Nutrición Hospitalaria

Original/*Investigación animal*

Epitope-based vaccine for the treatment of Der f 3 allergy

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Abstract

Introduction: mites allergic asthma is caused by exposure to home dust mite (HDM). Der f 3 is believed to be one of the major allergens in mites allergic asthma. The work was to identify the immune characteristics of Der f 3 epitope-based vaccine containing T cell and B cell epitopes.

Methods: T cell lines were generated from peripheral blood mononuclear cells of Der f 3 allergic patients. Three T cell epitopes and five B cell epitopes of Der f 3, which we identified previously, were selected to design a polypeptide (named Der f3-peptides). DNA constructions encoding these Der f 3-peptides were expressed in *Escherichia coli*. The T cell lines were stimulated with the peptides and tested for proliferative capacity and cytokine production.

Results: plasmid pET28a (+)-Der f 3-peptides was constructed and expressed in *E. coli* BL21, and the Der f3-peptides protein was purified and confirmed by Western blotting. The Der f 3-peptides were recognized by the T cell clones from allergic patients. SI value of Der f 3 group and Der f 3-peptides group were both higher than that of PBS group (P<0.05). The Der f 3 and Der f 3 peptides induced secretions of IL-4 and IL-5 were decreased compared with that of PBS group (P<0.05). The capacity of IgE-binding to Der f 3-peptides (41.25±5.67) μg/ml was decreased dramatically compared with that of Der f 3 (83.60 ± 10.92) μg/ml (P < 0.05).

Conclusions: our results demonstrate that several major T cell epitopes and B cell epitopes of Der f 3 can be valuable for designing the peptide-based immunotherapeutics for the mites allergic asthma.

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Key words: *Antigens. Dermatophagoides. Cytokines. Epitopes. T-lymphocyte. Immunotherapy.*

VACUNA BASADA EN EPÍTOPO PARA EL TRATAMIENTO DE LA ALERGIA DER F 3

Resumen

Introducción: el asma alérgica está causada por la exposición a los ácaros del polvo casero (HDM). Der f 3 se cree que es uno de los principales alérgenos en los ácaros del asma alérgica. El trabajo consistió en identificar las características inmunológicas de la vacuna basada en epítopo-Der f 3 que contienen las células T y las células B.

Métodos: se generaron líneas de células T a partir de células mononucleares de sangre periférica de pacientes alérgicos a Der f 3. Tres epítopos de células T y cinco epítopos de células B de Der f 3, que hemos identificado previamente, fueron seleccionados para diseñar un polipéptido (denominados péptidos Der f 3). Construcciones de DNA que codifican estos péptidos Der f 3 se expresaron en *Escherichia coli*. Las líneas de células T se estimularon con los péptidos y se utilizaron en el ensayo por su capacidad proliferativa y la producción de citoquinas.

Resultados: el plásmido pET28a (+) - Der f 3-péptidos se construyó y se expresaron en *E. coli* BL21, y la proteína de Der f 3-péptidos se purificó y se confirmaron mediante transferencia de Western. Los Der f 3-péptidos fueron reconocidos por los clones de células T procedentes de pacientes alérgicos. Valor SI de Der f 3 grupo y f grupo 3-péptidos Der eran tanto mayor que la del grupo de PBS (P <0, 05). El Der f 3 y Der f 3 péptidos indujeron secreciones de IL-4 e IL-5 se redujeron en comparación con la del grupo PBS (P <0,05). La capacidad de unión a IgE a Der f 3-péptidos (41, 25 ± 5, 67) μg/ml se redujo drásticamente en comparación con el de Der f 3 (83,60 ± 10,92) μg/ml (P <0,05).

Conclusiones: nuestros resultados demuestran que varios de los principales epítopos de células T y de células B de Der f 3 pueden ser valiosos para el diseño de agentes inmunoterapéuticos basados en péptidos para los ácaros del asma alérgica.

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Palabras clave: *Antígenos. Dermatofagoides. Citoquinas. Epítopos. Linfocitos-T. Inmunoterapia.*

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Introduction

Dust mite are a major source of inhalant allergens that induce allergic asthma which mainly include *Dermatophagoides farinae* and *Dermatophagoides pteronyssinu*[1-3]. Der f3 belongs to the group-3 allergens isolated from *D. farinae* and has strong allergenicity. It has been shown that Der f3 is a serine protease with trypsin-like activity, though its physiological role in mites is still unknown. It can activate the kallikrein-kinin system involved in various allergic reactions in human plasma[4,5]. Moreover, Der f3 could combine the specific IgE in serum in patients with allergic asthma at a rate of 16% - 100%[6], suggesting that Der f3 is a valuable antigen to design peptide vaccine for the treatment of asthma.

