Nutrición Hospitalaria

Nutr Hosp. 2015;32(6):2771-2776
ISSN 0212-1611 • CODEN NUHOEQ
S.V.R. 318

Original / *Investigación animal*

Prokaryotic expression and bioactivity evaluation of the chimeric gene derived from the group 1 allergens of dust mites

Xiaodong Zhan[1,2], Chaopin Li[1], Wei Guo[1] and Yuxin Jiang[1]

[1]*Deparment of Medical Parasitology, Wannan Medical College, Wuhu 241002 (Anhui), People's Republic of China.* [2]*College of life science, Anhui Normal University, Wuhu 241002 (Anhui), People's Republic of China.*

Abstract

Background: **we successfully reconstituted the gene from group 1 allergens of dust mites, and obtained a body of shuffled genes. In order to verify the prediction on the chimeric gene, we tentatively cloned R8 into the vector that was prokaryoticly expressed, purified and assessed for its bio-activities.**

Methods: **the expressed product was detected by SDS-PAGE and the target protein was purified. The purified protein R8 was detected by ELISA. 75 BALB/c mice were divided into 5 groups, namely PBS, rDer f1, rDer p1, R8 and asthma group. The mice were treated with dust mite allergens at 0, 7, 14 day by intraperitoneal injection and inhaled challenge as aerosol on day 21 for 7 days. Specific allergen immunotherapy was performed using rDer f1, rDer p1 and R8 allergens respectively. The level of IFN and IL- 4 in BALF was detected by ELISA.**

Results: **the chimeric gene R8 was expressed with a band of approximately Mr 35000. Compared with groups of rDer f 1 and rDer p 1 [(80.44±15.50) and (90.79±10.38) μg/ml respectively], IgE binding capacity of the protein R8 (37.03±12.46) μg/ml was statistically lower (P<0.001). The level of IFN in sera of R8 group [(343.43±38.79) pg/ml] was higher than that of the PBS and asthma groups [(393.93±50.68) and (208.44±46.11) pg/ml respectively] (P<0.01), but no statistical difference to that of the rDer f 1 and rDer p 1 groups (P>0.05). IL-4 level in R8 group was lower markedly than the others (P<0.05 or P<0.01).**

Conclusions: **chimeric protein R8 derived from the group 1 allergens of dust mites has been expressed with low allergenicity and high immunogenicity.**

(*Nutr Hosp*. 2015;32:2771-2776)

DOI:10.3305/nh.2015.32.6.9732

Key words: *Dust mite. Allergen. Chimeric protein . Allergenicity. Immunogenicity.*

EXPRESIÓN PROCARIOTA Y EVALUACIÓN DE LA BIOACTIVIDAD DEL GEN QUIMÉRICO DERIVADO DEL GRUPO 1 DE LOS ALÉRGENOS DE LOS ÁCAROS DEL POLVO

Resumen

Antecedentes: **se reconstituyó con éxito el gen del grupo 1 alérgenos de los ácaros del polvo, y obtuvo un conjunto de genes barajadas. Con el fin de verificar la predicción en el gen quimérico, hemos clonado tentativamente R8 en el vector que se expresó prokaryoticly, purificó y se evaluó por sus actividades-bio.**

Métodos: **el producto expresado se detectó por SDS-PAGE y la proteína diana se purificó. La proteína purificada R8 se detectó por ELISA. Setenta y cinco ratones BALB/ c se dividieron en 5 grupos, a saber: PBS, rDer f1, rDer p1, R8 y el grupo de asma. Los ratones fueron tratados con alérgenos de ácaros del polvo a los 0, 7, 14 días mediante inyección intraperitoneal y inhaladas desafío como aerosol en día 21 durante 7 días. La inmunoterapia específica para el alérgeno se realizó utilizando rDer f1, rDer p1 y alérgenos R8, respectivamente. El nivel de IFN e IL-4 en BALF se detectó por ELISA.**

