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Preparation of Der p 1 specific monoclonal antibodies and use in a two-site-ELISA to detect Der p 1 allergen

Tzu-I Chien[1], Yi-Guang Chen[2], Bor-Luen Chiang[1,2]

Graduate Institutes of [1]Immunology and [2]Clinical Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan, ROC

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It has been well documented that the mite *Dermatophoides pteronyssinus* (Der p) is the major allergen in house dust in Taiwan. The purpose of this study is to develop a sandwich immunometric assay which can be used for the standardization of mite allergen extracts or house dust, for assessing the procedures of mite avoidance, and for identifying species in epidemiological studies. Der p 1 allergen (both recombinant and native Der p 1) was purified and a panel of Der p 1 specific monoclonal antibodies was developed. The monoclonal antibodies were biotinylated and a two-site enzyme-linked immunosorbent assay (ELISA) with peroxidase-conjugated streptavidin was developed for the detection of Der p 1 allergen. The results suggest that these monoclonal antibodies could be applied both in affinity columns for the purification of native Der p 1 antigen and in sandwich-ELISA for epidemiological study.

Key words: *Dermatophoides pteronyssinus* (Der p) 1 allergen, monoclonal antibodies, sandwich-ELISA

House dust mite is one of the major allergens in house dust. It can be classified into different species according to the morphology [1,2]. *Dermatophagoides pteronyssinus* (Der p) is the major species of the house dust mite in Taiwan area. Immunochemical and molecular studies have already identified more than 10 groups of allergens from Der p. The predominant group 1 allergen is the most common etiology of atopic diseases, and can cause IgE antibody responses in 80% to 95% of patients allergic to mites [3].

Monoclonal antibodies (mABs) have been demonstrated to be a powerful tool in allergy research. They have been used in epitope mapping, in the purification and quantification of allergens in crude extracts, and in the measurement of allergen levels in environmental samples. Use of monoclonal antibodies allows the efficient standardization of allergic extracts [4-9]. It has been recently suggested that the major allergen content or potency of allergen preparations could be more appropriately expressed in weight units than that in biologic units.

In this study, Der p 1 allergen was purified and a panel of Der p 1 specific monoclonal antibodies was then developed. One of these monoclonal antibodies was biotinylated and used to develop a two-site enzyme-linked immunosorbent assay (ELISA) with peroxidase-conjugated streptavidin for the detection of Der p 1 allergen. This assay is highly specific, highly sensitive and suitable for screening large numbers of samples. This assay may be useful in the standardization of mite allergen extracts or house dust, in assessing procedures for mite avoidance, and in the identification of species in epidemiological studies.

Corresponding author: Dr. Bor-Luen Chiang, Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, No. 1, Chang-Teh Street, Taipei, Taiwan, ROC

Materials and Methods

Affinity purification of fusion proteins (recombinant Der p 1)

To purify the proteins used in establishment of Der p 1 specific monoclonal antibodies, plasmids containing pGEX-Der p 1 sequences were transformed into *E. coli.* Cells were grown overnight at 37 °C in Luria broth (L broth, Merck, Darmtadt, Germany) containing ampicillin (100 μg/mL). On the second day, the cultures were diluted 1:10 in fresh L broth containing 100 μg/mL ampicillin, and grown for about 1.5 h at 37 °C to an OD600 = 0.5-1.0 unit. After addition of 0.1 mM isopropyl-b-D-thiogalactoside (IPTG, Promega Corp., Madison, WI, USA), cells were incubated at 37 °C for an additional 2 to 3 h and then were pelleted and resuspended in 1/100 culture volume of Tris-buffered saline (TBS). The cell suspension was then added to a Braun Bottle containing glass beads equal to half of

