Expression of recombinant allergen, Der f 1, Der f 2 and Der f 4 using baculovirus-insect cell systems

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Submitted: 17 May 2016
Accepted: 11 November 2016

Arch Med Sci 2018; 14, 6: 1348–1354
DOI: https://doi.org/10.5114/aoms.2018.79005

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Abstract

Introduction: Specific immunotherapy is critical for alleviating symptoms associated with house dust mite allergy, such as asthma and rhinitis. However, this approach relies on crude extracts, which are often not of sufficient quality or purity and are not standardized. The use of recombinant allergens may enable safer, more effective treatment.

Material and methods: Using our previously constructed plasmids pET28a(+)-Der f 1, pET28a(+)-Der f 2 and pET28b(+)-Der f 4 as templates, the gene fragments coding for the allergens Der f 1, Der f 2 and Der f 4, respectively, of the dust mite *Dermatophagoides farinae* were amplified by PCR. Next the PCR-amplified DNAs were recovered, cloned into pFastBacHT A, and transformed into *Escherichia coli* DH10Bac. The resulting vectors were co-transfected into *Spodoptera frugiperda* Sf9 cells for expression. The recombinant allergens were purified by Ni^{2+} affinity chromatography, and identified by SDS-PAGE and ELISA.

Results: The recombinant allergens were successfully expressed and purified from a baculovirus expression system introduced into Sf9 cells, which were verified as being of the correct predicted molecular weights by SDS-PAGE. Furthermore, the reactivity to recombinant allergens rDer f 1, rDer f 2, and rDer f 4 was 85.2%, 88.9%, and 44.4%, respectively, in 27 children with asthma and *D. farinae* allergy.

Conclusions: Recombinant allergens from dust mites can be successfully generated using a baculovirus-insect expression system. Furthermore, these recombinant allergens can be used to detect mite sensitivity in sera, highlighting their utility in future work to understand and develop treatment for mite allergy.

Key words: *Dermatophagoides farinae*, baculovirus expression system, recombinant allergen, cloning, gene expression.

Introduction

Since 1978, when house dust mite allergens were shown to be a major cause of type 1 IgE-mediated allergies [1], these allergens have been used in specific immunotherapy (SIT) to alleviate symptoms. SIT uses increasing exposure to an allergen over months and years to sensitize a patient and reduce the severity of asthma, dermatitis, or rhinitis [2]. A major limitation of SIT is its reliance on natural allergen extracts, which comprise an unpredictable mixture of both allergenic and non-al-

lergenic components that results in both poor efficacy and side effects [3]. Despite continuous attempts to improve the quality of natural allergen extracts by standardization, problems persist, such as batch-to-batch variations, lack or poor representation of allergens, poor immunogenicity of certain allergens, varying allergenic activities, and presence of non-allergenic materials and contaminations. Furthermore, the administration of natural allergen extracts can induce severe and life-threatening side effects and hence requires cumbersome up-dosing schemes based on either multiple injections or hospitalization for rush protocols, which prevent the broad applicability of SIT [4].

Fortunately, recombinant DNA technology provides the potential to produce previously scarce allergens in large quantities, enabling us to overcome many, if not all, of the problems associated with the use of their natural counterparts, such as insufficient quality, allergenic activity, and poor immunogenicity. Because the most important allergens from mites have been cloned, sequenced, and expressed, and their three-dimensional structures and allergenic epitopes have been identified, there exists a solid foundation for recombinant allergen production. Indeed, high-level expression systems have been developed to produce recombinant allergens in prokaryotes such as *Escherichia coli* as well as in a variety of eukaryotes: the yeasts *Saccharomyces cerevisiae* [5] and *Pichia pastoris* [6]; baculovirus-insect cell systems; mammalian cell systems; and plant systems [7].

