

Purification of Group 2 *Dermatophagoides pteronyssinus* Allergen and Prevalence of Its Specific IgE in Asthmatics

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Key Words

Dermatophagoides pteronyssinus · Der p 2 · Bronchial asthma

Abstract

Group 2 allergens are a major cause of sensitization in patients allergic to house dust mites. This study was performed to determine the prevalence of hypersensitivity to group 2 allergens (Der p 2) of *Dermatophagoides pteronyssinus* (Dp) in asthmatic patients in Taiwan. To facilitate the analysis of Der p 2-specific IgE, we raised a panel of monoclonal antibodies (MoAbs) to Der p 2 antigens. Purified Der p 2 was obtained after MoAb affinity column purification. There were 82 asthmatic patients (41 adults and 41 children) with hypersensitivity to Dp who were analyzed for hypersensitivity to Der p 2. All of them were both skin test- and serology test-reactive to Dp. Using purified Der p 2, 87.8% (72/82) of patients had a skin-test-positive reaction. Six adults (6/41) and 4 children (4/41) had negative skin tests for Der p 2. Ten families (both parents and children were asthmatics) of the 82 patients were selected for Der p 2 skin testing and Der p 2-specific IgE determination using immunoblot analysis. Results showed that 90% (18/20) of patients' skin reactions to Der p 2 and serum contained specific IgE to Der p 2. Because 87.8% (85.4% of adults and 90.2% of children)

of the asthmatic patients with Dp hypersensitivity were allergic to Der p 2, its role in the pathogenesis of asthma in Taiwan appears to be important. Purified Der p 2 allergens can be further used for allergen skin testing and immunotherapy.

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Introduction

Sensitization to house dust mites is an important risk factor for the development of bronchial asthma [1, 2]. A previous study demonstrated that house dust mites are highly prevalent in Taiwan [3]. Although many species of mites have been identified, *Dermatophagoides pteronyssinus* (Dp) accounted for 52.1% of the total number of mites and was found in every house of asthmatic patients in Taiwan [4]. More than 80% of asthmatic patients are allergic to Dp as determined by skin testing and Dp-specific IgE measurement [5].

Although multiple IgE-binding allergens have been identified in extracts of house dust mites, Der p 1 and Der p 2 are generally considered the major allergens. Der p 1, a 24-kD component of mite feces, and Der p 2, a 14-kD component of mite bodies, bind IgE in the majority of mite-allergic patients and account for up to 75% of the total IgE reactivity to mites [6–11].

Table 1. Peptide fragments contained in fusion proteins

S ₂ R		F1
1–32		F2
1–69		F3
1–76		F4
1–84		F5
20–32		F6
20–45		F7
22–45		F8
22–76		F9
22–129		F10
33–61		F11
42–84		F12
65–84		F13
69–115		F14
69–129		F15
105–129		F16

In atopic patients, the published frequency of IgE reactivity to Der p 2 varies from 27 to 81% [12, 13]. A comparison of different age groups of allergic patients showed that 89% of children were serologically reactive to Der p 2, whereas only 58% of adults were reactive [14]. There are a number of potential reasons for this variation, including the study of patients from different environments and varied clinical manifestations. However, the precise mechanism is still unclear.

Our study investigated the frequency of recognition of Der p 2 by both skin prick testing and IgE determination using Western blot analysis. Both adults and children, particularly in families with the same environmental conditions, were included to clarify whether age plays a role in Der p 2 IgE formation.

Materials and Methods

Subjects

Eighty-two allergic, asthmatic patients who attended the allergy clinic at Cathay General Hospital-Taipei were recruited for this study. Allergic asthma was defined as a history of reversible obstructive airway disease associated with exacerbation resulting from allergen exposure and concomitant skin reactivity to exacerbating allergens. All patients were sensitive to mites and had positive skin tests and mite-specific IgE as determined using the Pharmacia CAP System (Uppsala, Sweden). Sera were drawn and stored at –70 °C before testing. The study was approved by the Human Research Ethics Committee of Cathay General Hospital and informed, written consent was obtained from all participants.