Asthma is a chronic airway inflammatory disease characterized by lung leukocyte and eosinophilic infiltration accompanied by mucus hypersecretion and airway hyperresponsiveness[7]. To date, allergen specific immunotherapy (SIT)[8] is the only etiology therapy that can change the allergic disease process. Many studies about SIT have focused on the epitope-based vaccine[9,10] which connects multiple B cell and (or) T cell linear antigen epitopes. The epitope-based vaccines have many advantages. They can overcome virulence return or spread and induce more efficient presentation when detected and combined by MHC molecules compared with traditional vaccines[11,12]. These findings suggest that use of several major T cell epitopes and B cell epitopes from one major component of a certain allergen may be necessary for the immunotherapy of allergic diseases.

In the present study, a recombinant protein containing Der f 3-peptides was prepared. It contains three T cell epitopes and five B cell epitopes from Der f 3, based on a difference among the types of restriction molecules capable of presenting these peptides. In order to verify its immune efficacy, the proliferative response of PBMC from allergic patients, cytokine level and the IgE binding with Der f 3-peptides were measured after stimulation with Der f 3-peptides. Our study provides useful information for the SIT of the allergic asthma.

Materials and Methods

Donors

Sixteen donors (twelve are positive and four are negative) who participated in this study were diagnosed on the basis of case histories and skin prick test, and recruited from Respiratory Medicine of Yijishan Hospital, Wannan Medical College, Wuhu 241002, Anhui, China (Table I). The Medical Ethics Committee of Wannan Medical College approved the sample collection.

Construction and expression of recombinant prokaryotic expression plasmid pET-28a(+)-Der f 3-peptides

Recombinant Der f 3 (rDer f 3) protein was expressed and stored in our laboratory. Three T cell epitopes and five B cell epitopes were connected and chemically synthesized by Sangon Biotech, Shanghai, China (Fig.1). The Der f 3-peptides gene was amplified by PCR using the primers: 5'GGATCCAUGGGTGATTGTCCATATCAAATTTCCTTG3' and 5'CTCGAGTTAATCAACAACTGGT CCGCCAGAATCACC3'. PCR was performed at 94ºC for 4 min, followed by 35 cycles of amplification at 94ºC for 30 s, 55ºC for 30 s and 72ºC for 1 min. The PCR product and pET-28a (+) vector were both digested with *BamH* I and *Xho* I enzymes and ligated with T4 ligase to generate the recombinant expressing plasmid pET-28a (+)-Der f3-peptides (Fig. 2). DNA sequencing was validated by Sangon Biotech, Shanghai, China (Fig. 2). pET-

Table I
Basic data of the allergic sera measured by Der f3 for this study

Number	Age	Sex	Result	Number	Age	Sex	Result
1	22	Woman	+++	9	24	Man	+++
2	27	Woman	++++	10	28	Woman	++++
3	25	Woman	+++	11	26	Woman	++++
4	26	Man	++++	12	29	Man	+++
5	30	Man	++++	13	24	Woman	-
6	22	Woman	++++	14	26	Man	-
7	21	Man	++++	15	21	Man	-
8	24	Man	++++	16	28	Man	-