the volume of the cell suspension and Aprotinin (Sigma Chemical Co., St. Louis, MO, USA) to 1%, 10 mg/mL of DNase (Boehringer Mannheim Biochemical, Mannheim, Germany) with $MGC1_2$ to 5 mM, phenylmethylsulfonyl fluoride (PMSF, Sigma), to 0.5 mM, and Tween-20 to 1%. Cells were homogenized for 1 min intervals three times in the homogenizer (Braun, Hamburger, Germany), stopping after each minute to add PMSF to 0.5 mM and chilling the bottle on ice. The lysate was removed to an ultracentrifuge tube and ethylene diamine tetraacetic acid (EDTA) was added to 0.25 mM followed by centrifugation at 20000 rpm for 30 min at 4 °C. The supernatants were added to a hydrated column of glutathione agarose beads (Sigma). The agarose beads were washed and the excess TBS was drained overnight. Fusion proteins were eluted by competition with free glutathione by washing for three 30 min intervals with 1 bead volume of 50 mM Tris-HCl (pH 8.0) containing 5 mM reduced glutathione (final pH7.5, freshly prepared). The eluted solutions were dialyzed against PBS (pH 7.4) overnight and stored at -70 °C.

Purification of native Der p 1

Native Der p 1 antigen was purified from crude mite medium by affinity chromatography with a Sepharose (Pharmacia Diagnostic AB, Uppsala, Sweden) column chemically coupled with Der p 1 specific mAbs. The crude mite medium was dissolved in Tris-HCl pH 7.6 and passed through the affinity column, and then washed with Tris-HCl pH 7.6 until the protein absorption (280 nm) reached baseline. Der p 1 was eluted with NH_4OH into tubes containing 300 mL of Tris-HCl pH 6.8 buffer to neutralize the eluate. Der p 1 eluted in this manner from multiple runs was pooled, dialyzed against 1 x PBS, and stored at -70 °C. The purity of Der p 1 proteins (both recombinant and native Der p 1) was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein yields were calculated from the absorbance at 280 nm using BSA as the standard.

Immunization of mice

To develop hybridomas specific for Der p1 antigen, Balb/c mice were immunized with 25 μg Ag in 100 μL PBS with complete Freund's adjuvant subcutaneously. Ten days after the first immunization, the mice were boosted by intraperitoneal injection with 50 μg Ag in 100 μL PBS mixed with incomplete Freund's adjuvant. Then 25 μg Ag in 100 μL PBS was injected with incomplete Freund's adjuvant by subcutaneous and intraperitoneal injection alternately. Seven days after each injection, the concentration of specific antibodies in mice sera was monitored by ELISA. Intravenous injection with 25 μg Ag in 100 μL PBS only was used as the final boost just 3 days before a hybridoma fusion.

Establishment of Der p 1-specific monoclonal antibodies

Single cell suspensions of spleen cells were prepared and fused with F-0 myeloma cells affected with polyethylene glycol. A ratio of 3:1 spleen cells to myeloma cell ratio of 3:1 was used and cells were plated at 2×10^5 per well in 96 well plates in Iscove's modified Dulbecco's medium (IMDM, Gibco/BRL, Gaithersburg, MD, USA) medium containing antibiotics, and hypoxanthin-aminopterine-thymidine (HAT, Sigma). Hybridoma supernatants were screened by ELISA, and cell lines were cloned in soft agar or by limiting dilution (0.5-1.0 cells/well). Then ascites fluid produced in pristane-primed Balb/c mice was collected.

ELISA for anti-Der p 1 antibody

Polystyrene microtiter plates were coated with 100 μL per well of Der p 1 at a concentration of 1 μg/mL in PBS, pH 8.0 at 4 °C overnight. The wells were thoroughly washed with PBS, pH 7.4, containing 0.05% Tween-20. After antigen coating, the plates were blocked for 45 min at 37 °C with 200 μL of blocking buffer consisting of 1% bovine serum albumin (BSA) and 0.05% Tween-20 in PBS, pH 7.4. This buffer was also used to dilute the antibodies. The collected supernatants of hybridomas or ascites were added in optimal dilutions to the solid-phase-bound antigen with the volume of 100 μL and incubated for 45 min at 37 °C. The murine mAbs were detected by peroxidase-labeled goat anti-mouse IgG (γ chain) or IgM (μ chain) which were diluted with blocking buffer. Then, 100 μL of 2,2'-azino-bis (3-ethyl-benzthiazoline-6-sulfonic acid; ABTS) was added to each well at a concentration of 0.55 mg/mL in 1 mL (1x) citrate buffer, pH 4.2, containing 10 μL 3% H_2O_2. The optical density was measured at 420 nm with an ELISA reader.