Preparation of Mite Extracts

Standardized extracts of Dp and *Dermatophagoides farinae* (Df; 10,000 BU/ml, 0.28 mg/ml) were purchased from Greer Laboratory (Lenoir, Calif., USA) for both skin testing and immunoblot analysis. For Der p 2 purification, Dp was obtained from Allergon Pharmacia (Uppsala, Sweden). Mites were extracted by homogenizing after resuspending in phosphate-buffered saline (PBS) containing aprotinin (0.1 IU/ml; Sigma Chemicals, St. Louis, Mo., USA) and phenylmethylsulfonyl fluoride (1 mmol/l; Sigma). After centrifugation at 10,000 g for 30 min, the supernatant was dialyzed against 0.05 mol/l of ammonium carbonate, pH 8.0. It was then freeze-dried in aliquots and stored at 4 °C until use. The protein content was determined by Lowry's method, using bovine serum albumin as standard.

Recombinant Der p 2 and Monoclonal Antibody Preparation

Constructs expressing peptide fragments of Der p 2 as fusion proteins from pGEX vectors were prepared by Dr. K.Y. Chua at the University of Singapore (table 1). The polypeptides were fused to glutathione S-transferase (GST), encoded by the Sj26 gene of the pGEX vector, and were affinity-purified from glutathione agarose. The purified recombinant Der p2 (rDer p 2), analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), appeared as a fusion protein of 42 kD with a fraction of GEX protein, 26 kD.

Monoclonal antibodies (MoAbs) against Der p 2 were prepared as previously described [15]. Spleen cells obtained from Balb/c mice immunized with the rDer p 2 were fused with murine plasmacytoma NS-1 cells in the presence of polyethylene glycol (molecular weight 1,500 daltons; Merck, Hohenbrunn, Germany). Antibody-producing hybrid cells were screened using the enzyme-linked immunosorbent assay (ELISA) and recombinant GST-Der p 2 and GST alone. Hybridomas producing Der p 2-specific MoAbs were subcloned at least twice by limited dilution and further expanded. The isotype of the MoAbs was determined by ELISA with class-specific antisera (Southern Biotechnology Associates, Birmingham, Ala., USA).

ELISA

ELISA was performed as previously described [15]. The antigens used were coated separately onto wells of polyvinyl microtiter plates (Costar, Cambridge, Mass., USA) by the addition of 50 µl of a 0.5 µg/ml solution of recombinant proteins in carbonate buffer, pH 9.6. After blocking with 1% skimmed milk, supernatant solutions from the cultured hybridoma cells were added to each well and incubated for 1 h at 37 °C. After washing with PBS containing 0.05% Tween 20 (PBST), peroxidase-conjugated goat antimouse IgG (whole molecule, Bio-Rad, Richmond, Va., USA) was added to each well and was incubated for 1 h at 37 °C. Wells were washed again with PBST and 50 µl of substrate solution [Bio-Rad, Peroxidase Substrate Kit 172-1064, containing 2,2-di-(3-ethyl benzthiazoline-6-sulfonic acid) and hydrogen peroxide] was added to each well for 30 min at 37 °C. The color change was detected by measuring the absorbance at a wavelength of 415 nm using the Titertek Multiskan (Flow Laboratories, McLean, Va., USA). Supernatant solutions of hybridoma cells containing MoAbs against fungal proteins were used as negative controls. Two readings from two separate determinations were averaged for the result. ELISA activity was attributed to supernatants that had absorbencies greater than 1.2 times the absorbance obtained with the negative controls.