Evaluation criteria of positive response: possible reaction (+), weak positive reaction (++), middle strong positive reaction (+++), strong positive reaction (++++), negative (-).
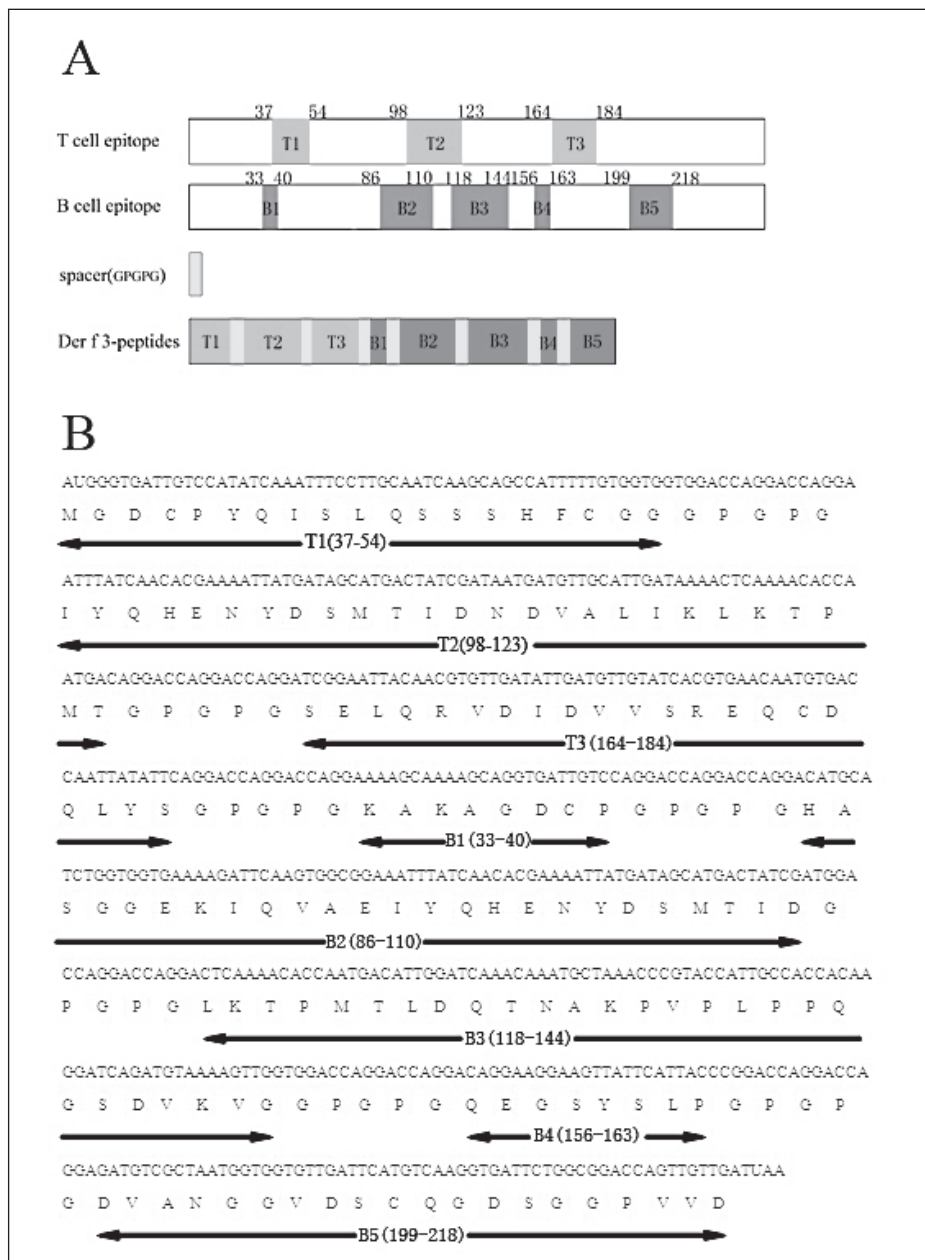
A

T cell epitope
```
        37    54      98      123   164      184
        [T1]          [T2]          [T3]
```

B cell epitope
```
        33 40         86  110 118 144 156 163  199      218
        [B1]          [B2]    [B3]  [B4]        [B5]
```

spacer(GPGPG)

