Original Article

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Generation and purification of monoclonal antibodies against Der f 2, a major allergen from *Dermatophagoides farinae*

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Summary **Monoclonal antibodies (mAbs) are needed for the quantitation of environmental allergens for precise diagnosis and immunotherapy. In this study, we produced and purified monoclonal antibodies against Der f 2, one of the major allergens of the house dust mite *Dermatophagoides farina*, in order to develop an assay for the detection of this allergen. BALB/c mice were immunized four times with the protein Der f 2 together with an adjuvant after which splenocytes were collected and fused with SP2/0 (myeloma cells) in the presence of polyethylene glycol (PEG). The fused cells were selected in the presence of Hypoxanthine-Aminopterin-Thymidine (HAT) and then Hypoxanthine-Thymidine (HT) medium. Positive cells were screened with ELISA and subcloned by limited dilution at least three times to achieve stable mAb-producing clones. Four stable mAb-producing clones were obtained. One clone with IgG1 isotype and another with IgG2b isotype were chosen to produce large amounts of mAb by inoculation of the cells into the abdominal cavity of mice. Ascites were collected and the mAbs were purified using protein A affinity chromatography. Testing of the ascites by ELISA showed the titration of IgG1 and IgG2b to be higher than $1/10^6$ dilution. The specificity of both antibodies was confirmed by immunoblotting. Thus, we produced two mAb clones against Der f 2 that can be used to create a precise quantitative method to identify allergen components in dust samples and facilitate further study in Der f 2 component-resolved diagnosis (CRD).**

Keywords: *Dermatophagoides farina*, environmental allergen, house dust mite monoclonal antibody

1. Introduction

House dust mite (HDM) is one of the most common causes of respiratory allergic diseases such as allergic asthma and allergic rhinitis (*1*). HDM allergens can be found in dust samples from the bedding of 95% of Chinese households (2). The higher average relative humidity in the south of China, especially in the coastal cities, may be a factor in promoting exposure to mites and, thus, result in an increasing prevalence of asthma (*3*). Because of the warm climate (average temperature: 22°C) and humidity (average relative humidity: 76%), more than 90% of children with asthma have positive skin test responses to HDM in Taipei, China (*4*). *Dermatophagoides pteronyssinus* (D. *pteronyssinus*) (65.3%) and *Dermatophagoides farina* (D. farina) (20.6%) are two of the most common groups of dust in the household environment (*5,6*). Currently, there are nearly 30 kinds of allergenic components in HDM that have been cloned and identified (*7*). Approximately 80% to 100% of HDM allergic patients respond to group 1 and group 2 allergens (*8*).

Allergen avoidance is widely recommended to reduce the symptom severity of allergic diseases in sensitized individuals (*9-11*). Therefore, it is important to monitor the amount of allergens in dust samples to evaluate the correlation between the environmental allergens and the occurrence and severity of allergic diseases (*12*).

Allergen immunotherapy (AIT) is the only

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treatment approach that can improve the immune response of patients to allergens (*13*). AIT with HDM extract is one of the most common treatments for mite allergy. Furthermore, several new strategies such as the administration of recombinant and genetically modified allergens are being explored for their use in new types of AIT (*14*). However, the amount of each allergen component in both the crude extract and other new types of AIT is not exactly known, which could lead to serious side-effects in the course of treatment (*15*), especially in patients who have experienced anaphylactic reactions. It is important to detect the concentration of each allergen component exactly in the HDM extract. However, efficient tools for quantification of allergen components are still limited.

MAbs against specific allergen components could be used in their quantification as well as for the further improvement of HDM allergy immunotherapy. In 2013, a mAb against Der p 2 was generated for the development of a Der p 2 ELISA (*16*) that was used to monitor environmental mite infestation and estimate the number of mites in house dust samples. However, currently, there is no commercial antibody kit against the allergen Der f 2. In this study, we produced and purified mAbs against Der f 2, which might be used in the quantification of this allergen component.

