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Construction and expression of *Dermatophagoides pteronyssinus* group 1 major allergen T cell fusion epitope peptide vaccine vector based on the MHC II pathway

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Abstract

**Background and aims:** Dermatophagoides pteronyssinus is one of the important house dust mites responsible for allergic asthma that can be tentatively managed by specific immunotherapy. The present study was to construct a vector encoding T-cell epitopes of major allergen group 1 of *Dermatophagoides pteronyssinus* as a vaccine delivered by MHC class II pathway.

**Methods:** the nucleotide sequences of the 3 target genes were synthesized, including TAT, IhC and the recombinant fragment of Der p 1 encoding 3 T-cell epitopes. After amplification of the 3 target fragments by PCR and digestion with corresponding restriction endonucleases, the recombinant gene TAT-IhC-Der p 1-3T was ligated using T4 DNA ligase and inserted into the prokaryotic expression vector pET28a(+) to construct the recombinant plasmid pET-28a(+)−TAT-IhC-Der p 1-3T, which was confirmed by digestion with restriction endonucleases and sequencing. The recombinant vector was transformed into *E. coli* strain BL21 (DE3) and induced with IPTG, and the induced protein TAT-IhC-Der p1-3T was detected by SDS-PAGE. After purification, the recombinant protein was confirmed by Western blotting and its allergenicity tested using IgE-binding assay.

**Results:** the recombinant plasmid pET-28a(+)−TAT-IhC-Der p1-3T was successfully constructed as confirmed by restriction endonuclease digestion and sequencing, and the expression of the recombinant protein TAT-IhC-Der p1-3T was induced in *E. coli*. Western blotting verified successfull purification of the target protein, which showed a stronger IgE-binding ability than Der p1.

**Conclusion:** we successfully constructed the recombinant expression vector pET-28a-TAT-IhC-Der p1-3T expressing a T-cell epitope vaccine delivered by MHC II pathway with strong IgE-binding ability, which provides strong IgE-binding ability than Der p1.

**Conclusión:** hemos construido con éxito el vector de expresión recombinante pET−28a−TAT−IhC−Der p1-3T que expresa una vacuna de epítopo de células T administrada por vía MHC II con fuerte capacidad de unión a IgE. Este trabajo proporciona una base para seguir
Introduction

Allergic asthma is a Type I allergy, primarily characterized in clinic by reversible airway obstruction, bronchial inflammation and mucus hypersecretion or the similar symptoms\(^1,2\) and commonly caused by abnormal immunoreactivity against allergens such as one important species — *Dermatophagoides pteronyssinus* (Der p). The allergens are found positive in about 80\% asthmatics allergic to *Der p*\(^1,6\). Currently, specific immunotherapy (SIT) is considered the only etiological therapy that can ameliorate the allergic symptoms for long period\(^7-10\), yet responsive stimulation of T cells is essential for desensitization and reduction or inhibition of allergen specific IgE\(^2,11,12\). Previous experiment on the use of human T cells epitope peptide from Fel d 1 allergen for SIT demonstrated a change of the reaction of T cells to these peptides\(^13\). The potential mechanism may be that the allergen was up-taken through antigen presenting cells (APC), and antigen peptide was generated by hydrolysis of the lysosomal protease. After the MHC II from endoplasmic reticulum was protected by the invariant chain (Ii) and enters the lysosomes, Ii was hydrolyzed, leading to formation of the MHC II molecule with peptide that are finally presented to CD4+ T cells\(^14\). Antigen presentation through the MHC II pathway is recognized as a potential technique to the treatment of allergic diseases\(^15\). Studies have shown that construction and prokaryotic expression of MHC II-peptide fusion protein can promote effective presentation of the peptides\(^16\), because the sequence of TAT, with 11 amino acids (GYGRKKRRQRRR) derived from the human immunodeficiency virus (HIV), can transfer to the proteases from extracellular into intracellular\(^17\), whereas the IhC is short peptide of the first 110 amino acids from human lysosomal Ii, and can target protein at the endosome lysosomes\(^18,19\). Thus, the vaccine constructed on TAT-IhC-antigen fusion protein basis may effectively alleviate the symptoms of patients with asthma\(^20\).

