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Constructing a hybrid molecule with low capacity of IgE binding from *Chenopodium album* pollen allergens

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ABSTRACT

Allergen specific immunotherapy is the only remedy to prevent the progression of allergic diseases. Nowadays, using of recombinant allergens with reduced IgE-binding capacity is an ideal tool for allergen immunotherapy. Therefore, in this study we focused on a hybrid molecule (HM) production with reduced IgE reactivity from *Chenopodium album* pollen allergens. By means of genetic engineering, a head to tail structure of the three allergens of the *C. album* pollen was designed. The resulting DNA construct coding for a 46 kDa HM was inserted into an expression vector and expressed as hexahistidine tagged fusion protein in *Escherichia coli*. IgE reactivity of the HM was evaluated by western blotting, inhibition ELISA and *in vivo* skin prick test and its immunogenic property was tested by proliferation assay. The recombinant HM was expressed and purified by nickel-affinity chromatography. Comparison of the recombinant HM with a mixture of three recombinant allergens, as well as natural allergens in the whole *C. album* pollen extract via immunological experiments revealed that it has a much lower potential of IgE reactivity. Furthermore, *in vivo* skin prick tests showed that it has a significantly lower potency to induce cutaneous reactions than the mixture of recombinant wild type allergens and whole extract while, it had been preserved immunogenic properties. Our results have demonstrated that assembling three allergens of *C. album* in a hybrid molecule can reduce its IgE reactivity.

1. Introduction

Type I allergy, a genetically determined hypersensitivity disease which has affected more than a quarter of the population in industrialized areas of the world [1,2]. To date, the only curative treatment strategy for Type I allergy is allergen-specific immunotherapy (SIT), which currently has been performed through subcutaneous or mucosal administration of allergens with the natural sources to the patients in order to switch to an allergen-specific unresponsiveness condition [3,4]. However, this strategy has some disadvantages. Currently, many pollen extracts, which were used in SIT were associated with some side effects in clinical settings due to their complicated composition. Therapeutic allergen extracts may contain no important allergens; the ratio of the individual components in the extract may be greatly valid and possibly induce a new IgE specificity [5,6]. The use of recombinant allergens can resolve many of these problems. In addition, a few reports on SIT with recombinant wild type allergens have recently been published demonstrating the efficacy of these molecules [7,8]. However, recombinant wild type allergens may lead to IgE mediated side effects such as urticaria, allergic asthma and anaphylactic shock [9]. Therefore, production of recombinant allergens that display reduced allergenic activity is an approach that diminishes IgE mediated side effects.

According to clinical data, one of the most important problems in allergic patients is that they are sensitized to more than a few allergens from one or several sources. In addition, many mono-sensitized patients acquire additional allergies gradually with increasing of the age [10]. It has recently been demonstrated that molecules which consist of several copies of one or more homologous allergens, or even consist of immunologically unrelated allergens has shown the capability to be used for the allergy treatment in the patients who are sensitive to the several allergen components [11,12]. It seems that the use of hybrid molecules can

Abbreviations: SPT, skin prick test; HM, hybrid molecule; Mixture of rAllergens, A mixture with equimolqr of rChe a 1, rChe a 2 and rChe a 3.

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overcome many existing problems in immunotherapy with allergenic extract or recombinant wild type allergens.

In *Chenopodiaeae* family, *Chenopodium album* is one of the most recognized allergenic members. *Chenpodiaseae* pollens are the main cause of sensitization in desert and semi-desert areas like Kuwait, Saudi Arabia and Iran [13]. *C. album* pollens were found in atmosphere of our country, Iran, especially from June to early October. Previous studies in Mashhad, northeast of Iran, have demonstrated the susceptibility to *C. album* pollen allergens up to 63.4% of population [14]. Che a 1, Che a 2 and Che a 3 are known allergens in *C. album* pollen, of which the first two have been identified as the major allergens whereas Che a 3 is a minor allergen in the patients who are sensitive to *C. album* pollen. Che a 1 is a single N-glycosylated polypeptide whit 168-amino acids length and 18 kDa molecular mass. Che a 2 consists of a 131-amino acids chain and displays up to 75% and 82% identity with pollen and food profilins, respectively. Che a 3, a polypeptide chain with 86-amino acids length, belongs to the polcalcin family. It displays acidic p*I* and an apparent molecular mass 9.5 kDa [15–18].

Since, *C. album* pollen is considered as an important cause of respiratory allergy in Iranian population and most patients are frequently co-sensitized to immunologically unrelated allergens of this pollen, we were thus encouraged to design a HM from three allergens of *C. album* for therapeutic purposes. Here, we also present an approach that allows the combination of allergen molecules. The chimeric DNA for these allergens expressed as a HM in *Escherichia coli*, and its immunological characteristics were studied by independent techniques.