2. Materials and Methods

2.1. *Materials*

Recombinant Der f 2 protein was generated and purified in a routine process similar to a method described elsewhere (*17*). BALB/c mice were obtained from the Medical Laboratory Animal Center (Guangdong, China. License No.: SCXK 2013-002). Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA) were purchased from Sigma-Aldrich Co (St. Louis, America). The Sp 2/0 myeloma cell line was obtained from CAS Shanghai Life Science Cell Resource Center (Shanghai, China). HAT medium, HT medium, penicillin, streptomycin, fetal bovine serum (FBS), RPMI-1640 medium plus L-glutamine were purchased from Life Technologies Inc (New York, USA). Horse anti-mouse IgG-HRP conjugate was purchased from Cell Signaling Technology (Shanghai, China). Polyethylene glycol (PEG 4000) was bought from Sigma-Aldrich Co. HiTrap Protein A (5 × 1 mL Lot: 17-0402-01) was bought from GE Healthcare (Freiburg, Germany).

2.2. *Immunization of mice*

All procedures performed in this study involving animals were in accordance with the ethical standards of the institution and have been approved by a research ethics committee of The Second Affiliated Hospital of Guangzhou Medical University. Five mice were subcutaneously (*s.c.*) immunized with purified Der f 2 protein emulsified with CFA at the first injection. The total amount of protein was 36 μg/mouse. After two weeks, the mice were given a second subcutaneous injection of 36 μg/mouse in IFA followed by a third injection in IFA two weeks later in the same manner. After another two weeks, serum was obtained from the tail and the titer of Der f 2 specific IgG antibodies was investigated. The mice with highest titers of positive antibodies were then selected for administration of the final intraperitoneally (*i.p.*) injected protein without adjuvant and were sacrificed three days later.

2.3. *Fusion*

Peritoneal cells were obtained from a healthy non-immunized mouse and cultured in 96-well plates as primary feeder cells. Spleen cells from the immunized mice were fused with SP2/0 cells in the presence of PEG 4000. The fused cells were suspended in HAT medium containing 10% FBS and 1% penicillin-streptomycin and distributed into prepared 96-well plates. Culture supernatant was semi-replaced with fresh HAT medium on the 4th, 7th, and 10th day after fusion. After ten days of culture in HAT medium, the hybridoma cells were transferred to HT medium.

2.4. *Screening of antibody-producing cells*

Wells of a 96-well plate were coated with 2 ng of purified Der f 2 protein and then incubated at 4°C overnight. After washing four times with PBS containing 0.05% tween 20, the plates were blocked with 2% BSA solution. 100 μL of hybridoma supernatant was added to each well and incubated for 1 h at 37°C. After plate washing, secondary antibody was added (horse anti-mouse IgG at 1/4,000 dilution) to each well and incubated for another hour at 37°C. After washing, the plate was developed with TMB and the absorbance was checked using an ELISA Reader (Thermo Scientific, Varioskan Flash) at 450 nm.

2.5. *Subcloning and immunoglobulin isotyping of mAb-producing hybridomas*

The hybridomas that generated the highest antibody titers were selected for subcloning by limited dilution. Briefly, cell suspension was distributed at concentrations of 10, 5, or 2 cells/well in 100 μL medium and cultured in 96-well plates containing feeder cells. The clone supernatants were tested using ELISA after ten days in culture. In order to obtain stable antibody-secreting clones, the positive clones were subcloned at least three times using the limited dilution method (*18*). The isotype of the mAb was qualitatively analyzed using a commercial ELISA kit (Sino Biological.inc, SEK003),

following the manufacturer's guidelines.

2.6. *Mass production and verification of mAbs*

Six-eight weeks old female mice were *i.p.* with 0.5 mL of priming reagent per mouse. Ten days later, 1 × 10^6 stable mAb-producing cells suspended in 0.5 mL RPMI-1640 medium were injected into the abdominal cavity of each mouse. Production of ascites as noted by abdominal distension was observed a few days after inoculation and was collected after seven to ten days.

