application; for example, the hepatitis surface antigen, Pres could induce a strong allergen-specific IgG response. This induced the IgG response, which could have a beneficial effect on the allergic patients by blocking the IgE binding to the allergen, which subsequently reduced the IgE-mediated allergen presentation and decreased the IgE Abs production.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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