Insect cells have proven to be good hosts for the production of recombinant proteins. Insects are natural hosts for baculovirus, and the baculovirus expression system has been used for protein overproduction since the late 1980s. This expression system can introduce many of the post-translational modifications that are necessary for protein function but are often lacking in proteins produced in prokaryotic expression systems. Typically, the *Autographa californica* multiple capsid nucleopolyhedrovirus (AcMNPV) is introduced to host cells such as *Spodoptera frugiperda* ovarian cell lines or a *Trichoplusia ni* egg-derived cell line. All of the recombinant allergens produced using this system show IgE-binding capacity and behave with similar biological activities to the native proteins. In the case of the mite allergen Lep d 2 and the yeast allergen Mal f 1, no functional differences were observed between protein produced in prokaryotic and baculovirus expression systems [8, 9]. Additionally, both systems produce comparable amounts of immunoreactive recombinant proteins.

The group 1 and group 2 allergens are the most potent found in dust mites, binding over 50% of the IgE antibody that binds to house dust mite extracts. Three other allergens, groups 4, 5, and 7, each bind about 10% of the titers and are called mid-tier allergens [10]. To explore the structure and functions of these mite allergens, we cloned, sequenced, and expressed Der f 1, Der f 2, and Der f 4 in *E. coli*. The IgE binding reactivity of their expression products was detected as 97.4% (38/39) for rDer f 1 [11], 95.7% (44/46) for rDer f 2 [12], and 40.74% (11/27) for rDer f 4 [13], using sera from different populations. Although these binding rates are consistent with the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (http://www.allergen.org/), the binding activity was weak (using color in reactivity). The present study sought to generate recombinant versions of some of the most potent allergens from the house dust mite *Dermatophagoides farinae* – Der f 1, Der f 2, and Der f 4 – by using baculovirus expression systems. The IgE binding reactivity for these recombinant allergens was determined in the same sera, called "component-resolved diagnosis (CRD)", to understand their potential future applicability to SIT.

Material and methods

Sera samples

Serum samples from children with allergic asthma were provided by the Department of Laboratory, the first affiliated hospital of Shanghai Jiao Tong University. Patients had not received treatment before blood sampling. Asthma diagnosis followed the WHO criteria: all children had cough, asthma, obvious wheeze, rhonchus and moist rales in the lung, and other clinical manifestations; and X-ray examination showed increased lung markings or lung hyperinflation and infection [14]. The diagnosis of allergy to dust mite allergens was established on the basis of a suggestive clinical history and positive serum IgE (sIgE) to *D. farinae* extracts as determined by the Allergy Screening test panel for atopy (Mediweiss Analytic, Moers, Germany). The inclusion criteria were: (1) allergy to *D. farinae* extracts with more than 1 year of follow-up, and (2) levels of sIgE to *D. farinae* extracts higher than 3 kU/l at the time of inclusion in the study. A total of 27 pediatric patients (16 male and 11 female), mean age: 6.6 ±3.6 years, were enrolled as serum-positive for *D. farinae*, and then used for detection of specific IgE-binding to recombinant fusion allergen. Additionally, the sera of 5 non-atopic healthy children (3 male and 2 female), mean age 6.0 ±2.2 years, were treated as negative controls. The study protocol was approved by the Ethics Committee for Clinical Investigation of Yancheng Health Voca-

tional and Technical College, and patients' guardians provided informed consent.

Primer design

DNA primers were designed and synthesized based on the published sequence of Der f 1 (GenBank Accession No. EU095368), Der f 2 (GenBank Accession No. FJ436110), and Der f 4 (GenBank Accession No. KJ400030). Full-length sequences of these genes were expected to be 963 bp, 441 bp, and 1578 bp, respectively. The primers sequences are listed in Table I.

Construction and identification of the recombinant plasmids pFastBacHT A-Der f 1, pFastBacHT A-Der f 2, pFastBacHT A-Der f 4

Using our previously constructed plasmids pET28a(+)-Der f 1 [15], pET28a(+)-Der f 2 [16], and pET28b(+)-Der f 4 [13] as templates, the gene fragments Der f 1, Der f 2, and Der f 4 were amplified by PCR. The reaction systems were as follows: 5 µl of 10 × LA PCR Buffer, 0.5 µl of TaKaRa Pyrobest DNA Polymerase, 8 µl of dNTP, 1 µl of forward primers, 1 µl of reverse primers, 0.5 µl of plasmids pET28a(+)-Der f 1/pET28a(+)-Der f 2/pET28a(+)-Der f 4 (as appropriate), and 34 µl of ddH_2O; the total reaction volume was 50 µl. Thermal cycling was performed as follows: 2 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C; and a final step at 72°C for 10 min.