2. Material and methods

2.1. Serum samples and Skin prick test

Initially 17 patients, eight women and nine men (mean age of 31.76 ± 12.94 years; age range of 19–54 years), presenting to Immunology Research Center of Avicenna Research Institute of Mashhad were included in this study. The selected patients had histories of allergic conjunctivitis, allergic rhinitis, and/or bronchial asthma and positive skin prick test (SPT) to *C. album* pollen extract. Serum samples were collected from these patients and stored at −70 °C until usage. None of the patients received glucocorticosteroid, anti-histamine treatment or SIT. Sera were also obtained from 4 non-atopic volunteers who were negative on SPT with *C. album* pollen extract (Table 1). SPTs were performed on the ventral side of the subjects' forearms according to Dreborg method [19]. Histamine phosphate at 10 mg/ml and glycerin were used as positive and negative controls, respectively. Reactions with a wheal of 3 mm greater than the negative control, surrounded by an erythema, were considered positive. SPTs were performed with HM and a mixture of rChe a 1, rChe a 2 and rChe a 3 with several concentration. The Human Ethics Committee of the Mashhad University of Medical Sciences (Mashhad, Iran) approved the study, and written informed consent was obtained from all study participants before the study commenced.

2.2. Production of pollen extract

C. album pollen extract was prepared as previously described [13]. Briefly, pollens were collected from *C. album* plants from Bu-Ali garden of Mashhad, Northeast of Iran. In the first step of pollen extract production, pollens mixed with the cold acetone (1/10 w/v) and incubated at 4 °C with agitation. Acetone was removed by suction and then, phosphate-buffered saline (PBS, 0.15 M, pH 7.4) containing 20 mM EDTA (1:20 w/v) was added to the dried pollens followed by overnight agitation at 4 °C. After that, it was clarified by centrifugation at 7150 × *g* for 20 min at 4 °C, and the clear supernatant containing the solubilized proteins was dialyzed for 16 h at 4 °C against Tris–HCl 0.02 M, pH 8. The filtrate was then lyophilized and stored at −70 °C. The protein content was determined by Bradford's method [20].

2.3. Amplification of the sequence encoding the hybrid molecule by polymerase chain reaction

Total RNA was extracted from 0.8 g of mature non-defatted *C. album* pollen, according to the method of Chomczynski and Sacchi [21]. The first cDNA strand was synthesized from 2.5 μg of total RNA using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentase, Life Sciences, Lithuanian).

The cDNA coding for the hybrid molecule consist of three *C. album* identified allergens, Che a 1, Che a 2 and Che a 3 were constructed by using overlapping extension polymerase chain reaction (PCR). A chimeric DNA was designed as Che a3–Che a1–Che a2 containing Che a 3 at the 5′ end, Che a 1 at the central region and Che a 2 at the 3′ end of the HM (Fig. 1). The first step to make this chimeric DNA was amplification of the Che a 1 and Che a 2 coding region from first strand cDNA by conventional PCR using a pair of primers a_1-5′ and a_1-3′ for Che a 1 and a_2-5′ and a_2-3′ for Che a 2. The nucleotide sequences of the sense and antisense primers were described in Table 2. The PCRs were performed separately in 20 μl reaction volume containing 5 μg *C. album* cDNA, 12.5 pmol of each primer, 3 μl $MgSO_4$ (25 mM), 1.2 μl dNTPs (10 mM), 2 μl 10× reaction buffer, and 2.0 units of *pfu* polymerase (Fermentase, Life Sciences, Lithuanian). RT-PCR cycling parameters were as follow: initial denaturation at 95 °C for 3 min, then 35 continuous cycles of denaturation at 95 °C for 30 s, annealing temperatures for Che a 1 and Che a 2 were at 55 °C and 57 °C, respectively, for 1 min, extension for 2 min at 72 °C and the final extension for 3 min at 72 °C. Agarose Gel DNA Purification Kit (Bioneer, Korea) was used to obtain purified PCR products. Subsequently an overlapping extension PCR was designed to combine Che a 1 and Che a 2 genes in which exploited previous purified PCR products as template. The annealing temperature was optimized according to the melting temperature (T_m) of overhang's region of a_2-5′ primer. The PCR reaction contained 15 μl of each purified Che a 1 and Che a 2 PCR products, 3 μl $MgSO_4$ (25 mM), 1.2 μl dNTPs (10 mM), 2 μl 10× reaction buffer and 2.0 units of *pfu* polymerase. The PCR thermocycling parameters were as follows: initial denaturation at 95 °C for 3 min, 10 continuous cycles of denaturation at 95 °C for 30 s, annealing temperature for 1 min at 50 °C, extension for 2 min at 72 °C and final extension for 10 min at 72 °C. The desired linked DNA fragment for Che a 1–Che a 2 was isolated from agarose gel and then was amplified by a_1-5′ and a_2-3′ primers. The cDNA coding for Che a 3 allergen was amplified by a_3-5′ and a_3-3′ primers. The *Not I* restriction enzyme site was added to a_3-3′ primer. The PCR cycling conditions for Che a 3 were very similar to Che a 2 with slightly modification. The annealing temperature was optimized at 60 °C. For construction of a chimeric DNA from Che a 3–Che a 1–Che a 2, secondary overlapping extension PCR was performed with Che a 3 PCR products and the linked DNA fragment for Che a 1–Che a 2 as templates. The annealing temperature was adapted to overhang at a_3-3′ primer (1 min at 50 °C). The other thermocycling parameters for this reaction were as same as the first overlapping extension PCR. After this reaction, the chimeric DNA fragment containing Che a 3, 1 and 2 was amplified by a_3-5′ and a_2-3′ primers. It should be noted that three allergens of a *C. album* were amplified separately with specific primers via conventional PCRs. All of the PCRs were performed by a Corbett Research Thermocycler (Corbett, Australia).