Der f 2 protein was resolved through SDS-PAGE and then transferred onto a PVDF membrane. The membrane was then blocked with 2% BSA and incubated for 2 h at room temperature. Either cell supernatants or ascites were diluted and added as primary antibody. After the incubation and washing steps, goat anti-mouse IgG antibody (1/4,000) was added as the secondary antibody. The membrane was developed in ECL reagent and images were acquired using ImageQuant LAS 4000 mini.

2.7. *Purification of Der f 2 monoclonal antibody*

The ascites was diluted with normal saline and precipitated by 50% ammonium sulfate. The precipitate was dissolved and dialyzed into 20 mM phosphate-buffered saline (PBS) followed by antibody purification using protein A chromatography according to the manufacturer's recommendation. In brief, the resin was equilibrated with PBS pH 7.4. After protein loading, the resin was washed with at least a 20-fold column volume of PBS pH 7.4. The target protein was eluted using 100 mM citric acid buffer (pH 4.0) and the mAb concentration was monitored by UV absorption at 280 nm.

3. Results

3.1. *Serum titration of the immunized mice*

Serum from three of the immunized mice was diluted 1:5,000, 1:10,000, 1:20,000 and 1:40,000 and tested for titers of Der f 2 specific IgG antibody. Control serum of non-immunized mice was tested at a 1:10,000 dilution. All the serum from the immunized mice had antibody titers that could be detected at a dilution of 1:40,000 (Table 1) and the mouse with the highest titer was selected for fusion.

Table 1. OD 450 nm of serum titers in immunized mice

Immunized Mice	1:5,000	1:10,000	1:20,000	1:40,000	Ctrl
1*	1.858	1.561	1.174	0.679	0.079
2	1.508	1.075	0.717	0.341	/
3	1.019	0.900	0.643	0.421	/

Ctrl: negative control using the serum from non-immunized mouse with the a dilution of 1:10,000. *Immunized mouse 1 was selected for fusion.

3.2. *Isotype of the mAb and ascites titration*

After fusion, 130 clones were found to have antibody titers that were significantly higher than the negative control and eight of them were selected for limited dilution (Table 2). After the first subcloning, four stable clones were retained for further limited dilution. Two of the four stable clones, which were classified as either IgG1 or IgG2b isotype (Table 3), were selected for ascites induction for mass production of mAb. ELISA results showed that the antibody titer in the ascites was higher than $1:10^6$, indicating a mAb with high potency (Table 4).

3.3. *Characterization and purification of the mAbs*

The high specificity of the mAbs against Der f 2 was confirmed by immunoblotting assay in both the supernatant of the mAb-producing clones and the ascites before purification (Figure 1A). The ascites was first precipitated by 50% ammonium sulfate and then purified by protein A chromatography. The highest

Table 2. Absorbance of the selected clones after fusion and after the first subcloning detected by ELISA

Clones selection after fusion		Clones selection after the first subcloning	
Clones	OD at 450 nm	Clones	OD at 450 nm
neg.ctrl	0.059	neg.ctrl	0.090
pos. ctrl	3.496	pos. ctrl	4.147
1B12	4.921	1-1B12[a]	5.519
1G4	1.081	1-1G4[b]	0.114
3A12	2.257	3-1A12[a]	4.163
3E1	3.108	3-1E1	4.406
3G5	2.406	3-1G5[b]	0.927
3A7	1.325	3-1A[7]	1.570
3F9	1.381	3-1F9[b]	0.180
4B10	1.092	4-1B10[b]	0.081

neg.ctrl: negative control, serum from non-immunized mouse. pos.ctrl: positive control, serum from immunized mouse. [a]: The individual clones selected for mAb mass production are labeled as 1-1 and 3-1. [b]: The four clones that showed decreasing antibody production.

Table 3. Isotype identification as determined by absorbance at 450 nm

Clones	IgG1	IgG2a	IgG2b	IgG3	IgM	Control
1-1	2.089	0.071	0.169	0.086	0.043	0.043
3-1	0.293	0.165	1.251	0.398	0.220	0.155

Table 4. Titration of Der f 2 mAbs in ascites as shown by absorbance at 450 nm

Clones	$1:10^2$	$1:10^3$	$1:10^4$	$1:10^5$	$1:10^6$	$1:10^7$	blank
1-1	/	4.865	4.513	2.450	0.573	0.112	0.045
3-1	5.645	3.467	1.957	0.943	0.216	/	0.044

/: means not done because of too high in $1:10^2$ and too low in $1:10^7$.