Resultados: **el gen quimérico R8 se expresó con una banda de aproximadamente Mr 35000. En comparación con los grupos de rDer f 1 y rDer p 1 [(80,44 ± 15,50) y (90,79 ± 10,38) μg/ml, respectivamente], la capacidad de unión a IgE de la proteína R8 (37,03 ± 12,46) μg/ml fue estadísticamente inferior (P<0,001). El nivel de IFN en el suero del grupo R8 [(343,43 ± 38,79) pg /ml] fue mayor que el de la PBS y los grupos de asma [(393,93 ± 50,68) y (208,44 ± 46,11) pg/ml, respectivamente] (P <0,01), pero no hubo diferencia estadística a la de los grupos rDer f 1 y rDer p 1 (P> 0,05). La IL-4 en el grupo R8 fue menor significativamente que las otras (P <0,05 o P <0,01).**

Conclusiones: **la proteína quimérica R8 derivados de los grupos 1 alérgenos de los ácaros del polvo se ha expresado con baja alergenicidad y alta inmunogenicidad.**

(*Nutr Hosp*. 2015;32:2771-2776)

DOI:10.3305/nh.2015.32.6.9732

Palabras clave: *Ácaros del polvo. Alérgeno. Proteína quimérica. Alergenicidad. Inmunogenicidad.*

Correspondence: Chao-pin Li.
Department of Medical parasitology.
Wannan Medical College.
Wuhu 241002 (Anhui), People's Republic of China.
E-mail: cpli001@126.com

Recibido: 4-VIII-2015.
Aceptado: 6-IX-2015.

Abbreviations

IPTG: isopropyl-β-D-1-thiogalactopyranoside
BALF: bronchial alveolar lavage fluid
Der f 1: *Dermatophagoides farinae* 1
Der p 1: *Dermatophagoides pteronyssinus* 1
SIT: specific immunotherapy
RAST: radioallergosorbent test
ELISA: enzyme-linked immuno sorbent assay

Introduction

Group 1 allergens of dust mites is currently recognized as primary allergen leading to allergic diseases[1], and such allergens are found to be bound with serum IgE in more than 80% of the patients allergic to dust mites[2,3], in which *Dermatophagoides farinae* 1 (Der f 1) and *Dermatophagoides pteronyssinus* 1 (Der p 1) are common prevalent and one of the important roles in inducing asthma and other allergic diseases[4]. In recent years, the group 1 allergens, being the major airborne allergen, attracted a lot of research interest in allergic diseases. Allergen specific immunotherapy (SIT) is the current available strategy that can sustainably ameliorate the allergic symptoms[5]. However, traditional vaccines are associated with a series of defects, including complex allergen components, difficulty in standardization, and easy to produce local or systemic adverse reactions[6,7]. Therefore, the promising SIT on security basis should be the allergen with high purity and hypoallergenicity. Fortunately, introduction of DNA shuffling and gene selection, a technology based on molecular directed evolution, has opened the door to obtain the allergic protein that is modified and reliable for such purpose[8,9], and has been widely applied to development of bio-pharmaceutics and vaccines as well as gene therapy. Nevertheless, few reports are available on application of such technology to preparation of proteins, with hypoallegenicity and high immunogenicity, from the group 1 allergens of dust mites. In our previous laboratory work, we successfully reconstituted the gene from group 1 allergens of dust mites, including *Der f* 1 and *Der p1*, and obtained a body of shuffled genes, which were subjected to on-line bioinformatics analysis for the changes of their amino acid sequences at the epitopes of T and B cells. Online prediction showed that T cell epitopes were dominant, whereas B cell epitopes were less in some of the recombined proteins in which the chimeric *R8* gene was particular representative. In order to verify the prediction on the chimeric gene, we tentatively cloned *R8* into the vector that was prokaryoticly expressed, purified and assessed for its bio-activities with ELISA for supportive evidences for immunotherapy of asthma and other allergic reactions of type I disease on SIT basis.

Materials and methods

Materials

Experimental animals, bacterial strains and plasmids

A total of 75 BALB/c mice, weighing 18-22 g, were purchased from the Center of Comparative Medicine, Yangzhou University (Jiangsu, China). *E.coli* line BL21, the pUCm-T recombinant plasmid containing the *R8* chimeric gene and the pET28a (+) empty plasmid were preservations of our laboratory.

Allergens and sera from dust mite allergic patients

Prokaryoticly expressed recombinant allergens rDer f 1 and rDer p1 were purified and preserved in our laboratory. Blood samples were obtained under the consent from 12 asthmatic inpatients, whose skin prick was positive and radioallergosorbent test (RAST)>kU/L, in the affiliated Yijishan Hospital of Wannan Medical College, Wuhu, Anhui, China.