5 µl of the PCR product were analyzed by agarose gel electrophoresis (1.0%) and visualized with ImageMaster VDS. After the PCR-amplified DNA was recovered with a MiniBEST Agarose Gel DNA Purification Kit Ver 2.0 (TaKaRa Code No. D823A), it was then cloned into pFastBacHT A (Invitrogen) with a solution of the DNA Ligation Kit (TaKaRa Code No. D6020A). *Escherichia coli* JM109 cells (TaKaRa Biotech Co. Led, Code No.D9052) were then transformed with the recombinant plasmids pFastBacHT A-Der f 1, pFastBacHT A-Der f 2, and pFastBacHT A-Der f 4. Positive clones were selected by blue/white screening on Luria-Bertani (LB) plates and submitted to PCR identification.

Transposition and identification of the recombinant baculovirus

The *E. coli* DH10Bac (MAX Efficiency DH10Bac Competent Cell, Invitrogen) was transformed with the recombinant plasmids pFastBacHT A-Der f 1, pFastBacHT A-Der f 2, and pFastBacHT A-Der f 4, respectively, and grown at 37°C in L-broth containing K^+, TET^+, GEN^+, X-gal, and IPTG. After 48 h culture, the white clones were picked and grown on another L-broth containing K^+, TET^+, GEN^+, X-gal, and IPTG. After 48 h, positive clones were selected by blue/white screening on Luria-Bertani (LB) plates and submitted to PCR identification. The forward primers were 5′-CTGGGGTGATAGCGGATAC-3′ for amplification of Der f 1, 5′-GGTGTTTTGGCTTGCGC-3′ for amplification of Der f 2, 5′-CTGTCTGACATCTACGTTGG -3′ for amplification of Der f 4, and the reverse primer was M13R (5′-CAGGAAACAGCTATGACC-3′).

Transfection and amplification of recombinant baculovirus

For transfection, the insect cell *Spodoptera frugiperda* Sf9 was grown on Grace medium, and the recombinant baculovirus was added. After 4–6 h, the viruses were collected and centrifuged, and the supernatant was collected. Supernatant was mixed with 2% fetal bovine serum (FBS) and named P1 viral stock. The P1 viral stock was grown on Grace medium containing Sf9 cells for further transfection, and the product was named P2 viral stock. Similarly, the P2 viral stock was used to infect the Sf9 cells. After 4 h, the virus was collected and named P3 viral stock.

Purification and renaturation of recombinant fusion protein

P3 viral stock was centrifuged at 4000×g for 10 min, then the cell pellet was treated with 40 ml of 1× binding buffer (His•Bind Purification Kit, Novagen company, Cat. No. 70665), placed on ice, then sonicated (5 cycles for work 3 s and stop 3 s, 200 W). Samples were then centrifuged at 5000×g for 30 min. Both supernatant and precipitate were

Table I. Primer sequences for PCR reaction

Genes	Primers	Primer sequences (5′→3′)	Enzyme sites underlined
Der f 1	Forward	CGGGATCCACGTCCAGCTTCAATCAAAAC	*Bam*H I
	Reverse	GGGGTACCTCACATGATTACAACATATGG	*Kpn* I
Der f 2	Forward	GGGGATCCGATGATTTCCAAAATCTTGTGCC	*Bam*H I
	Reverse	CGGGTACCTTAATCACGGATTTTACCATG	*Kpn* I
Der f 4	Forward	CCTGCCATGGACTCTAAATTCTCTAACC	*Nco*I
	Reverse	CCAACTCGAGTTAAGATTCAACACGAGCACCG	*Xho*I

submitted to purification through a His•Bind column (Novagen company, Cat. No. 70971). Next, 10 μl of the protein samples were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5%) and CBB-R250 staining.

The purified inclusion bodies were resuspended in PBS buffer (pH 8.0) containing 8 mol/l of urea and 5 mmol/l of DTT, stirred slightly at 4°C overnight, and centrifuged at 4000 rpm for 10 min. The supernatant was filtered by a microfiltration membrane with a pore size of 0.22 μm. This protein product was further diluted 1 : 4 with buffer (pH 8.0, containing 50 mmol/l of PBS, 0.3 mmol/l of NaCl, 10 mmol/l of imidazole solution, 5% glycerol), which was mixed for 4 h at 4°C. The product was renatured by gradient dialysis with a renaturation solution containing 2 mol/l to 0.2 mol/l of urea, and 0.2 mol/l of EDTA. The elution peak at each stage was collected and kept at –86°C.