Preparation of Der p 2 by MoAb Affinity Column and Affinity Chromatography

MoAbs were purified from ascites fluid with a protein A column and coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) at a concentration of 4 mg/ml. The gel matrix was then reacted with ethanolamine and washed with sodium acetate and sodium bicarbonate buffer before it was packed into a glass column, according to the manufacturer's direction. The immunosorbent column (1.5 × 15 cm) was preequilibrated with PBS, and 40 mg of crude mite extract was loaded onto the column at a flow rate of 5 ml/h. Unabsorbed proteins were removed by washing with PBS, and the column was developed with two bed volumes of elution buffer (0.1 mol/l citric acid, pH 3.0) at a flow rate of 2 ml/min. The pH of the protein eluted from the column was immediately adjusted to 7.0 with 1 mol/l Tris buffer (pH 10), and then dialyzed against 0.05 mol/l ammonia bicarbonate pH 8.0 and lyophilized. The affinity chromatography-purified protein was further analyzed using SDS-PAGE after dissolving in the assay buffer as described above.

Skin Prick Test and Measurement of Specific IgE

Purified Der p 2 was used for skin prick testing at the concentration of 0.06 µg/µl after the end point titration by using 10-fold dilutions. Both purified Der p 2 and commercially available Dp were used in this study. The skin was prick-tested with a disposable needle (Hollister-Stier). Each allergen extract was applied 20 mm apart on the volar surface of the forearm. Fifteen minutes later, the reactions were read and the sizes (in millimeters) of wheals and flares were recorded. Reactions of 3 × 3 mm or greater were considered positive.

Measurement of specific IgE to Dp was performed using Pharmacia CAP System. The assay procedure was automatically performed and the results were expressed as kU/l (with calibration against the WHO standard for IgE). The concentration range was from 0.35 to 100 kU/l. The titers of antigen-specific IgE were further classified as follows: class 1: 0.35–0.7 kU/l, class 2: 0.7–3.5 kU/l, class 3: 3.5–17.5 kU/l, class 4: 17.5–50.0 kU/l, class 5: 50–100 kU/l and class 6: 100 kU/l.

Table 2. Immunological reactions of MoAbs against Der p 2 with *Dermatophagoides* spp.

MoAbs	ELISA activity (absorbance at 415 nm)			Immunoglobulin isotype
	rDer p 2	extract of Dp	extract of Df	
C1	2.264	2.594	0.798	IgG1, kappa
C2	1.265	0.888	0.674	IgG1, kappa
C3	2.441	2.282	0.598	IgG1, kappa
C4	1.246	0.830	0.656	IgG1, kappa
C5	2.132	2.056	0.250	IgG1, kappa
P40	0.125	0.139	0.121	IgG1, kappa

Whole mite extracts obtained from Greer Laboratories, Lenoir, N.C., USA.

P40 represents an MoAb against the 68-kD allergen of *Penicillium notatum*, which was used as a negative control antibody in ELISA.

Immunoblot Analysis

Immunoblot analysis of Dp components recognized by human IgE antibodies was done according to the standard method [13]. The total protein concentration used for the SDS-PAGE was 200 µg in 20 µl. Dp extract in SDS-PAGE sample buffer (containing 2% SDS and 5% 2-mercaptoethanol) was boiled for 5 min, separated on a 12.5% SDS-polyacrylamide gel and then transferred electrophoretically onto a polyvinylidene difluoride (PVDF) membrane (0.45 µm, Millipore, Bedford, Mass., USA). After being blocked with 1% skim milk in Tris-buffered saline (TBS, 20 mmol/l of Tris, 500 mmol/l of NaCl, pH 7.5), the blots were incubated with diluted serum samples [1 in 5 dilution in TBS containing 0.05% Tween 20 (TBST) and 1% skim milk] for 16 h at 4 °C. After washing with TBST, the blots were incubated with alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies (Pharmingen, San Diego, Calif., USA) for 1.5 h at room temperature. After washing, the blots were developed in a substrate solution of carbonate buffer (0.1 mol/l NaHCO₃, 1.0 mol/l MgCl₂, pH 9.8) containing 0.35 mol/l BCIP (5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt) and 0.37 mol/l NBT (*p*-nitro blue tetrazolium chloride) for about 30 min. After washing with water, the deposition of the purple stain demonstrating antigen-antibody binding was recorded photographically. For negative controls, serum samples obtained from cord blood were used.