Reagents

Dermatophagoides farinae injection was purchased from the Shanghai Fudan Forward Pharmaceutical Co., Ltd (Shanghai, China). SK2072 PCR amplification kit, SanPrep column DNA gel extraction kit, the SanPrep column PCR product purification kit, 200 bp DNA marker (BSM0633), prestained polyacrylamide gel electrophoresis standard (RM008 and RM009), Ni^{+}-NTA affinity chromatography, T_4 DNA ligase and restriction endonuclease *Xho* I and *Bam*H I were products of Sangon Biotech (Shanghai, China). Human IgE, mouse IFN-γ and IL-4 ELISA detection kit were purchased from R&D company (Switzerland).

Methods

Construction and identification of prokaryoticly expressed recombinant vector

PCR amplification was performed by using *Der f* 1 as specific primer and pUCm-T recombinant plasmid containing chimeric *R8* as a template. The PCR products were inserted into the pET28a (+) empty vector after double digestion with restriction endonuclease *Bam H* I and *Xho* I, respectively at 16˚C for 18 h. Then, the product was transferred into *E.coli* line DH5α, which was applied to LB plate containing kanamycin at and cultured at 37˚C overnight. The positive colonies were randomly picked to undergo plasmid extraction and further PCR amplification as well as double enzyme digestion. The amplified products from PCR as well as the enzymatically digested products were separated by 2% agarose gel electrophoresis. Sequencing of the positive clones was performed by Sangon Biotech Co., Ltd (Shanghai, China).

Expression and purification of the recombinant plasmid

Recombinant vector pET 28a (+) *R8* was initially transformed into *E.coli* BL21 (DE3) as the previously described procedures[10]. The monococcus was picked and cultured in LB liquid medium in shaking incubator at 37˚C overnight. Then the culture was adjusted to absorbance value (A_{600} =0.5-0.6) by proportion 1:100 till final concentration of 1 mmol/L, in which isopropy-β-D-thiogalactoside (IPTG) was added. The solution was further cultured in shaking incubator at 37˚C for about 4 hours, followed by centrifugation at 12 000 × g for 30 seconds. The bacteria were collected and loaded onto the 12.5% SDS-PAGE, and stained with Coomassie brilliant blue. The remaining supernatant was purified by Ni^{+}-NTA affinity chromatography column as the user instructions.

Determination of the specific IgE binding capacity

The ELISA board was coated with 500 ng solution containing *R8*, rDer f1 and rDer p1 by each well at 4˚C overnight. Then the board was washed 5 times with TBST buffer, and blocked at 37˚C for 1 h. Twelve aliquots of sera obtained from the patients allergic to dust mites were added into each well by100 μl(1:5), and incubated at 37˚C for 1 h. After rinsing of the plate 5 times, 100μl(1:1000)goat anti-human IgE antibody labeled with HRP was added into each well and incubated at 37˚C for 2 h. Then the plate was washed 5 times, and substrate TMB solution was added and reacted at 37˚C for 20 min. The reaction was terminated by adding 50μl stop buffer into each well, and A_{450} was measured.

Preparation of mouse asthma model and specific immunotherapy

Seventy-five BALB/c mice were randomized into five groups(*n*=15 for each), namely: PBS group (negative control), rDerf1 group, rDerp1 group, *R8* group and asthma group (positive control). PBS group were treated exclusively with PBS, and the remaining groups were sensitized with *Der f* 1 by intraperitoneal injection in dose of 100 μl (containing 10 μg of *Der f* 1 dissolved in PBS and 4 mg $Al(OH)_3$) for each mouse from day 0, 7 and 14 d, respectively. By day 21 through 27, each mouse was challenged with by aerosol inhalation of Der f 1 suspension in dose of 0.5 g/ml, 30 min a day. Mice in groups of rDer f 1, rDer p 1 and R8 were correspondingly given specific immunotherapy with rDer f 1, rDer p 1 and R8 in dose of 100 μg/ml via intraperitoneal injection 30 min before inhalation challenge from day 25 to 27. PBS group were exclusively administered with PBS (containing 4% $Al(OH)_3$) suspension by intraperitoneal injection and aerosol inhalation.