Dot blotting analysis

Dot blotting was performed by transferring samples to nitrocellulose paper with a 96-well microfiltration apparatus. Samples (100 μL) containing 5 μg of Der p 1 were applied to the nitrocellulose paper by suction, and blocked with 5% fat-free milk powder in TNT (containing Tris, NaCl, and Tween-20). After incubation with supernatants or ascites of hybridomas overnight, the filters were washed and bound mouse IgG or IgM were detected by phosphatase-conjugated anti-mouse

IgG or IgM. Finally the filters were again washed with TNT, and developed with the nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) detection reagent.

Immunoprecipitation

An equal volume of spent mite media was added to the ascites containing anti-Der p 1 IgG mAb, mixed and left at 4 °C for 1 h on an orbital shaker. Then, 20 µL of protein A-sepharose beads was added and shaking was done for another 1 h at 4 °C. The mixture was spun at 7,500 rpm for 30 sec, and the supernatant was then carefully aspirated with a fine-tip Pasteur pipet. The pellets were washed by resuspending in 1 mL of high salt solution, low salt solution, RIPA buffer, and washing buffer. Each wash step was followed by spinning at 7,500 rpm for 30 sec. The immune complex of anti-Der p 1 IgG and Der p 1 was prepared using immunoprecipitation and was estimated by 12% SDS-PAGE.

Purification of IgG from ascites

Packed immunopure immobilized protein G gel was put into the column to be and equilibrated with 5 column volumes of binding buffer (0.1 M sodium acetate, pH 5.0). To ensure that the proper pH was maintained for optimal binding, ascites fluid was diluted at least 1:1 with binding buffer. The diluted ascites were applied to the protein G column and allowed to flow completely into the gel. The protein G column could bind with the Fc regions of IgGs. The column was washed with 10 mL binding buffer. Eluted the bound IgG with 6 mL elution buffer. Collected eluate in 1 mL fractions to increase the concentration of samples. The pH of the protein fractions was immediately raised to neutral by addition of 250 µL PBS. Elution of bound proteins could be monitored by absorbance at 280 nm. The elution with the highest titer was used for desalting. Eluate was collected in 1 mL fractions from the desalting column as described above. The purified IgG fraction of the highest absorbance was analyzed by 12% SDS-PAGE and its concentration calculated using BSA as the standard.

Biotinylation

The purified IgG from ascites fluid was dialyzed against 0.1 M sodium phosphate, pH 7.2. Then, 1 mg BAC-SulfoNHS (biotinamidocaproate-N-hydroxy-sulfosuccinimide ester, Sigma) was dissolved with 6 µL DMSO, and 0.1 M sodium phosphate buffer was added to a final volume of 0.1 mL followed by thorough vortexing. Then, 38 µL of the solution was immediately added to 1 mL of the IgG solution with gentle stirring for 30 min at room temperature. The biotinylated protein was separated from unreacted fraction using by a gel-filtration column. The column was equilibrated with 30 mL PBS and reaction mixture was then applied to the column, and the eluate was collected in 1 mL fractions. The presence of protein was monitored by measuring absorbance at 280 nm.

Two-site immunometric assay

Polystyrene microtiter plates were coated overnight with anti-Der p 1 IgG mAb at a concentration of 10 µg/mL in a carbonate coating buffer at 4 °C. After washing, the plates were blocked for 45 min at 37 °C with 200 µL of blocking buffer consisting of 3% BSA and 0.05% Tween-20 in PBS, pH 7.4. Der p 1, Der p 2, GST, and dust samples were added to each of the plates at fourfold dilutions varying from 3.9 ng/mL to 1,000 ng/mL at 37 °C. After incubation, the plates were washed four times and biotinylated mAb 5C63 was added at a concentration of 1 µg/mL, and the plate was incubated at 37 °C for 45 min. After incubation and washing, the biotinylated antibody was detected using by streptavidin-peroxidase development system as described above.