Specific IgE-binding to recombinant allergen

As described in our previously reported protocol [13], the IgE-binding ability of these recombinant proteins rDer f 1, rDer f 2 and rDer f 4 was detected by enzyme-linked immunosorbent assay (ELISA).

Results

Construction and identification of the recombinant plasmids pFastBacHT A-Der f 1, pFastBacHT A-Der f 2, and pFastBacHT A-Der f 4

Using our previously constructed plasmids pET28a(+)-Der f 1 [15], pET28a(+)-Der f 2 [16], and pET28b(+)-Der f 4 [13] as templates, the cDNAs encoding the allergens Der f 1, Der f 2, and Der f 4 were amplified by PCR and linked into the vector pFastBac HT A. The obtained plasmids pFastBacHT A-Der f 1, pFastBacHT A-Der f 2, and pFastBacHT A-Der f 4 were confirmed by PCR and agarose gel electrophoresis (Figure 1).

Purification of recombinant fusion protein

The recombinant plasmids pFastBacHT A-Der f 1, pFastBacHT A-Der f 2, and pFastBacHT A-Der f 4 were transformed in *E. coli* DH10Bac and positive clones were selected. Bacmids were extracted and transfected into insect Sf9 cells. After three generations of culture, the insect cells were collected and subjected to ultrasonic fragmentation and protein purification by Ni^{2+} affinity chromatography. SDS-PAGE showed clear bands (Figure 2), which were consistent with the molecular weights of 34.5 kDa, 14.1 kDa, and 57.9 kDa for Der f 1, Der f 2, and Der f 4, respectively, as predicted by ProtParam Tools on the ExPaSy website.

Specific IgE binding to recombinant allergens

To assess the allergenicity of recombinant allergens, ELISA was performed using sera from 27 mite-allergic pediatric patients with asthma and 5 non-allergic control individuals. Positive IgE reactions to rDer f 1, rDer f 2, and rDer f 4 were detected in sera of 85.2% (23/27), 88.9% (24/27), and 44.4% (12/27) of asthma patients using a cut-off of 3 standard deviations above the mean value from healthy controls (Figures 3 A–C). A combination of rDer f 1 and rDer f 2 had a positive rate of 100%.

Discussion

This study reports, for the first time, the production of recombinant allergens from dust mites by using baculovirus-insect cell systems. The dust mite allergens Der f 1, Der f 2, and Der f 4 were successfully produced in insect Sf9 cells.

In the 27 children with asthma and hypersensitivity to *D. farinae*, 85.2%, 88.9%, and 44.4% were found to react positively to rDer f 1, rDer f 2, and rDer f 4. Two recombinant allergens, rDer f 1 and rDer f 2, synthesized in *E. coli* elicited positive radioallergosorbent test (RAST) responses in 88% and 80%, respectively, with sera from infants