Measurement of the specific IFN-γ and IL-4

Twenty-four hour after the final excitation, all mice were subjected to tracheotomy. The tracheae were exposed and irrigated with 1 ml of cold PBS by three repeats to recover the bronchial alveolar lavage fluid (BALF). The recovery rate was ensured greater than 80%. Then the BALF was centrifuged at 3000×g for 5 min, and the supernatant was transferred into another clean EP tube. Contents of IFN-γ and IL-4 in BALF were detected by ELISA as the manufacturer's protocol, and A_{450} was measured.

Statistical analysis

Data are expressed as the mean ± SD, and were analyzed using SPSS (version 16.0, Chicago, IL, USA). One-way ANOVA was used for statistical analysis to determine the differences among groups. Two samples were compared using the *LSD-t* and Tamhane's T2 method.

Results

Identification of the prokaryoticly expressed recombinant vector

PCR amplification of the colony showed that chimeric *R8* was successfully prokaryoticly expressed in the pET28a (+) vector, and the inserted fragments were consistent with the expected size (Fig.1). Positive cloning and sequencing revealed that the fragment size was 909 bp.

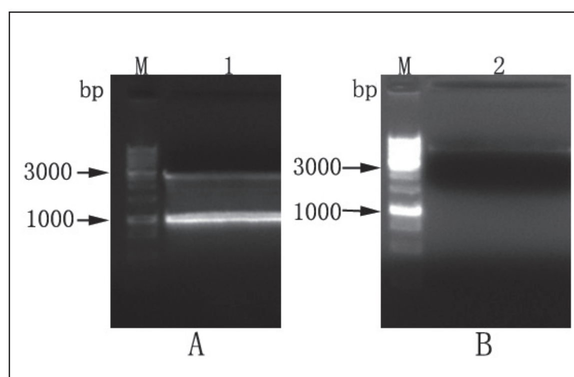

Fig. 1.—Double digestion of chimeric gene R8 (A) and expression vector pET28a(+) (B)
M: DNA marker, 1: Double digestion of recombinant plasmid pUCm-T-R8; 2: Double digestion of Expression vectors pET28a(+).

Induced expression and purification of the recombinant plasmids

The recombinant plasmids pET28a - *R8* were induced by 1mmol/L IPTG and verified with SDS-PAGE, and a molecular mass occurred at approximate M_r 35000 that was verified after Ni^+ column purification (Fig.2).

Determination of the IgE binding capacity

The binding capacity of chimeric protein *R8* with IgE was verified in the 12 aliquots of sera from the patients allergic to dust mites. The results indicated that IgE had significantly lower binding power with R8 (37.03±12.46 g/ml) compared to rDer f1 (80.44±15.50 g/ml) and rDer p1 (90.79±10.38 g/ml) ($P<0.01$, Fig.3).

Mouse asthma model by specific immunotherapy

ELISA was performed to determine the content of IFN-γ and IL-4 in BALF for evaluating the effect of specific immunotherapy with R8 in the mouse models. IFN-γ content was not significant between the rDer f1 group (322.98±30.36 pg/ml) and rDerp1group (314.97±33.89 pg/ml) and *R8* group (343.43±38.79 pg/ml) ($P>0.05$), yet the difference was significant between PBS group (393.93±50.68 pg/ml) and asthma group (208.44±46.11 pg/ml) ($P<0.01$, Fig.4A). IL-4 content was different in rDer f1 group (43.97±10.13 pg/ml) and PBS group (45.43±9.33 pg/ml) from *R8* group (37.0±14.00 pg/ml) ($P<0.05$), as well as in rDer p1 group (55.06±9.68 pg/ml) from asthma group (74.29±9.70 pg/ml) ($P<0.01$, Fig.4B).