Results

Preparation of recombinant and native Der p 1

We were unable to cleave recombinant Der p 1 from the glutathione fusion protein. All recombinant Der p 1 was thus expressed as fusion proteins containing glutathione-S-transferase. The results of analysis of both native and recombinant Der p 1 proteins by SDS-PAGE are shown in figure 1. The molecular weight of recombinant Der p1 antigen was in the region from 45 kd to 66 kd, however, the protein also had some degraded bands. Native Der p 1 protein was purified by affinity column and the molecular weight was located in the region from 21 kd to 31 kd. We further checked if the degraded protein of recombinant Der p 1 still contained its epitope on ELISA using immunized mice serum. The dose response curves for recombinant and native Der p 1 assays are illustrated in figure 2. The OD value was closer when coating 8 µg/mL of two kinds of Der p 1, and large differences were found on serial dilution of the allergens. The optimal concentration of native Der p 1 was 1 µg/mL.

Establishment of Der p 1-specific monoclonal antibodies

The mice serum was collected after each boost with

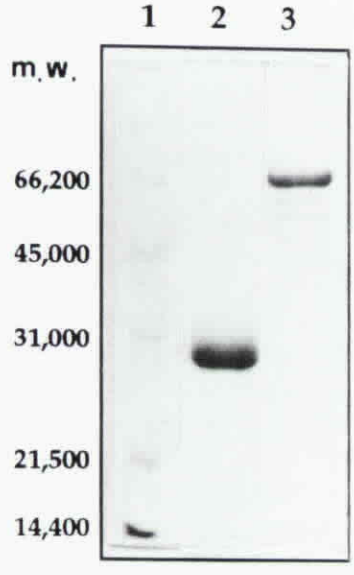

Fig. 1. The purified native and recombinant Der p 1 proteins were demonstrated by SDS-PAGE. Lane 1 = marker; lane 2 = purified native Der p1; lane 3 = recombinant Der p1; m.w. = molecular weight

Der p1 Ag. Serum was used to detect the titer of anti-Der p 1 Ab by ELISA (Fig. 3). The greater the number of times was boosted, the higher titer of antibody was noted. Cell fusion between spleen cells of Balb/c mice immunized with native Der p 1 and the mouse myeloma cells generated a panel of hybridomas specific for Der p 1. Nine monoclonal antibody-producing clones were chosen for further study. They secreted antibodies that

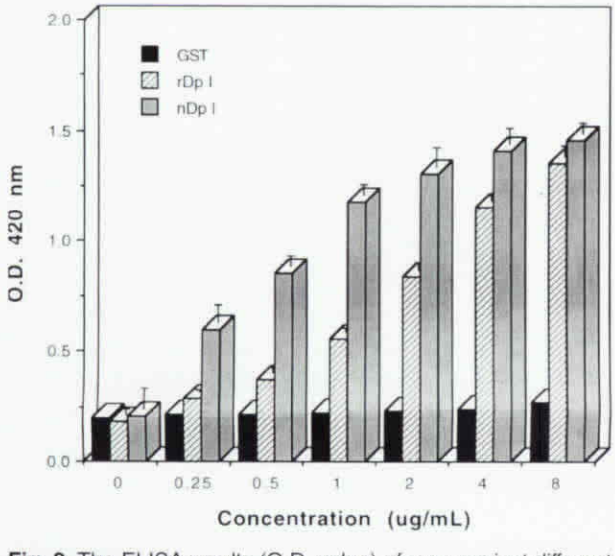

Fig. 2. The ELISA results (O.D. value) of sera against different preparations of Der p 1 allergens. GST = glutathione-S-transferase; rDp 1 = recombinant Der p1; nDp 1 = native Der p1

