

Identification of IgE-binding components of citrus red mite in sera of patients with citrus red mite-induced asthma

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Background: Our previous investigations demonstrated that citrus red mite (CRM) antigen could cause IgE-mediated bronchoconstriction in exposed farmers working on citrus farms.

Objective: This study was performed to confirm IgE-binding components and major allergens within the CRM antigens.

Methods: Ten subjects who had been diagnosed as having CRM-induced asthma were enrolled. Serum-specific IgE antibodies to CRM antigens were measured by using an ELISA. To identify IgE-binding components and major allergens, SDS-PAGE, 2-dimensional PAGE, IgE-immunoblot analysis, and amino acid sequencing of major allergens were performed. **Results:** All the asthmatic subjects had high specific IgE antibodies to CRMs. Twelve percent SDS-PAGE analysis showed more than 10 protein bands ranging from 6 to 64 kd. SDS-PAGE and IgE-immunoblot analysis with each individual serum showed 5 IgE-binding components (11, 24, 35, 40, and 64 kd), with 2 (24 and 35 kd) of them bound in more than 50% of the study subjects. Two-dimensional PAGE and IgE-immunoblot analysis demonstrated that the major allergen at 24 kd had 2 bands with different isoelectric points of 4.75 and 5.1. Thirty-five kilodaltons had one band with an isoelectric point of 4.75. All amino acid sequencing of the 2 major allergens was performed, which was not homologous with any previously characterized allergens.

Conclusion: Five IgE-binding components and 2 major allergens (24 and 35 kd) were identified within the CRM antigen. The N-terminal amino acid sequence of the 2 major allergens (24 and 35 kd) was determined. (J Allergy Clin Immunol 2001;107:244-8.)

Key words: Citrus red mite, IgE-binding component, major allergen, amino acid sequencing

There have only been a few reports of occupational asthma to *Tetranychidae* species in other parts of the

Abbreviations used

CRM: Citrus red mite
HDM: House dust mite
IEF: Isoelectric focusing
RT: Room temperature
TBST: TRIS-buffered saline

world.¹⁻³ Previous studies have revealed that citrus red mite (CRM; *Panonychus citri*) is the most prevalent inhalant allergen in farmers working on citrus farms in Korea,⁴ whereas pyroglyphid mites (*Dermatophagoides* species) are the most important inhalant allergens in other parts of this country.⁵ The previous reports on CRM-induced asthma suggested that IgE-mediated response is a major pathogenic mechanism responsible for the development of CRM-induced asthma.^{4,6} To the best of our knowledge, there has been no published data dealing with IgE-binding components or identification of major allergens within the CRM antigen. In this study, to confirm IgE-binding components and to identify major allergens within the CRM antigen, SDS-PAGE, 2-dimensional PAGE, IgE-immunoblot analysis, and amino acid sequencing of the major allergens were applied by using the sera of patients with CRM-induced asthma.

METHODS

Subjects

All the subjects had complained of aggravation of their lower respiratory symptoms when working on citrus farms; their mean occupational duration was 20 years, ranging from 10 to 30 years. They all showed either reversible airway obstruction after inhalation of bronchodilator, airway hyperresponsiveness to methacholine, or both. Their bronchial sensitizations were confirmed by means of specific bronchial challenge with CRM extract (Table I).

Airway reversibility and methacholine bronchial provocation testing

FEV₁ was measured with a computerized spirometer (Multi-spiro-SX), and the highest value of triplicate FEV₁ was adopted. Subjects with FEV₁ lower than 70% of predicted value inhaled 2 breaths of short-acting β_2 -agonist, and airway obstruction was assumed to be reversible if FEV₁ was at least 15% higher than before inhalation. The methacholine bronchial provocation test was done according to the method of Chai et al.⁷ Airway hyperresponsiveness to methacholine was defined if the provocative concentration of methacholine that caused a 20% fall in FEV₁ was less than 25 mg/mL.

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Supported by a research grant (HMP-98-M-1-0001) from the Ministry of Health and Welfare, Republic of Korea.

Received for publication June 20, 2000; revised September 25, 2000; accepted for publication September 29, 2000.

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0091-6749/2001 \$35.00 + 0 1/81/112123

doi:10.1067/mai.2001.112123

TABLE I. Clinical characteristics of patients with CRM-induced occupational asthma

Patient No.	Sex	Age (y)	Latency (y)	Symptom severity*	FEV ₁ [†] (% predicted)	Rhinitis	Skin response [‡] (mm)	Total IgE (IU/mL)	Specific IgE [§]
1	M	30	7	IV	29	+	10 (6)	1140	2.42
2	F	57	10	IV	37	+	5.5 (4)	112	0.91
3	M	56	15	IV	56	+	3 (2)	446	2.31
4	F	55	16	IV	49	+	5 (5)	156	0.60
5	M	66	20	IV	59	–	4 (3)	245	1.91
6	F	59	15	IV	58	+	3.5 (3.5)	1580	1.96
7	M	33	13	III	83	+	5 (4)	970	1.02
8	M	38	9	III	95	+	7 (4)	465	1.69
9	F	45	8	II	98	+	7 (6)	320	0.63
10	F	44	15	I	103	+	8 (6)	1340	2.07

*Symptom severity: II, mild persistent; III, moderate persistent; and IV, severe.