Der f 3-peptides
```
[T1][T2][T3][B1][B2][B3][B4][B5]
```

B

```
AUGGGTGAUUGUCCAUAUCAAAUUUCCUUGCAAUCAAGCAGCCAUUUUUGUGGUGGUGGACCAGGACCAGGA
 M  G  D  C  P  Y  Q  I  S  L  Q  S  S  S  H  F  C  G  G  G  P  G  P  G
           ◄───────────────── T1(37-54) ─────────────────►
```

```
AUUUAUCAACACGAAAAUUAUGAUAGCAUGACUAUCGAUAAUGAUGUUGCAUUGAUAAAACUCAAAACACCA
 I  Y  Q  H  E  N  Y  D  S  M  T  I  D  N  D  V  A  L  I  K  L  K  T  P
           ◄──────────────────── T2(98-123) ────────────────────►
```

```
AUGACAGGACCAGGACCAGGAUCGGAAUUACAACGUGUUGAUAUUGAUGUUGUAUCACGUGAACAAUGUGAC
 M  T  G  P  G  P  G  S  E  L  Q  R  V  D  I  D  V  V  S  R  E  Q  C  D
   ──►        ◄──────────────── T3 (164-184) ────────────────────►
```

```
CAAUUAUAUUCAGGACCAGGACCAGGAAAAAGCAAAAGCAGGUGAUUGUCCAGGACCAGGACCAGGACAUGCA
 Q  L  Y  S  G  P  G  P  G  K  A  K  A  G  D  C  P  G  P  G  P  G  H  A
   ──►        ◄───── B1 (33-40) ─────►                             ◄──
```

```
UCUGGUGGUGAAAAGAUUCAAGUGGCGGAAAUUUAUCAACACGAAAAUUAUGAUAGCAUGACUAUCGAUGGA
 S  G  G  E  K  I  Q  V  A  E  I  Y  Q  H  E  N  Y  D  S  M  T  I  D  G
           ──────────────────────── B2 (86-110) ──────────────────►
```

```
CCAGGACCAGGACUCAAAACACCAAUGACAUUGGAUCAAACAAAUGCUAAACCCGUACCAUUGCCACCACAA
 P  G  P  G  L  K  T  P  M  T  L  D  Q  T  N  A  K  P  V  P  L  P  P  Q
                   ──────────────────── B3 (118-144) ──────────────►
```

```
GGAUCAGAUGUAAAAGUUGGUGGACCAGGACCAGGACAGGAAGGAAGUUAUUCAUUACCCGGACCAGGACCA
 G  S  D  V  K  V  G  G  P  G  P  G  Q  E  G  S  Y  S  L  P  G  P  G  P
           ──────────────────►       ◄───── B4 (156-163) ─────►
```

```
GGAGAUGUCGCUAAUGGUGGUGUUGAUUCAUGUCAAGGUGAUUCUGGCGGACCAGUUGUUGAUUAA
 G  D  V  A  N  G  G  V  D  S  C  Q  G  D  S  G  G  P  V  V  D
   ◄──────────────────────── B5 (199-218) ──────────────────────►
```

Fig. 1.—Design of Der f3-peptides containing T cell epitopes and B cell epitopes of Der f3.
(A) Schematic representations of T cell epitopes and B cell epitopes of Der f3, spacer and Der f3-peptides. The numbers of amino acids are shown above respectively. (B) Nucleotide and amino acid sequences of Der f3-peptides. The position of each T cell and B cell determinant is indicated below the amino acid sequence. The spacer is indicated too.

28a (+)-Der f 3-peptides transformed *Escherichia coli* cells [BL21 (DE3)] stain was grown in LB medium at 37ºC to an optical density absorbance at 600 nm of 0.4–0.6 and then induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 37ºC. The *E. coli* cells were collected by centrifugation. After washing the cells with washing buffer [10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 0.1 M NaCl], cells were resuspended in PBS containing 0.5M NaCL, 100 mg/ml phenylmethylsulfonyl fluoride (PMSF), 20mM Tris-HCl, 10% glycerol and 8M Urea, then disrupted by sonication. The sonicated solution was centrifuged at 12 000×g for 20 min at 4ºC, and the Der f 3-peptides inclusion body was dissolved. After filtering using a 0.45 mm cellulose acetate filter, the His-tagged Der f 3-peptides protein was purified using a Ni-NTA column according to the manufacturer's instructions and eluted with buffer (20mM Tris-HCl, 8M Urea, 500mM NaCl and 500 mM midazole). The purified protein was analyzed by electrophoresis on a 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel and visualized by coomassie brilliant blue (CBB). Purified Der f 3-peptides was dialyzed against distilled water and then lyophilized. The protein was dissolved in 8 M urea/PBS to a concentration of 10 mg/ml and stored at -20°C before use. Western blotting was used to confirm the specificity of the purified Der f 3-peptides protein with primary human anti-Der f 3 serum IgG previously prepared (The serum samples was obtained under the consent from the inpatients of

the Department of Respiratory Medicine of Yijishan Hospital, Wannan Medical College) as the primary antibody and peroxidase-conjugated rabbit anti-human IgG as the secondary antibody. The immunoblots were visualized using enhanced chemiluminescence (ECL) technology (Fig. 3).