In present study, we tentatively applied the sequences of TAT, IhC and Der p1, T cell epitopes, to fusion of the gene by molecular biology technique. The fused gene was defined as TAT-IhC-Der p1-3T, which was subjected to prokaryotic expression in vector pET28a (+)-TAT-IhC-Der p1-3T, and further expression and purification by IPTG induction. The purified product was tested for the allergenicity via observation on its binding capacity with IgE, with an attempt to lay a foundation for preparation of such clinical vaccine with high efficacy on MHC II pathways.

Methods

Bacterial strains and plasmids

*Escherichia coli* DH-5, BL21 (DE3) competent cells and PET-28a vector were preservations of our laboratory.

Design and synthesis of the primers

Primers were designed based on the nucleotide sequences of TAT (No: NP_057853.1), IhC (1-110AA) (No: K01144.1) and ProDer p1 (No: NP_11695.1) released in the GenBank, by using corresponding restriction sites.

The primers for TAT (e-ncoding 11AA) included the upstream primer 5'-GATCTTTACGGTCTAAAGAGCTCGCCAGCTCGCGCGACGCTTTTTACGACCGTA-3' (BamH\(i\)) and the downstream primer 5’-TCGAGACTAGTGGATCCACGGCGACGCTCGCTCGCGCGACGCTTTTTACGACCGTA-3’ (Xho\(i\)). And the primers for IhC(1-110AA) were consisted of the upstream prime 5’-GAAGATCTATGGATGACCAAGCGCGACC-3’ (Bgl\(ii\)) and the downstream primer 5’-AAACTAGTGAGATCCCCCTCTGGGCAGG-GCTCC-3’ (Hind\(ii\)).

The three T cell epitopes matching with the nucleotide sequence of ProDer p1 (118-146, 175-196 and 206-264) reported previously were synthesized into a complete nucleotide sequence with a length of 324bp, which was defined as Der p1-3T). The primers for PCR amplification comprised 5’-TATGGATCC-GAAGATCTATGGATGACCAAGCGCGACC-3’ (BamH\(i\)) as upstream primer and 5’-AGCCTCGAGTGGTAAATCATATGCGGATT-3’ (Xho\(i\)) as downstream primer. Gene fusion, together with its synthesis of the fragments, was contracted by Sangon Biotech (Shanghai) Co., Ltd.

Main reagents

DL2000 DNA Marker and T4DNA ligase were purchased from Takara Biotechnology Co., Ltd (Dalian, }
Bgl II and (1-110AA) were subjected to double digestion with commercial kit, and pET28a (+)-TAT and IhC respectively in total 35 cycles, followed by extension for 4 min, 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, respectively in the conditions described above. The enzyme products were recovered, and the Der p 1-3T was connected to the pET28a (+)-TAT-IhC by T4 DNA ligase to create pET28a (+)-TAT-IhC-Der p 1-3T vector.

Identification of recombinant vector pET28a (+)-TAT-IhC-Der p 1-3T

Enzyme digestion was performed in a total volume of 80μl, which included 10×Tango buffer (8 μl) BamH I (2 μl), Xho I (2 μl), pET28a (+)-TAT-IhC-Der p 1-3T (24 μl) and ddH2O (44 μl) at 37°C for 3 h. The digested products were tested by agarose gel electrophoresis, and the positive clones were contracted confirmation and sequencing by Sangon Biotech (Shanghai) Co., Ltd.