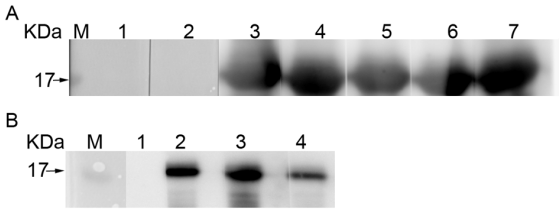

Figure 1. Characterization of Der f 2 mAbs by immunoblotting. A: Characterization of Der f 2 mAbs before purification. M: Marker. Lane 1: SP2/0 culture medium. Lane 2: Serum of non-immunized mouse. Lane 3: Serum of Der f 2 immunized mouse. Lane 4: Supernatant of Der f 2 mAb clone 3-1. Lane 5: Ascites of Der f 2 mAb clone 3-1. Lane 6: Supernatant of Der f 2 mAb clone 1-1. Lane 7: Ascites of Der f 2 mAb clone 1-1. **B:** Characterization of Der f 2 mAbs after purification. M: Marker. Lane 1: Serum of non-immunized mouse. Lane 2: Serum of Der f 2 immunized mouse. Lane 3: Purified Der f 2 mAb clone 1-1. Lane 4: Purified Der f 2 mAb clone 3-1.

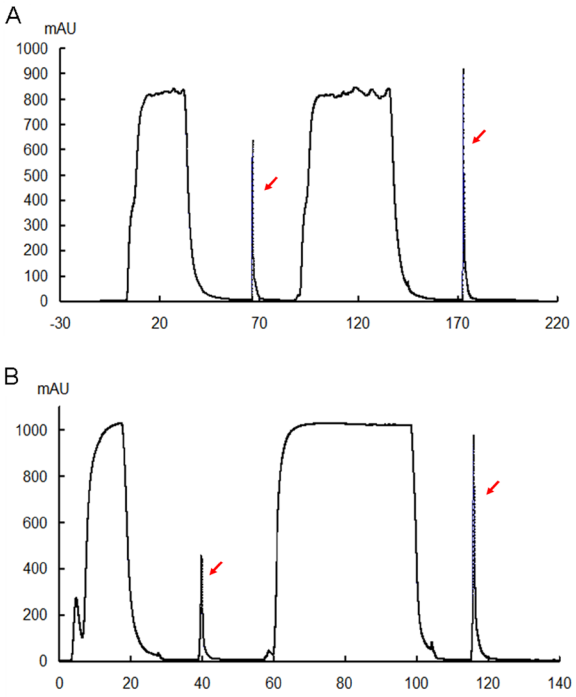

Figure 2. Purification of Der f 2 mAbs. A: Purification of Der f 2 mAb clone 1-1. **B:** Purification of Der f 2 mAb clone 3-1. The arrows indicate the elution peak of the target protein.

elution peaks were collected as purified antibodies (Figure 2), which were then verified by SDS-PAGE electrophoresis (Figure 3) and immunoblotting assay (Figure 1 B).

4. Discussion

In this study, we produced and purified two different isotypes of specific monoclonal antibodies against Der f 2 mite allergen. These antibodies could be used to develop an assay for the quantification of Der f 2 in environmental allergens.