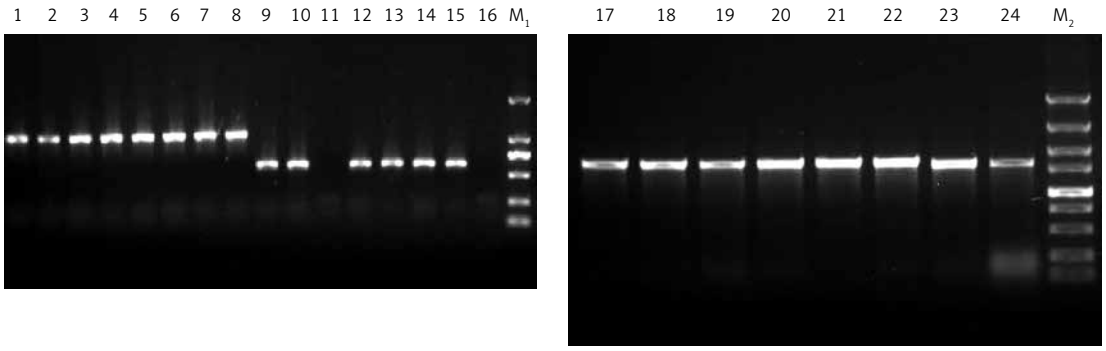

Figure 1. PCR products verifying the recombinant plasmids pFastBacHT A-Der f 1, pFastBacHT A-Der f 2, and pFastBacHT A-Der f 4. Lanes 1–8, Der f 1; lanes 9–16, Der f 2; lanes 17–24, Der f 4; lane M_1, DNA marker DL 2000; lane M_2, λ-Hind III DNA marker

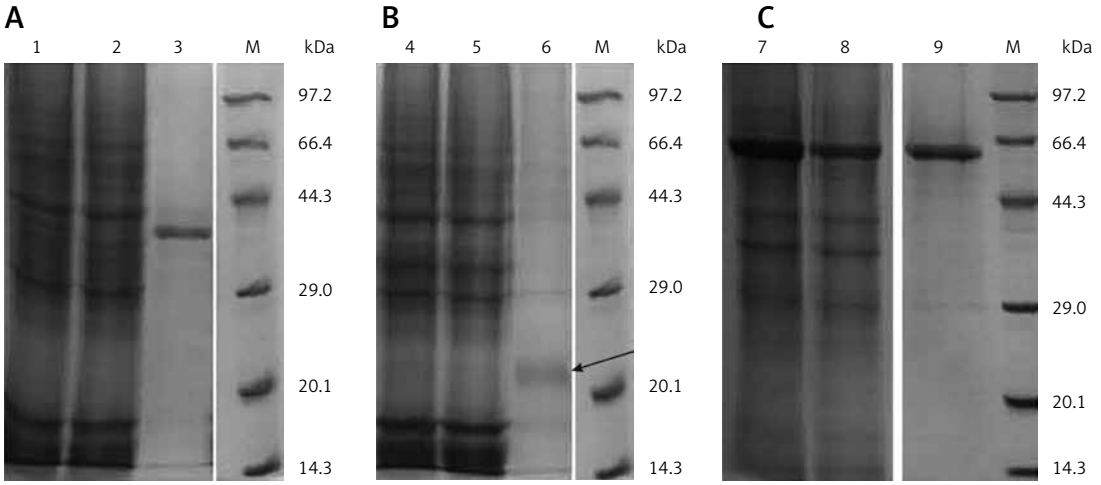

Figure 2. SDS-PAGE identification of the purified protein from affinity chromatography. **A** – Der f 1; **B** – Der f 2; **C** – Der f 4. Lanes 1, 4, 7, whole Sf9 cells infected by the recombinant virus. Lane 2, 5, 8, the flow-through of Sf9 cells infected by the recombinant virus. Lanes 3, 6, 9, the elution of Sf9 cells infected by the recombinant virus

with asthma; positive RAST responses to these antigens increased to 100% in children up to 5 years of age [17]. In contrast, the majority of sera with detectable IgE antibody to *D. farinae* also had IgE antibody to purified Der f 1 among children (29/42 = 69%) and adults (55/63 = 87%) [18].

In China, a cross-sectional survey was performed in 6304 patients suffering from asthma and/or rhinitis in 17 cities from 4 regions, and the overall prevalence of a positive skin prick test was 59.0% for *D. farinae* [19]. Another study indicated that, in 62 patients with asthma with/without rhinitis, 100% had specific IgE against natural Der f 1 and 95% against natural Der f 2 [20]. In those studies, the reactivity was determined by crossed radioimmunoelectrophoresis (CRIE) and western blotting. In contrast, our positive rates of 85.2% and 88.9% were determined by ELISA using rDer f 1 and rDer f 2 that were produced in baculovirus-insect cell systems.