Inducing the recombinant protein TAT-IhC-Der P 1-3T expression

The pET-28α(+) -TAT-IhC-Der P 1-3T recombinant plasmids were transferred to E.coli BL21 (DE3), the competent cells, and then applied to culture plate containing LB solid culture medium diluted in 100 mg/L Kana+ by single colony, which was cultured overnight at 37°C by 200 r/min. The ratio of cells to culture medium was adjusted to 1: 50, and set to 0.6 by OD600 measurement. IPTG (final concentration: 1 mmol/L) was added to further culture the cells for 4-5 h at 200r/min. Then, 1.5 ml bacteria liquid was taken, and centrifuged at 4°C by 10000 r/min for 5 min. The supernatant was removed, and 100 μl (2×) protein sample buffer was added and mixed thoroughly. The solution was boiled for 10 min, and 20 μl of it was taken and treated in 12.5% SDS-PAGE, followed by Kaumas brilliant blue staining before photographing.

Purification of recombinant protein TAT-IhC-Der P 1-3T

E. coli BL21 (DE3) strains containing pET-28α (+)-TAT-IhC-Der p 1-3T recombinant plasmid were cultured on a large scale; and the cells were collected after precipitation. Ni2+-NTA resin was used to isolate and purify the recombinant TAT-IhC-Der p 1-3T as the user instructions. , and the purity was determined by SDS-PAGE.

Identification of recombinant protein TAT-IhC-Der p 1-3T

BCA protein assay kit and BSA standard were used to determine the protein concentration according the user protocol. After quantifying the protein, 10 μg total protein was taken for separation by 12.5% SDS-PAGE...
GE, and then transferred to nitrocellulose membrane containing 1% BSA TBST (50 mmol/L Tris; pH 7.5; 150 mmol/L NaCl; 0.1% Tween-20), which was blocked for 2 h at room temperature. Der p 1 antibody diluted in 1: 500 was applied to the membrane that was cultured overnight at 4°C, followed by rinsing three times with TBST(10 min/each rinse). Second HRP-Goat anti-rabbit IgG (1: 5000 dilution) was added to the membrane, which was incubated for 40 min at 37°C, subjected to three rinses (10 min/each rinsing) and exposure by ECL technique.

**Determination of TAT-IhC-Der P 1-3T**

The binding capacity of TAT-IhC-Der P 1-3T with IgE was tested using ELISA as procedures described in previous literature. Der p 1 was initially applied to a 96-well plate (500 ng/each well) that was coated overnight at 4°C, and TBST was used to rinse the plate 5 times (100 μl/each well). Then the plate was blocked with TBST containing 1% BSA (150 μl/each well) for 1 h at 37°C. Sera from the asthmatics were added by dilution ratio of 1:8, and incubated for 1 h at 37°C. The plate was rinsed 5 times with TBST, and added with TMB substrate and reacted for 20 min at 37°C. The reaction was terminated by stop buffer (50 μl/each well), and measured at A_{450} nm.

**Results**

**Identification of recombinant vector pET28a (+)-TAT-IhC-Der p 1-3T**

The recombinant plasmid pET28a (+)-TAT-IhC-Der p 1-3T was doubly digested with BamH I and Xho I, and electrophoresis revealed a gene strip with approximate 800 bp (Fig. 1), which indicated that the recombinant plasmids were successfully constructed. By sequencing and NCBI-BLAST results, the size of the recombinants was 687 bp, which was consistent with that of TAT-IhC-Der p 1-3T gene.

**Expression of the recombinant protein TAT-IhC-Der P 1-3T**

In order to observe whether TAT-IhC-Der P 1-3T could induce expression, we transfected the recombinant vector pET28a (+)-TAT-IhC-Der p 1-3T with E. coli BL21 (DE3). After induction by IPTG at its optimal final concentration of 1 mmol/L and confirmation by SDS-PAGE, the strip indicating TAT-IhC-Der p 1-3T protein was exposed (Fig. 2), suggesting that this protein was successfully induced. The relative molecular mass was about 25000, which is consistent with the theoretical value. Single band was evident after treatment by Ni²⁺-NTA resin (Fig. 2).
Western blot detection of recombinant protein
TAT-IhC-Der p 1-3T

To verify the purified recombinant protein band for TAT-IhC-Der p 1-3T, Western blot was performed by using Der p 1 polyclonal antibody. The results demonstrated an evident strip for TAT-IhC-Der p1-3T instead of the control E. coli BL21 (DE3) (Fig. 3). The size of the recombinant was comparable with that on SDS-PAGE, which indicated that TAT-IhC-Der p 1-3T was successfully purified, and can be used for further test.