Results

Preparation and Characterization of MoAbs

After the fusion of spleen cells with NS-1 cells, hybridomas secreting antibodies against Der p 2 were screened using ELISA. The antibody-producing hybridomas were cloned using limited dilution. Five stable clones (C1–C5) were expanded. The immunological characteristics of these five MoAbs are listed in table 2. The immunoglobulin isotypes were IgG1 with κ-light chains. All MoAbs

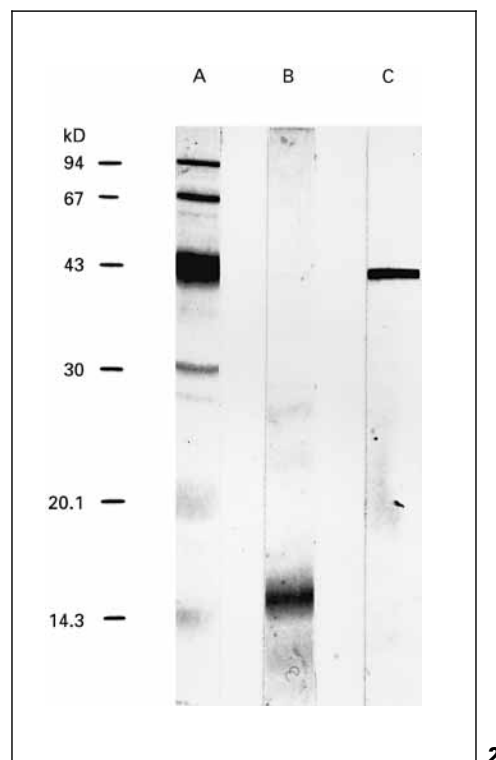
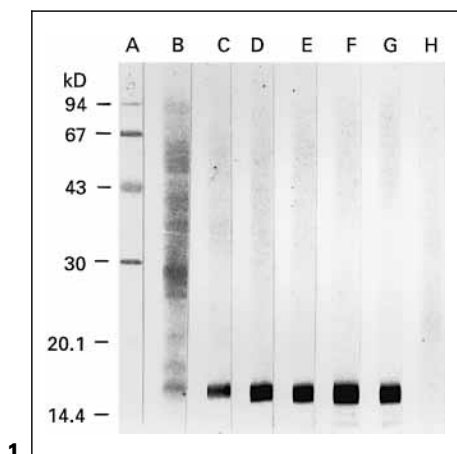


Fig. 1. Immunoblotting activity of MoAb C1–C5, to crude extract of Dp. Lane B represents the crude extract of Dp separated on gel and transferred onto a PVDF membrane. Protein was visualized using the Coomassie blue stain. Lanes C–G are the same protein reacted with MoAb C1–C5, respectively. Lane H was reacted with P40 as a negative control. Lane A contains the protein markers.

Fig. 2. SDS-PAGE pattern of purified Der p 2. Both MoAb and glutathione agarose affinity columns were used to purify Der p 2. Proteins were visualized by silver staining. Lane A contains protein markers. Lanes B and C contain native Der p 2 and rDer p 2, respectively.