[†]Percent predicted value of FEV₁ at initial visit.

[‡]Skin response is presented as mean wheal diameter formed by CRM; wheal diameter formed by histamine is presented in parentheses.

[§]Absorbance value to represent serum-specific IgE level by ELISA.

Preparation of CRM extract

CRM extract was obtained from the Citrus Research Center on Cheju Island, Korea. They were extracted into PBS (pH 7.5, 1:5 wt/vol) at 4°C overnight, followed by centrifugation at 5000 rpm. The supernatant was passed through a syringe filter and used for a bronchoprovocation test at a concentration of 1:10 wt/vol. For skin prick testing, the 1:5 wt/vol extract was mixed with an equal amount of sterile glycerin. The supernatant was dialyzed (cut-off molecular weight, 6000 d) against 4 L of PBS at 4°C for 48 hours and lyophilized at –70°C. The protein concentration, as determined by using the Bradford method, was 3.52 mg/mL.

Allergy skin prick testing

Skin prick tests with 11 commercially available inhalant allergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Aspergillus* species, *Alternaria* species, tree pollen mixture I and II, grass pollen mixture, mugwort and ragweed pollen, animal epithelium, and cockroach; Allergopharma Co), CRM extract (1:10 wt/vol), and histamine (1 mg/mL) were performed on the volar side of both forearms. After 15 minutes, the mean diameter of a wheal formed by the allergen was compared with that formed by histamine. If the former was the same or larger than the latter (A/H ratio, ≥1), it was considered positive.

Measurement of total serum IgE

The total serum IgE level was measured with a DPC kit, according to the manufacturer's directions.

ELISA for specific IgE antibody to CRM antigen

The presence of specific IgE antibody to CRM antigen was determined by using an ELISA according to the method described previously.^{4,6} Microtitre plates (Dynatech) were first coated with 100 µL/well CRM (1 µg/well) extracts, and left at 4°C overnight. Each well was washed 3 times with 0.05% PBS, and the remaining binding sites were blocked by incubation with 350 µL of 3% BSA-PBS for 1 hour at room temperature (RT). The wells were then incubated for 3 hours at RT with 50 µL of either the patient or control sera (undiluted) from 30 patients showing negative skin prick test responses to common inhalant allergens, as well as to CRMs. After washing 3 times with PBS, 50 µL of the 1:500 vol/vol biotin-labeled goat anti-human IgE antibody (Vector Co) was added to the wells

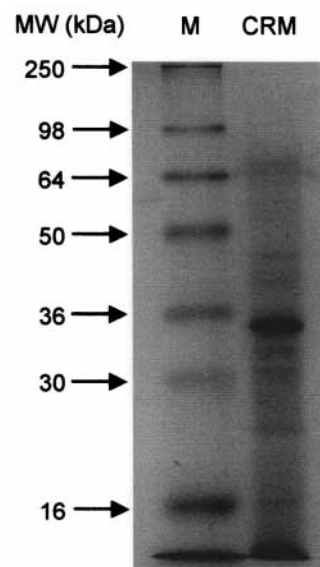


FIG 1. Protein staining of 12% SDS-PAGE of CRM extract. MW, Molecular weight.

and incubated for 1.5 hours at RT. The wells were then washed 3 times with PBS and incubated with 1:1000 vol/vol streptavidin-peroxidase (Sigma Co) for 30 minutes at RT before another washing step, which was followed by incubation with 100 µL *o*-phenylenediamine dihydrochloride for 10 minutes at RT. The positive cut-off value was decided as the mean ± 2-fold SD of the absorbance value of the controls.

SDS-PAGE and IgE-immunoblot analysis

SDS-PAGE and immunoblot analysis were done under reducing conditions according to the methods described previously.^{8,9} CRM antigen was mixed with sample buffer (0.5 mol/L TRIS-HCl [pH 6.8] of 1 mL, glycerol of 1.6 mL, 10% [wt/vol] SDS of 1.6 mL, 0.5% 2-mercaptoethanol of 0.4 mL, and distilled water of 3 mL), which was then adjusted to 1 mg/mL and heated in boiling water for 5 minutes. Ten microliters of standard marker (Novex) and antigen solution were added to a Novex precast TRIS-glycine gel (12%) for the separation of antigens. Electrophoresis was performed with Novex