TAT-IhC-Der P 1-3T ELISA IgE binding ability test

Recombinant protein TAT-IhC-Der p 1-3T was prokaryotically expressed and tested for its binding capacity with IgE in 16 aliquots of sera from patients sensitized by house dust mites. ELISA assay demonstrated a higher binding power of TAT-IhC-Der p 1-3T to bind to IgE over single Der p 1[(1.35±0.33) vs. (0.80±0.19)] (P<0.001). The findings showed that TAT-IhC-Der p 1-3T had excellent allergenicity.

Discussion

SIT is considered the only etiological therapy to modify the allergic disease process20, and can effectively induce the production of allergen specific IgG1 and IgG4, thus leading the reduced antibody levels of allergen specific IgE, and blocking the occurrence of type I hypersensitivity21. SIT not only reduces the allergic sensitization, but also the incidence of asthma after treatment22. An early study on SIT by using recombinant pollen allergen Bet V 1 with T cell epitopes being reserved while B cell epitopes being reduced had reduced the allergen specific IgE production, and prevented the allergens from binding to IgE and/or stimulated inability of body to induce antigen-specific T cells23. These findings suggest that optimal SIT should be involved in vaccines with hypoallergenic (reduced B cell epitope), enhanced immunogenic (increased T cell epitope vaccine)2. Prickett et al once proved the efficacy of CD4+T cell epitope peptide derived from Ara h 1 allergen by stimulating the mononuclear cells from peripheral blood of patients allergic to peanuts24, and Mackenzie et al. (2013) used the dominant T cell epitope peptide extracted from the ovalbumin of chicken egg for SIT, and found that this peptide had effectively inhibited the production of allergen specific IgE and eosinophilia25.

Exogenous antigen uptaken by APCs can be degraded and bound with MHC II, and activated after presenting to CD4+T cells, which leads to antigen specific immune responses. The effect of SIT by MHC II pathway has been confirmed26. However, the efficacy may be boosted if MHC II pathway can be presented through multiple T cell epitope peptide fusion protein.

In view of the TAT and IhC functions as well as the role of T cell epitopes in SIT, we tentatively constructed recombinant TAT-IhC-Der p 1-3T and prokaryotically expressed it in vector pET-28a-TAT-IhC-Der p 1-3T. After induction by IPTG and SDS-PAGE analysis, we found that this fusion allergen can be effectively expressed. Further Western blot assay proved that the allergens were successfully purified. Following experiments showed that the capacity of TAT-IhC-Der p 1-3T in binding with IgE is superior to Der p 1, and our findings are inconsistent with previous reports. Kamloo26 constructed a chimeric protein from the melittin, which contains Api M 1, Api M 2 and Api m3 T cell epitopes, and successfully removed the B cell epitopes from the antigen. And his following test proved that the chimeric protein had lower binding potential with IgE than the natural allergen. Contrarily, our TAT-IhC-Der p 1-3T protein had enhanced power of allergen, this is probably associated with the overlapping of T cell and B cell epitopes in Der p 123, and emergence of new epitopes of B cell configuration or linear epitope. Yet the exact mechanism remains unclear.

In summary, we successfully obtained the fusion protein that can be intracellularly transferred and anchored in the lysosomes, and enhance T cell epitope peptide. This work may lay a foundation for vaccine development in therapy of asthmatic patients caused by house dust mites, particularly the vaccine on SIT basis.

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References