There are currently 33 different house dust mite allergens listed in the IUIS nomenclature database; 31 have been described for *Dermatophagoides* spp. (www.allergen.org). The only requirement of the designation of a protein as an allergen is that it binds IgE in sera from 5% of people with a house dust mite allergy. Thus, it is critical to know the relative importance of the different allergens, and whether individuals respond differently to different allergens. Although the top-tier allergens Der f 1 and Der f 2 are widely studied, the mid-tier allergens like Der f 4 are less understood [10]. In the present study, the recombinant allergen for group 4 from *D. farinae* obtained using baculovirus-insect cell systems bound with sera from 44.4% of children with asthma and *D. farinae* allergy. These may be the first data indicating the prevalence of reactivity to the group 4 allergen of dust mites in China.

In conclusion, perhaps more important, component-resolved diagnosis (CRD), which is the overall approach used herein, with purified house dust mite allergen allowed us to discriminate patients who were mainly sensitized to the two major house dust allergens from others who exhibited reactivities to highly cross-reactive allergens (e.g., Der p 10) and to storage mites. A survey from Yonsei University College of Medicine (2015) using recombinant house dust mite allergens produced in *Pichia pastoris* (Der f 1) or *Escherichia coli* (5 allergens) investigated the IgE reactivity to the individual recombinant allergens and total extract of mite by ELISA. Der f 1 was recognized by 79.1%, Der f 2 by 79.1%, Der f 6 by 9.3%, Der f 8 by 6.2%, Der f 10 by 6.2%, and Der f 20 by 6.6% of the patient sera tested. The prevalence of IgE reactivity to total mite extract was 94.7%. A combination of Der f 1 and Der f 2 had a sensitivity of 87.6%, while specific IgE to Der f 2 alone was detected from 89.4% of sensitized respiratory allergy subjects and 92.3% were sensitive to the combination of the 2 major allergens Der f 1 and Der f 2 [21]. In another report, the IgE reactivity profiles to Der p extract were determined in a Middle European mite-allergic population by IgE immunoblotting and by using a panel of seven purified or recombinant Der p allergens (nDer p 1, nDer p 4, rDer p 2, rDer p 5, rDer p 7, rDer p 8, rDer p 10). More than 95% of the patients could be diagnosed with a combination of nDer p 1 and rDer p 2; therefore, the diagnostic tests containing the major mite allergens (i.e., Der p 1, Der p 2) may improve the diagnostic selection of patients for immunotherapy with Der p extracts [22, 23]. Since most allergen extracts used for mite-specific immunotherapy are mainly standardized with respect to their major allergens, we suggest the use of diagnostic tests based on recombinant major and mid-tier allergens.