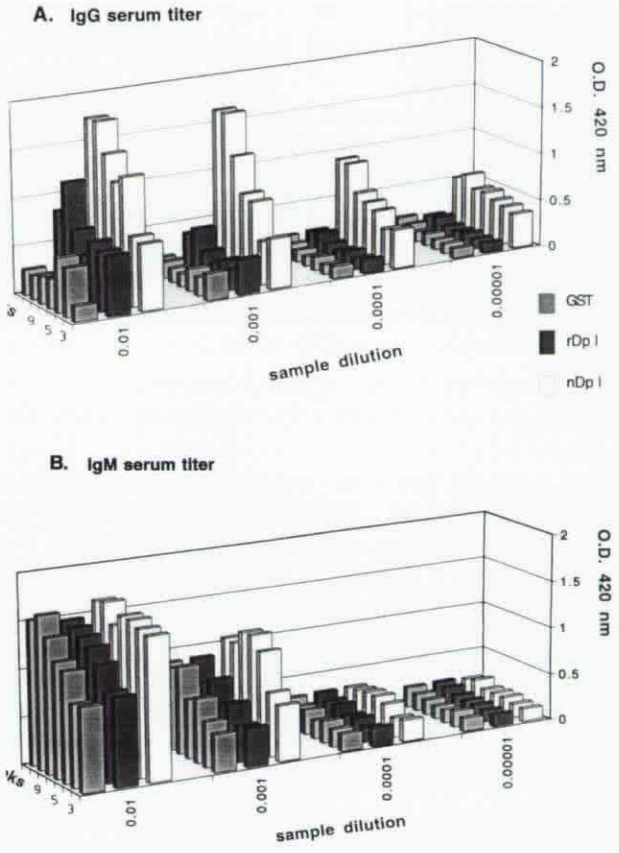

Fig. 3. Sera sequential titer of antibody in mice immunized with native Der p 1 antigen. The titer increased with the booster of immunization and reached the peak nine weeks after the first immunization. GST = glutathione-S-transferase; rDp 1 = recombinant Der p1; nDp 1 = native Der p1

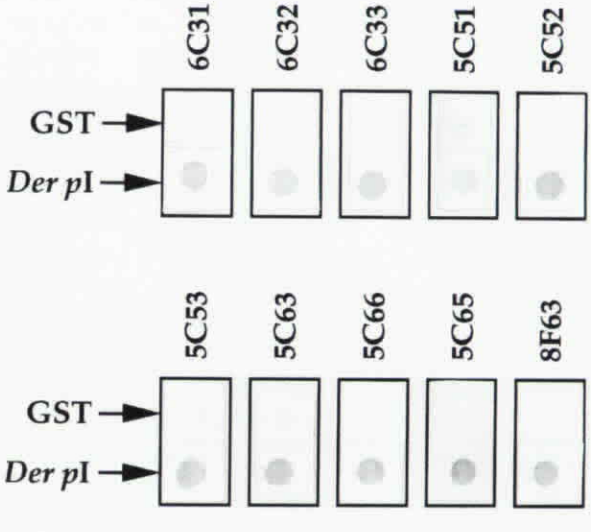

Fig. 4. The immunoblotting data suggested that monoclonal antibodies can specifically recognize Der p 1 antigen but not GST protein.

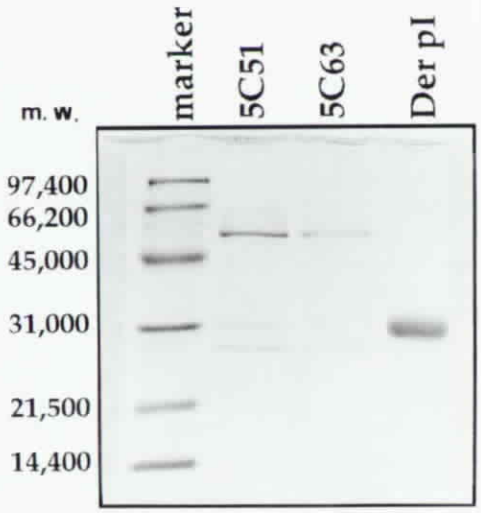

Fig. 5. Immunoprecipitation of Der p 1 antigen with monoclonal antibodies isolated from ascites of mice injected with monoclonal antibody hybridomas.

bound Der p 1, as determined by ELISA and dot blotting analysis (Fig. 4). Three of the hybridomas secreted IgM antibody (6C31, 6C32, and 6C33) and the others secreted IgG antibody (5C51, 5C52, 5C53, 5C63, 5C65, and 5C66).