APCs

PBMC were isolated from heparinized blood from negative ones by density centrifugation. EBV-B cells are established by cultivation of PBMC after in vitro infection of EBV, obtained from the culture of cell line B95-8 in the presence of 1 mg/ml cyclosporin A. EBV-B cells were cultured in RPMI 1640 supplemented with 10 to 12% FCS. EBV-B cells were treated with 50 mg/ml mitomycin C (MMC) for 30 min and then washed four times with RPMI 1640, after which they were used as APC.
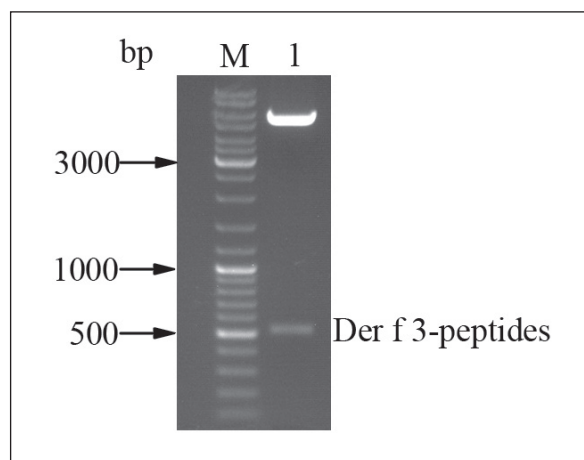
Fig. 2.—Recombinant plasmid Pet28a (+)-Der f3-peptides enzyme cut figure.
M: Maker; 1: Der f3-peptides.

Lymphocyte proliferation assays

To prepare human T-cell lines, 2×10^5 peripheral blood mononuclear cells (PBMC) from newly diagnosed mite-allergic asthmatic donors (judged by skin test, diameter of erythema >20 mm) were stimulated with Der f3 (5 mg: ml) for 7 days in LB medium were purified using nylon wool column as described. Then T-cell lines cells (2×10^5) were co-cultured for additional 7 days with Der f3-peptides (5 mg: ml), r-human IL-2 (10 U: ml, Sangon Biotech, Shanghai, China), and freshly prepared 2×10^5 MMC-treated PBMC as APC in the environment of 5% CO_2 at 37ºC. Cytokine production by T cell lines supernatants (0.1 ml/well) were collected from the proliferation assay plates 72 h after T cell stimulation and stored at -70ºC. The levels of IL-2, IL-4, IL-5 and IFN-γ were measured in duplicate using commercial enzyme-linked immunosorbent assay (ELISA) kits. Then CCK-8 (10 μl/well) was added into the hole and sample absorbance values at 450 nm wavelength were measured using ELIASA microplate reader and the stimulation indexes (SI) were calculated. SI value≥2.0 was judgmented as positive.

ELISA for allergen-specific IgE bind assays

1 mg of Der f3 and 1 mg of Der f3-peptides were coated on a 96-well black plate at 4°C for 16 h. Before blocking the plate with 200μl PBST and 10 g/L BSA at 37°C, TBST (50 mmol/L Tris, pH 7.5; 150 mmol/L NaCl; 0.1% Tween-20) was used to wash the plate 5 times. Then 100 ml of threefold diluted serum with TBST from 12 patients was transferred to individual wells and incubation for 1 h at 37°C. After washing the plate 5 times with TBST, HRP labeled IgE antibody (1:1000) against human was transferred to individual wells and incubated for 2 h at 37°C. After washing the plate with TBST, the reaction liquid TMB was added.
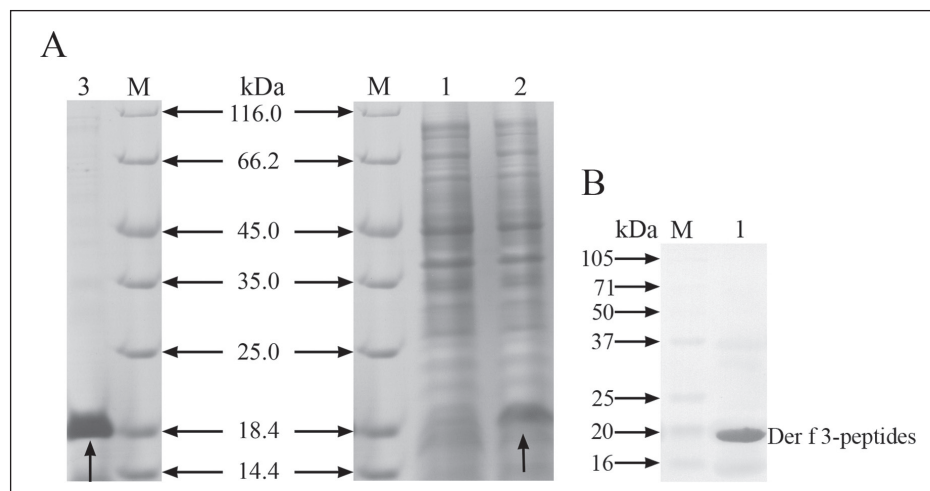