Table 3. Peptide mapping of the house dust mite Der p 2

MoAbs	ELISA activity (absorbance at 415 nm)					
	S ₂ R	F3	F5	F9	F15	GEX
C1	2.606 (16)	0.331 (2)	2.474 (13)	0.305 (2)	2.532 (16)	0.151 (1)
C3	2.588 (16)	2.248 (13)	2.419 (13)	1.464 (9)	1.292 (8)	0.164 (1)
C5	2.490 (15)	0.504 (3)	2.454 (13)	0.434 (3)	2.516 (16)	0.162 (1)
P40 ^a	0.162 (1)	0.176 (1)	0.192 (1)	0.168 (1)	0.160 (1)	0.188 (1)

^a An MoAb against the 68-kD allergen of *Penicillium notatum*.

showed strong ELISA activity to rDer p 2. When crude mite extract was used, C1, C3 and C5 showed strong ELISA activity to Dp; however, C2 and C4 showed only weak ELISA activity. All MoAbs showed weak reactivity to the Df extract. Negative ELISA activity to all extracts was found using mold MoAb P40 as control. A panel of peptide fragments was used to identify the epitope that was recognized by these MoAbs. Peptide fragments were coated onto polyvinyl microtiter plates followed by MoAb reaction (0.2 µg/ml for antigen and 0.1 µg/ml for MoAb). Results showed that C3 recognized F3 and F5 and C1 and C5 recognized F5 and F15 (table 3). MoAbs were used to

detect Der p 2 components on Dp PVDF blots. Results showed that all MoAbs reacted strongly with the crude mite extract of Dp. No immunoblot reaction was detected when control MoAb P40 was used. The blotting showed that all MoAbs reacted with 16-kD mite components (fig. 1).

Purification of Der p 2

rDer p 2 fused to GST was affinity-purified using glutathione agarose. Purified rDer p 2 was analyzed using SDS-PAGE and visualized by silver staining. It appeared as a fusion protein of 42 kD (fig. 2) and native Der p 2 was

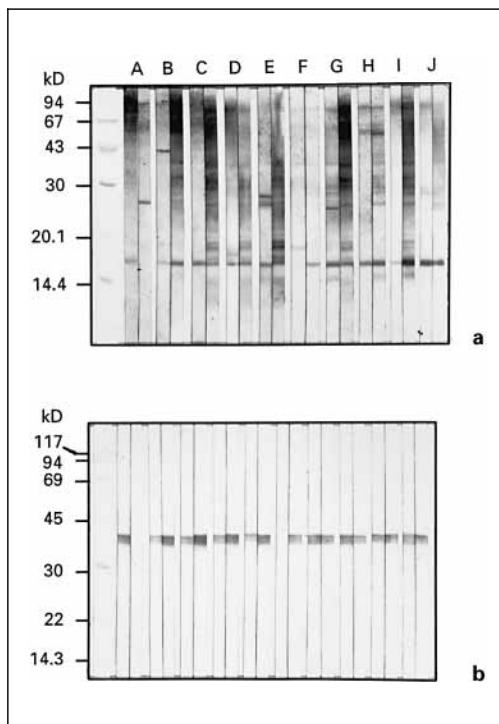


Fig. 3. SDS-PAGE immunoblot of Dp IgE-binding components. Dp blot (a) and Der p 2 blot (b) are shown. Sera from 10 families (A–J) were used to detect IgE-binding components of Dp on a polyvinylidene difluoride blot.

isolated from crude mite extract of Dp with the MoAb affinity column. After analysis with SDS-PAGE and visualization by silver staining, native Der p 2 appeared as a single major component with a molecular weight of 16 kD (fig. 2).

Skin Reaction and IgE Serologic Reactivity to Der p 2

A total of 82 asthmatic patients (41 adults with a mean age of 38 ± 6 years and 41 children with a mean age of 11 ± 4 years) underwent skin prick testing. All of them showed skin hyperreactivity to Dp; however, only 72 patients reacted to Der p 2. Six adults and 3 children showed negative responses to Der p 2. Twenty patients (10 families with 10 parents and 10 children) were selected for a comparison study of skin testing of specific IgE to Dp and Der p 2. The immunoblot assay was used to identify the specific IgE to Dp and Der p 2.