Sandwich-ELISA to detect Der p 1

Ascites collected from the mice were further purified with a protein G column and the results of immunoblotting assay suggest that the antibody could precipitate the Der p 1 protein (Fig. 5). The monoclonal antibody was further biotinylated before setting up the sandwich-ELISA assay to measure the level of Der p 1 allergen. The results demonstrated that sandwich-ELISA developed in this study could detect Der p 1 allergen but not Der p 2 allergen (Fig. 6).

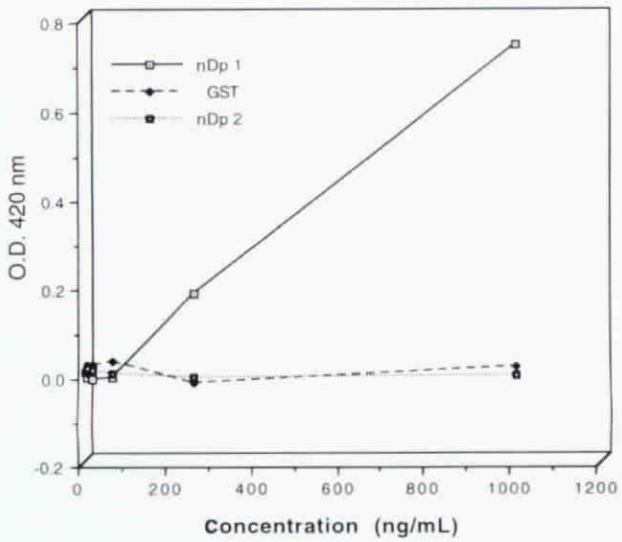

Fig. 6. Establishment of sandwich-ELISA for the determination of Der p 1 protein. The data suggested that the assay could specifically detect Der p 1 protein but not Der p 2 protein. GST = glutathione-S-transferase; nDp 1 = native Der p1; nDp 2 = native Der p2

Discussion

The prevalence of atopic diseases such as bronchial asthma, allergic rhinitis and atopic dermatitis has increased strikingly in recent years. House dust mite has been documented to be the major allergen accounting for atopic diseases such as bronchial asthma, allergic rhinitis and atopic dermatitis in many countries. Mite allergen has been found to be the most important aeroallergen both in Taiwan and worldwide [10]. Thus, evaluation and monitoring of the relationship between the environmental concentration of mite allergen and the severity of atopic diseases has become increasingly important. Although several studies concerning the environmental mite allergens have been reported [11, 12], more epidemiological data still are needed in the future.

Many researchers have suggested that purified recombinant allergen might be the major component of future immunotherapy. All the evidence suggests that more effort should be focused on the identification and application of allergens. The immune response to Der p allergen has been extensively studied in patients with different clinical symptoms and in patients being treated with immunotherapy. However, very few studies have been performed using purified allergens of major importance to human allergen patients because these purified major mite allergens are not available in sufficient quantities to allow such studies. It has been demonstrated that both genetic and environmental factors play a critical role in the pathogenesis of bronchial asthma. The monoclonal antibodies derived in this study can be used for either sandwich-ELISA to detect environmental mite allergen or for the purification of recombinant allergen for purposes of research and treatment [13]. However, the availability of monoclonal antibodies for the assay remains limited. In the future, the measurement of environmental mite allergen might become a standard environmental control for allergic diseases [14].

In conclusion, panels of monoclonal antibodies were generated in this study which can be used for future epidemiological and research studies. The result of this study also suggested that monoclonal antibodies to

allergens can serve as a useful tool for the purification of the allergens and can also be applied for the assay of environmental allergens.

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