Fig. 3.—The analyze results of SDS-PAGE and WB to Der f3-peptides protein.
(A) The SDS-PAGE electrophoresis result of unpurified and purified recombinant protein Der f3-peptides. M: Maker; 1: The E. coli BL21 without pET28a (+)-Der f3-peptides; 2: The E. coli BL21 with pET28a (+)-Der f3-peptides; 3: Purified protein of Der f3-peptides. (B) The WB result of Der f3-peptides. M: Maker; 1: Purified Der f3-peptides protein.

Twenty minutes later, the stop buffer (50µl/well) was added to stop the reaction. The amounts of IgE bound to the allergens were measured by the ELISA microplate reader at A450.

Statistical analyses

Differences among groups were analyzed using One-Way ANOVA analysis and data were demonstrated in $\bar{\chi} \pm s$. Statistical analysis was performed using the SPSS16.0 and the difference was considered statistically significant when p value was less than 0.05.

Results

Construction of plasmid pET28a (+)-Der f3-peptides and its expression in E. coli BL21

The coding sequence of Der f 3-peptides amplified by PCR was cloned into the *Bam*H I and *Xho* I sites of prokaryotic expression vector pET28a (+), resulting in the recombinant plasmid pET28a (+)-Der f 3-peptides. Enzymes digestion was used to confirm the construction (Fig. 2). *E. coli* BL21 (DE3) strain containing Der f 3-peptides expressed a protein with approximately 19kDa upon induction with IPTG, which was identified by SDS-PAGE (Fig. 3A). Accordingly, the Der f 3-peptides protein was bound with Ni-NTA purified column under denaturing conditions, and was purified using elution buffer containing 30 mM imidazole (Fig. 3A). The specificity of purified protein was confirmed by Western blotting as shown in Figure 3B.

Proliferative responses of Der f 3-peptides-induced T cell lines

SI value of Der f 3 group and Der f 3-peptides group were both higher than that of PBS group ($P<0.05$). The capacity of specific T proliferative response induced by Der f 3-peptides was not less than that induced by Der f 3 (Fig. 4).

The levels of cytokines from T lymphocytes stimulated with Der f 3 and Der f 3-peptides

Der f 3 group and Der f 3 peptides group induced higher levels of IFN-γ and IL-2 than PBS group. However, there was no statistical difference between those two groups ($P>0.05$). Moreover, the Der f 3 and Der f 3 peptides induced secretions of IL-4 and IL-5 were decreased compared with that of PBS group ($P<0.05$), whereas the difference between those two group has no statistically significant ($P>0.05$) (Fig. 5).
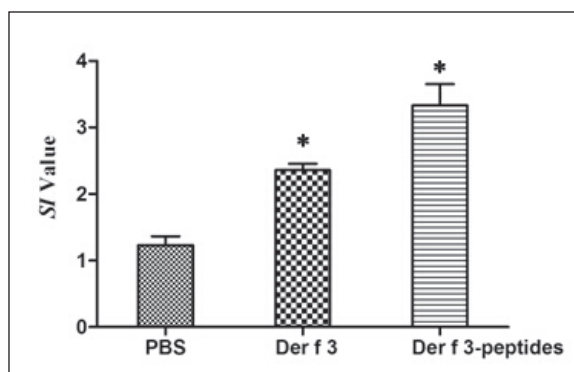

Fig. 4.—T lymphocyte proliferation experiment.
SI value of Der f 3 group and Der f 3-peptides group were both higher than that of PBS group. * P<0.05 compared with PBS group.