Results showed that 9 parents (9/10) and 9 children (9/10) were both skin test- and serologically reactive to Der p 2 (table 4). The patients who were skin test-negative to Der p 2 (A2 and F1) were also negative to rDer p 2 in the immunoblot assay (fig. 3b). When crude mite extracts

Table 4. Comparison of skin test and specific IgE to Dp and Der p 2 between parents and children

Parent, child	Sex	Age years	Skin test		Specific IgE	
			Dp	Der p 2	Dp, kU/l	Der p 2
A1	F	32	+	+	4	+
A2	M	10	+	–	4	–
B1	M	41	+	+	6	+
B2	M	20	+	+	6	+
C1	M	33	+	+	3	+
C2	F	6	+	+	6	+
D1	F	32	+	+	5	+
D2	M	9	+	+	3	+
E1	M	43	+	+	5	+
E2	M	14	+	+	6	+
F1	M	39	+	–	3	–
F2	M	11	+	+	4	+
G1	F	34	+	+	4	+
G2	M	11	+	+	5	+
H1	F	39	+	+	3	+
H2	M	9	+	+	6	+
I1	M	34	+	+	3	+
I2	M	11	+	+	6	+
J1	F	44	+	+	4	+
J2	F	16	+	+	4	+

1 = Parents; 2 = children.

were used for the immunoblot assay, there were still some IgE-binding proteins detectable (fig. 3a).

Discussion

Allergy to the Dp house dust mite is mediated by IgE to the major allergens Der p 1 and Der p 2 in the majority of mite-allergic patients. In recent years, standardized preparations of Dp, commercially available from several sources, have become widely used for the diagnosis. However, it has been reported that different Dp extracts reveal variability in levels of both Der p 1 and Der p 2 [16]. These extract differences may contribute to differences in skin test reactions in mite-allergic patients. The use of purified, standard Der p 1 and Der p 2 allergens might diminish this wide variability. In our study, we were able to obtain purified Der p 2 after MoAb affinity column purification. The purified Der p 2 was further used to evaluate the prevalence of Der p 2 hypersensitivity among Dp-allergic patients, including those with atopic dermatitis, allergic rhinitis and asthma.

Although it has been reported that more than 80% of asthmatic patients in Taiwan are allergic to Dp according to both skin testing and the presence of Dp-specific IgE [5], information on Der p 2 hypersensitivity in asthmatic patients is still lacking. Our results showed that most asthmatic patients presenting to the allergy clinic with Dp sensitivity had skin test reactivity to Der p 2 (87.8%, 72/82). These results further indicate that Der p 2 is a major allergen in Dp and could play an important role in the pathogenesis of asthma.

Although it has been suggested that mite hypersensitivity may evolve with age, during childhood and early adult life Der p 2 allergy is dominant but declines with continuing exposure to the same allergen [14]. Our study showed that there was a trend toward a slightly higher incidence of Der p 2 hypersensitivity in patients under 20 years of age. However, this was not significantly different when compared with patients over 20 years of age (90.2 vs. 85.4%). There was also no difference in the frequency of Der p 2 hypersensitivity in our 10-family study. Hypersensitivity to Der p 2 was similar between adults and children within the same family. There seem to be two possibilities for this discrepancy. The first is that patients presenting

to our allergy clinic might have more severe allergic symptoms. The second is that those adults who had specific Der p 2 IgE levels in the same range as the children had a long history of allergic disease and had been hypersensitized earlier in life. Longitudinal studies are necessary to clearly distinguish between these two possibilities.

In our study, the incidence of Der p 2 hypersensitivity in asthmatic patients did not decline with age, indicating that there was no alteration of immune reactivity to Dp during aging. The results suggest that our patients continued to be exposed to the Dp allergens, and no other allergens became important.

Because the prevalence of Der p 2 hypersensitivity in asthmatic patients is very high, the Der p 2 skin testing and specific IgE measurement could serve as markers of mite hypersensitivity in allergic asthmatics.

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