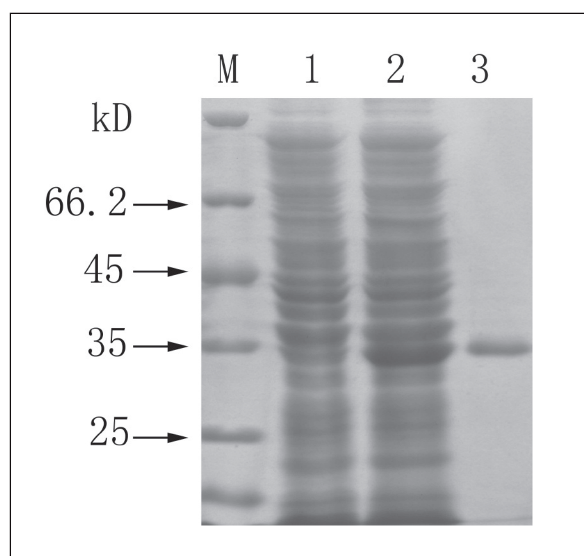

Fig. 2.—SDS-PAGE analysis for the expressed and purified R8 chimeric protein
M: Protein marker; 1: E. coli BL-21 cell line containing pET28a(+) induced by IPTG; 2: E. coli BL-21 cell line containing pET28a(+)-R8 induced by IPTG; 3: Purified protein R8.

Discussion

Type I hypersensitivity, including allergic asthma and allergic rhinitis, is primarily mediated by specific IgE antibody that is generated by plasma cells differentiated from B cells[11], it is also the result of imbalanced Th1/Th2 when an antigen is presented to CD4+ Th2 cells[12]. At present, strategies on treatment of type I hypersensitivity are involved in reducing the IgE production, yet increasing activity of Th1 cells.

Although ASIT is recognized as the promising strategy and has achieved better therapeutic effects in clinic, yet, safe and effective vaccine for SIT should be lower binding capacity with IgE[13]. Wallner *et al* once recombined 14 genes from family Betv1 by using DNA shuffling technology, and successfully selected two chimeric protein with low binding capacity with IgE but high immunogenicity[14]. Gafvelin *et al* also screened two chimeric genes, with high expression and ability to induce the protective IgG production as well as low binding power with IgE, from *Lepd 2* and *Glyd2* derived from group 2 dust mite allergen[5]. In the previous laboratory work, we successfully recombined two important proteins (Der f1 and Der p1) from group 1 dust mite allergens by using DNA shuffling, and obtained a chimeric R8 gene, an optimal protein for preparation of the vaccine, through sequencing in large-scale and online bioinformatics analysis (www.cbs.dtu.dk/services/). The results showed that the protein encoded by R8 can facilitate increase of T cell epitopes buy decrease of B cell epitopes as compared with simple use of Der f 1 or Der p 1.

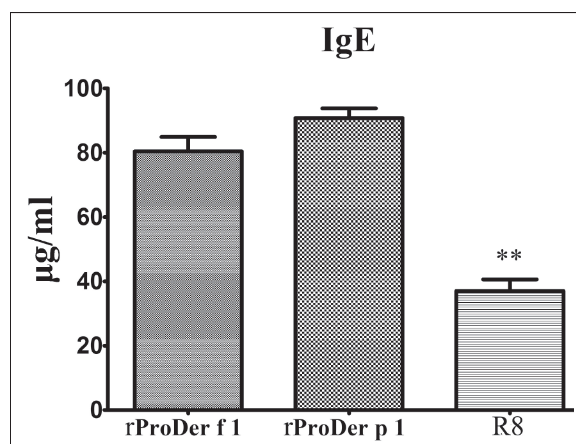

Fig. 3.—Binding reaction of chimeric protein R8 and IgE
** Compared with rDer f 1 and rDer p 1 groups' P<0.01.*