Capacity of IgE-binding to Der f 3-peptides

We found that the capacity of IgE-binding to Der f 3-peptides (41.25±5.67) µg/ml was decreased dramatically compared with that of Der f 3 (83.60±10.92) µg/ml ($P < 0.05$) (Fig. 6).

Discussion

Type I allergic diseases such as allergic asthma, allergic rhinitis and atopic dermatitis are increasing in prevalence worldwide, which has become one of global health problems today. Its total incidence rate reaches as high as 10% ∼ 30[13,14]. Allergic asthma is the hot topic in recent years and previous studies have proved that the imbalance of Th1/Th2 was a key factor contributing to asthma [15]. Th2-type cytokines, such as interleukin (IL)-4, IL-5, and IL-13, are thought to drive the accumulation of eosinophils in the lungs of asthmatic patients. Th1 cells release interleukin (IL)-2 and interferon (IFN)-γ which could inhibit the development of Th2 by inhibiting the antibody IgE induced by IL-4.

Epitope, also known as antigenic determinant, is refer to linear segments of antigen from molecules on the cell surface after antigen presented cells (APCs) treatment during specific immune response. Generally, epitope contains 5 - 7 amino acids or monosaccharide residues and less than 20 amino acid residues. It represents as immune active area of antigen molecule, which could be recognized and stimulate the body to produce antibodies or sensitized lymphocytes. It is strictly said that the specificity of the antibodies is not aimed at complete antigen molecules but the epitopes[16,17].Therefore, epitope is regarded as the basis of the antigenic protein, also the basic structure and functional unit which induces the specific immune response. T/B cell epitopes are clips which can be recognized by T cell surface receptors or antibody[12,18]. In our work, three T cell epitopes and five B cell epitopes
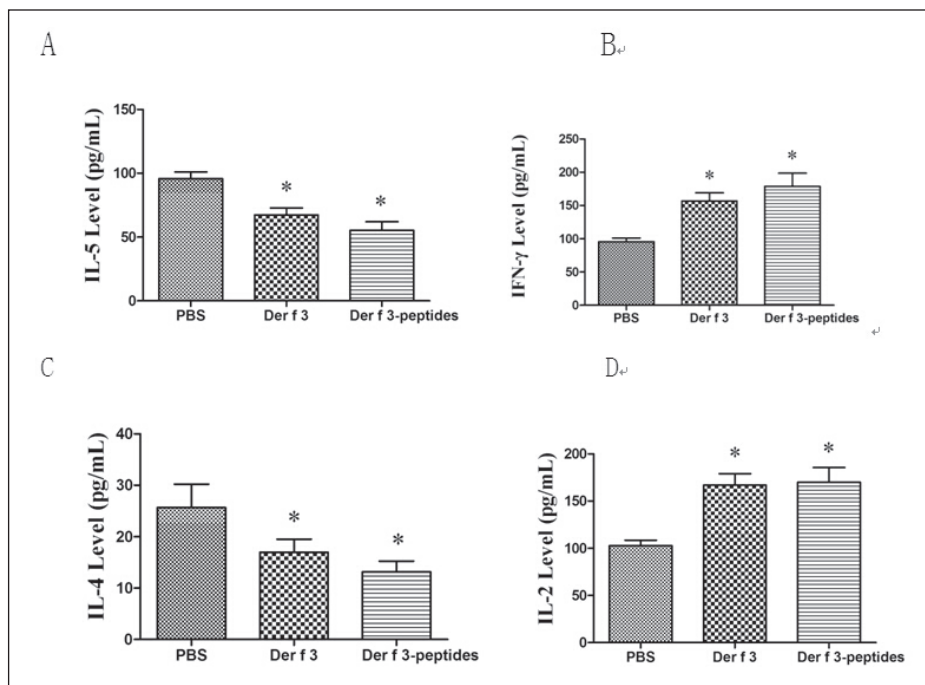
Fig. 5.—The levels of various cytokines in the T lymphocyte culture supernatant.
(A) The level of cytokines IL-5 in the T lymphocyte culture supernatant; (B) The level of cytokines IFN-γ in the T lymphocyte culture supernatant; (C) The level of cytokines IL-4 in the T lymphocyte culture supernatant; (D)The level of cytokines IL-2 in the T lymphocyte culture supernatant. * P<0.05 compared with PBS group.
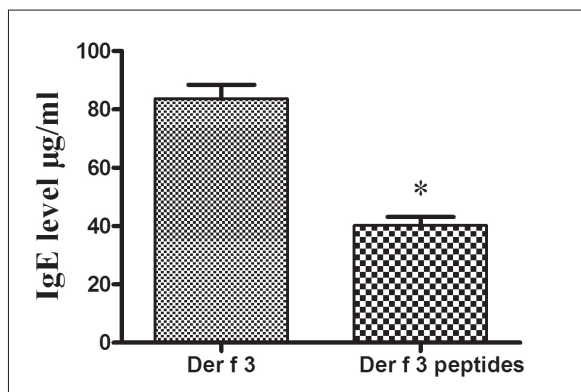