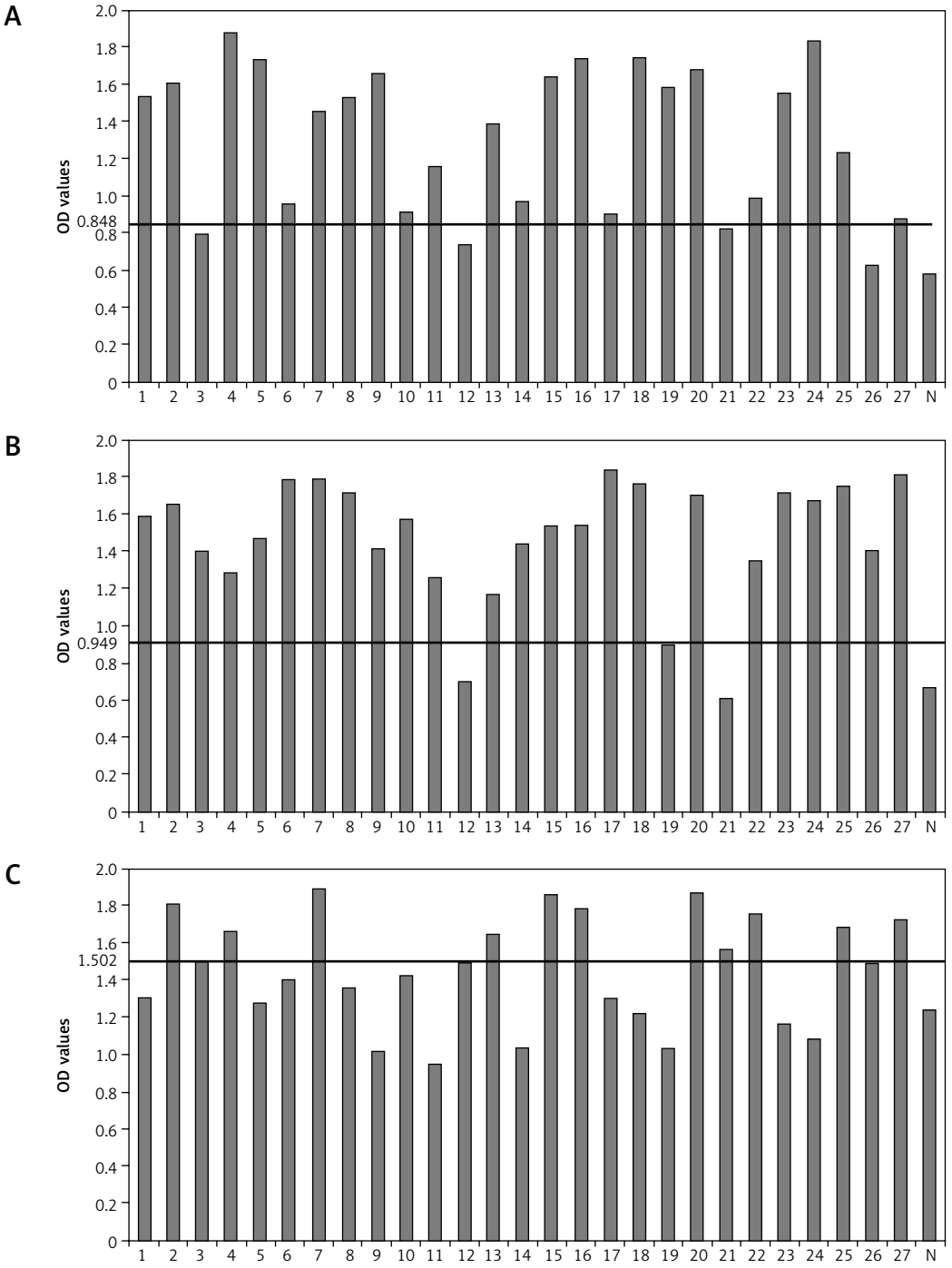

Figure 3. IgE-binding reactivity of the recombinant allergen, Der f 1, Der f 2 and Der f 4 using baculovirus-insect cell systems. **A** – IgE-binding reactivity of rDer f 1. Using rDer f 1 as a coating antigen, the optical density (OD) value for mite-insensitive healthy donors (n = 5, negative control) was 0.577; the cut-off ELISA value (mean ELISA value of healthy donors ±3 SD, 0.577 ±3 × 0.090) was 0.848, which is indicated as a horizontal line. The reactivity was considered to be positive if the OD values of the detected sera were higher than this cut-off ELISA value, so 23 individuals (85.2%) were concluded to be positive for sensitivity to the group 1 allergen. **B** – IgE-binding reactivity of rDer f 2. Using rDer f 2 as a coating antigen, the optical density (OD) value for mite-insensitive healthy donors (n = 5, negative control) was 0.673; the cut-off ELISA value (mean ELISA value of healthy donors ± 3 SD, 0.673 ±3 × 0.092) was 0.949, which is indicated as a horizontal line. The reactivity was considered to be positive if the OD values of the detected sera were higher than this cut-off ELISA value, so 24 (88.9%) were concluded to be positive for sensitivity to the group 2 allergen. **C** – IgE-binding reactivity of rDer f 4. Using rDer f 4 as a coating antigen, the optical density (OD) value for mite-insensitive healthy donors (n = 5, negative control) was 1.246; the cut-off ELISA value (mean ELISA value of healthy donors ± 3 SD, 1.246 ±3 × 0.085) was 1.502, which is indicated as a horizontal line. The reactivity was considered to be positive if the OD values of the detected sera were higher than this cut-off ELISA value, so 12 (44.4%) were concluded as being positive for sensitivity to the group 4 allergen

Acknowledgments

This work was supported by the National Sciences Foundation of China (NSFC 30060166, NSFC81001330, NSFC31272369, NSFC31572319). We thank Dr. Man Tian, the Respiratory Department at the Affiliated Nanjing Children's Hospital, Nanjing Medical University, Jiangsu, China, who provided sera for specific-IgE antibody detection.

Conflict of interest

The authors declare no conflict of interest.

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