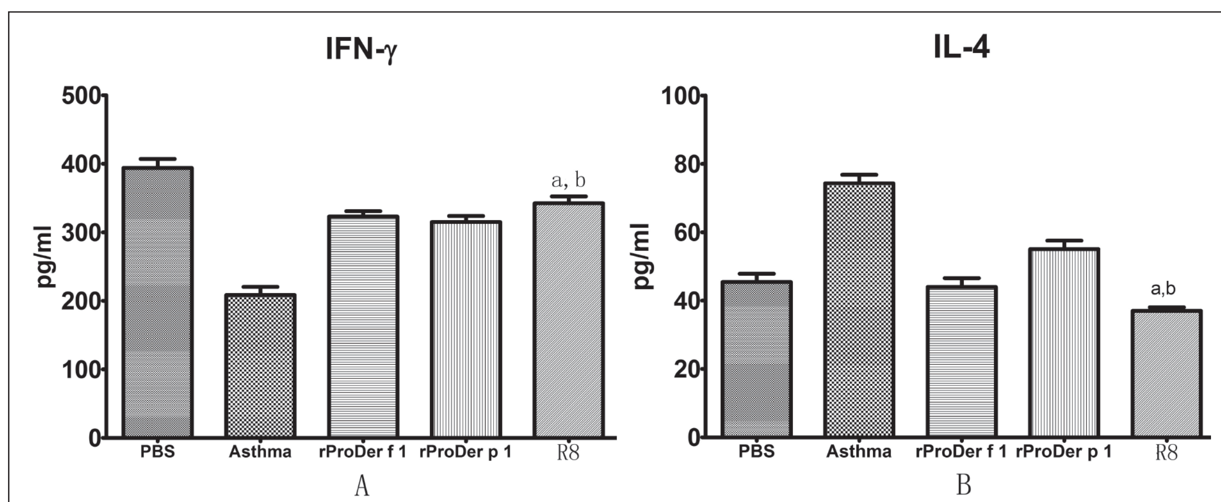

Fig. 4.—Cytokine levels o in BALF by ELISA determination
A: IFN-γ level in BALF from asthmatic mouse model after ASIT. B: IL-4 level in BALF from asthmatic mouse model after ASIT.
Fig. 4a.—a: Compared with rProDer f 1 and rProDer p 1 groups, $P > 0.05$; b: Compared with PBS and asthma groups, $P > 0.05$.
Fig. 4b.—a: Compared with rProDer f 1 and PBS groups, $P < 0.05$; b: Compared with rProDer p 1 and asthma groups, $P < 0.01$.

Previous study proved that the IgE binding capacity was similar to natural Der p 1 when rDer p 1 was obtained by simply transferring the gene encoding Der p1 into drosophila[15]. In order to verify whether R8 is hypoallergenic, we constructed a system for prokaryotic expression and purification of R8, and determined its capacity in binding with IgE in 12 aliquots of sera from patients allergic to dust mites by using ELISA technique. The results showed that the *R8* was hypoallergenic, and had significantly lower binding capacity with IgE than the two parental proteins. The possible reason may be that B cell epitopes are conformational[16], and recombining of the protein has led to destruction to the disulfide bonds, resulting in the change of spatial structure and inability of correct folding, which eventually causes destruction of the original conformation of B cells. Another explanation may be that the conformational IgE epitopes is blocked by the recombined gene that potentially contains propeptide capable of inhibiting the catalytic activity[17]. Although these factors may affect the binding ability of chimeric gene with IgE, yet particular mechanisms remain further determined. Some researches also confirmed that allergic process can be checked by decreasing IgE binding power [18-20], and this is consistent with our findings. In order to further verify whether the recombinant R8 protein can be highly immunogenic, we determined the the content of cytokines of IFN-γ and IL-4 with ELISA . The results showed that the mice intervened with R8 protein had significantly higher IFN-γ level than asthma group, whereas the difference was not significant as compared with the mice treated with the two parent proteins. This indicates that *R8* can stimulate the proliferation of Th1 cells. Contrarily, the mice treated with R8 had lower IL-4 level as compared with those treated with rDer f 1, rDer p 1 or PBS. These findings further indicate that R8 can correct the balance of Th1/Th2 and facilitate predominance of Th1. This may be associated with either preserved or aggregated epitopes on T cells from the two parental proteins in reconstruction, which is capable of boosting the the immunogenicity of *R8*.

In summary, we successfully obtained a R8 protein with hypoallergenicity and high immunogenicity, and applied it to treatment of experimental mice. The results showed that this protein is capable of correcting the imbalance of Th1/Th2, and has achieved optimal effects as ASIT. At present, we are verifying the safety and efficacies of this protein in ASIT, with an attempt to develop a vaccine for allergic asthma on ASIT basis.

Conclusion

Chimeric protein *R*8 derived from the group 1 allergens of dust mites has been expressed with low allergenicity and high immunogenicity.

Acknowledgements

This study was supported by a grant from the National Natural Science Foundation of China (No. 81270091, No.30872367, and No.81172790)

Declaration

This paper was published in Chinese under the title of “Prokaryotic Expression of Chimeric Gene Derived from the Group 1 Allergens of Dust Mites and Bioactivity Identification” in *Chin J Parasitol Parasit Dis* 2012; 30(4): 274-278.