Fig. 6.—The capacity of IgE-binding force of Der f3-peptides with Der f3.
*P<0.05 compared with Der f 3 group.

were successfully obtained using epitope prediction software and immunology experiment, which will lay a foundation for further research of low-toxic allergens with small molecule that can connect T cell epitopes and B cell epitopes in series.

Epitope vaccine is a new type of vaccine developed in recent years, which is expressed in vitro or synthesized using genetic technology[12,18-20]. In the early 1980s, epitope-based vaccine is considered to be an ultimate synthetic vaccine in the future due to its specificity, high security, easy generation, storage and use[21]. Human immune system includes humoral immunity and cellular immunity. Therefore, some epitope-based vaccines containing several B cell epitopes and T cell epitopes of certain allergens may become complete allergic proteins, and induce strong humoral immunity and cellular immunity. Compared with traditional vaccines, epitope-based vaccine has many advantages, combined with multiple genetic background of MHC molecules, it is able to induce efficient presentation. It can overcome the variation of pathogenic microorganisms and effectively deal with many adverse factors in immune response [22]. The major advantage lies in the ability to overcome the possibility of virulence recovery or spread caused by the traditional vaccine. In addition, epitope-based vaccine not only boosts the immunogenicity of antigen epitope, but also quantifies the antigen epitope accurately, which is in accordance with future development of prospective vaccines.

To develop low-toxic allergens with small molecule which connected T cell epitopes and B cell epitopes in series, we constructed the chimeric gene and prokaryotic expression vector pET28a (+)-Der f 3-peptides containing three T cell epitope and five B cell epitope from Der f 3. To avoid producing new antigen epitopes between two consecutive epitopes, a flexible short peptide sequence named GPGPG was inserted into every two epitopes[23] .The chimeric protein Der f 3-peptides was expressed successfully by IPTG and identified by Western Blotting. The chimeric protein Der f 3-peptides enhanced T cell proliferation (similar to Der f 3) detected by modified MTT method, increased IL-2/IFN-γ secretion and decreased IL-4 and IL-5 production compared with that of Der f 3 (P<0.05). Furthermore, the capacity of IgE-binding with Der f 3-peptides was decreased significantly compared with that of Der f 3. These work indicate that a low-toxic allergen with small molecule which connects T cell epitopes and B cell epitopes in series is effective in immunotherapy for allergic asthma, and also provide

new idea for further research of vaccines for even wider clinical treatment of allergic asthma.

Epitope peptide has strong immunogenicity in spite of its low molecular weight, and can induce protective immune responses to complex allergens. Epitope-based vaccine has achieved encouraging results, however, most studies are still at the experimental stage because many problems remain unsolved: 1) Technology of permutation and combination of epitopes plays a key role in the construction of epitope-based vaccine, yet such technology is not mature. 2) How to avoid the influence between neighbouring epitopes needs further exploration. 3) How to choose the effective, safe adjuvants which could affect the efficacy of epitope-based vaccine profoundly needs further exploration. The epitope-based vaccines have already shown promising expectation despite fewer epitope-based vaccines for clinical use at present. We believe more technologies will be utilized to screen and identify more efficient and predominant epitopes, which should provide broader prospects for diagnosis and therapy of allergic diseases.

Conclusions

Our results suggest that several major T cell epitopes and B cell epitopes from Der f3 could be chosen in the design of peptide based immunotherapy for the management of mites allergic asthma in subjects having various types of HLA class II molecules.

Acknowledgements

Disclosure of conflict of interest

None.

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