In our study, the fusion rate between the splenocytes and SP2/0 cells was approximately 79% as screened

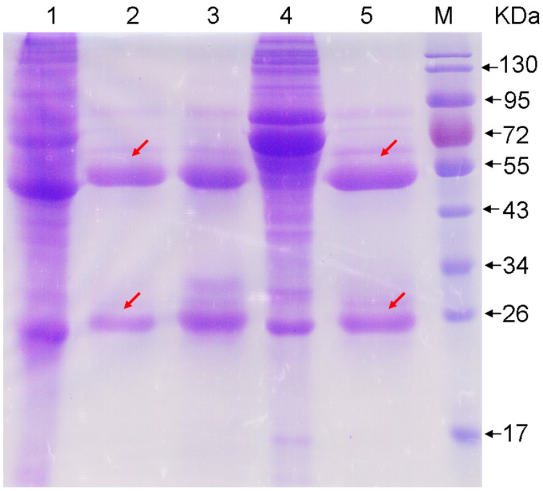

Figure 3. The mAbs after purification. Lane 1: Der f 2 mAb clone 1-1 before purification. **Lane 2:** Purified Der f 2 mAb clone 1-1 collected from the first part of elution peak. **Lane 3:** Purified Der f 2 mAb clone 1-1 collected from the second part of elution peak. **Lane 4:** Der f 2 mAb clone 3-1 before purification. **Lane 5:** Purified Der f 2 mAb clone 3-1. **M:** Markers. The arrows indicate the heavy chain and light chain of the purified antibodies, individually.

by ELISA, which demonstrated the efficiency of PEG 4000 in the production of monoclonal antibodies. Eight clones with the highest ODs were selected for limited dilution. However, after the first subcloning, four of the clones showed a gradual decrease in antibody production, while the other four clones grew well. This situation has been described during other mAb production procedures and could be due to chromosomal instability (*19*).

BALB/c mice are most commonly used for ascites production because this strain of animals is syngeneic with the myeloma cells that are most frequently used for fusion (*20*). Mice were first given a priming agent by *i.p.* injection. After seven days, the hybridoma cells were inoculated by *i.p.* injection. Ascites induction is a life-threatening procedure due to tumor growth, metastatic spreading and infiltrative growth. Mineral oil was first tried as the priming agent but failed to prevent negative side effects related to the growth of the hybridoma cells and resulted in too much bleeding in the abdominal cavity. Thus, we used IFA for priming agent (*21*), which significantly alleviated the negative side effects of tumor growth and increased the amount of ascites production. We also analyzed the differences in ascites induction in different ages and genders of mice and we found that the yield of ascites was greater in 10-12 week-old mice than 6-8 week-old ones, while the gender had no significant influence on ascites production.

During the last several decades, the prevalence of allergic disease has steadily increased in developed countries. Among atopic patients in China, approximately 87% are sensitive to one or more species of HDM (*22*) indicating that it is the main allergen for these patients.

Another study from Taiwan showed that more than 90% of children with asthma had positive skin test responses to HDM (*23*). The high humidity and high ambient temperature in the southern coastal regions of China is suitable for the growth of dust mites. HDM is abundant in the dust samples collected from carpets, mattresses and air-conditioners. There is convincing evidence that avoidance of mite allergen can effectively reduce allergic symptoms (*24*). Thus, it is very important to develop an effective tool to monitor the concentration of allergen components in the indoor environment. The high titer, highly specific antibodies that have been produced in this work could be used to develop assays that can help the physician educate patients in creating an environment with lower concentrations of mite allergen, which would therefore reduce patient exposure to HDM in their residences.

Although HDM allergen is a major cause of respiratory allergic disease, there are still unresolved challenges in both the specific diagnosis and effective treatment (*17*). Purified allergens are helpful for CRD and immunotherapy of HDM allergy (*25*). Treatments for HDM allergy include HDM avoidance and AIT, which is a widely recommended strategy and well documented (*26*). AIT is effective when appropriate doses of allergens are administrated (*27*). Several studies showed that an optimal maintenance dose needed for AIT through the subcutaneous route is in the range of 5-20 μg of the major allergen found in aeroallergens (*28-31*). Therefore, it is quite important to develop a tool for the precise quantitation of allergen components in the allergen extract or the recombinant protein to ensure that it contains an effective therapeutic dose of each of its individual allergen components.

In summary, to better evaluate the correlation between symptom relief and HDM avoidance, we produced and purified two different isotypes of monoclonal antibodies against Der f 2 that might facilitate CRD as well as identify the effective doses of this HDM major allergen component when used in component-resolved immunotherapy.

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