Disclosure of conflict of interest

None.

References

1. Thomas WR, Smith WA, Hales BJ, et al. Characterization and immunobiology of house dust mite allergens. *Int Arch Allergy Immunol* 2002; 129(1):1-18.
2. Chapman MD, Platts MillsTA. Purification and characterization of the major allergen from antigen P1. *J Immunol* 1980; 125(2): 587-592.
3. Heymann PW, Chapman MD, Aalberse RC .Antigenic and structural analysis of group II allergens DerfII and DerpII from house dust mites (Dermatophagoides spp). *J Allergy Clin Immunol* 1989; 83(6):1055-1067.
4. Milin E, Diaz AM. Allergy to house dust mites and asthma. *PR Health Sci J* 2004; 23(1):47-57.
5. Gafvelin G, Parmley S, Neimert Andersson T, et al.. Hypoallergens for allergen specific immunotherapy by directed molecular evolution of mite group 2 allergens. *J Biol Chem* 2007; 282(6):3778-3787.
6. Ball T, Sperr WR, Valent P, et al. Induction of antibody responses to new B cell epitopes indicates vaccination character of allergen immunotherapy. *Eur J Immunol* 1999; 29(6):2026-2036.
7. Movrare R, Elfman L, Vesterinen E, et al. Development of new IgE specificities to allergenic components in birch pollen extract during specific immunotherapy studied with immunoblotting and Pharmacia CAP System. *Allergy* 2002; 57(5):423-430.
8. Stemmer WP. DNA shuffling by random fragmentation and reassembly recombination for molecula revolution[J]. *ProcNatl Acad Sci USA* 1994; 91(22):10747-10751.
9. Crameri A, Raillard A, Bermudez E. DNA shuffling of a family of genes from diverse species accelerates directede volution. *Nature* 1998; 391(6664):288-291.
10. Sambrook J, Russell DW. Molecular Cloning A Laboratory Manual. 3rd ed. New York Cold Spring Harbor Laboratory Press 2001; 1217-1265.
11. Chen WF, Jin BQ. Medical Immunology. 4th ed. Beijing Peoples Medical Publishing House, 2004; 191-195 (in Chinese).
12. Prescott SL. Maternal allergen exposure as a risk factor for childhood asthma. *Curr Allergy Asthma Rep* 2006; 6(1):75-80.
13. Akdis CA, Blaser K. By passing IgE and targeting T cells for specific immunotherapy of allergy. *Trends Immunol* 2001; 22(4):175-178.
14. Wallner M, Stcklinger A, Thalhamer T, *et al*. Allergy multivaccines created by DNA shuffling of tree pollen allergens. *J Allergy Clin Immunol* 2007; 120(2):374-380.
15. Jacquet A, Haumont M, Massaer M, *et al*. Biochemical and immunological characterization of a recombinant precursor form of the house dust mite allergen Derp1 produced by cells. *Clin Exp Allergy* 2000; 30(5):677-684.
16. Lombardero M, Heymann PW, Platts-Mills TA, *et al*. Conformational stability of B cell epitopes on group I and group II *Dermatophagoides* spp. allergens. Effect of thermal and chemical denaturation on the binding of murine IgG and human IgE antibodies. *J Immunol* 1990;144(4):1353-1360.
17. Takai T, Kato T, Yasueda H, *et al*. Analysis of the structure and allergenicity of recombinant pro- and mature Derp1 and Der f1 major conformational IgE epitopes blocked by prodomains. *J Allergy Clin Immunol* 2005; 115(3)555-563.
18. Valenta R, Niederberger V. Recombinant allergens for immunotherapy. J Allergy Clin Immunol 2007; 119(4): 826-830.
19. Velickovic TC, Jankov RM. Design and modifications of allergens for improving specific immunotherapy. *Inflamm Allergy Drug Targets* 2008; 7(4):270-278.
20. Pulsawat P, Piboonpocanun S, Sirivichayakul S, *et al*. Production and immunogenicity of hypoallergenic codon-optimized DNA vaccine encoding mature Derp1 allergen. *J Investig Allergol Clin Immunol* 2010; 20(7)):582-590.