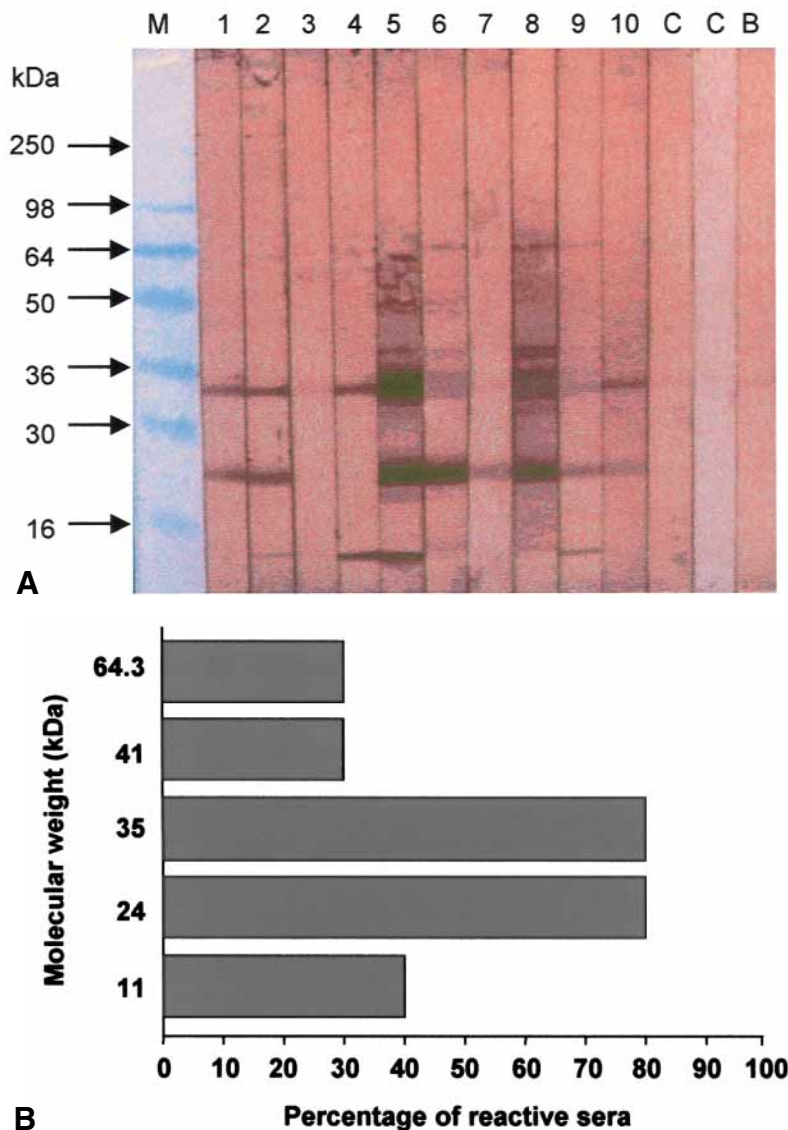


FIG 2. A, Binding pattern of IgE-binding components of CRM extract in 10 individual patients (*lanes 1-10*), control sera (*lane C*), and buffer (*lane B*). *M*, Marker protein. **B,** Percentage of sera of CRM-allergic individuals showing specific IgE binding to the different proteins of CRM.

Mini-cell for 90 minutes at 125 V. The gel was fixed and stained with Coomassie brilliant blue. For immunoblot analysis, transfer of the proteins onto nitrocellulose membrane (pore size 0.45 μ m; Bio-rad Labs) was performed on a Novex Western transfer apparatus in a transfer buffer (25 mmol/L TRIS base, 192 mmol/L glycine in 20% methanol) at 200 mA for 90 minutes. The blotted nitrocellulose membrane was blocked by 5% BSA in TRIS-buffered saline (TBST) for 1 hour. The patient and control sera were diluted 1:1 vol/vol with 3% BSA-TBST. Each membrane was then incubated with patient and control sera overnight at RT and washed with TBST. Affinity-purified biotinylated goat anti-human IgE antibody (Vector Labs) was diluted 1:500 with TBST and used as a secondary antibody solution. After washing with TBST, streptavidin-alkaline phosphatase (Sigma Chemical Co) was diluted 1:1000 with TBST and used to incubate the membrane for 30 minutes and then detected with a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium system (BCIP/NBT system, Sigma Co) as a substrate solution.

Two-dimensional PAGE

Forty micrograms of CRM extract mixed with the isoelectric focusing sample buffer (20 mol/L arginine, 20 mol/L lysine, and 15% glycerol) was applied on precasted native isoelectric focusing gel (Novel). The gel was run at 100 V for 60 minutes, 200 V for 60 minutes, and 500 V for 30 minutes. After running, the gel was removed from the cassette and fixed in fixing solution (3.46% sulfosalic acid and 11%-46% trichloroacetic acid) for 30 minutes. Each lane was cut into 4-mm widths and incubated in equilibration buffer (5% 2-mercaptoethanol, 62.5 mmol/L TRIS-HCl, 2.3% SDS, 10% glycerol, and 20% ethanol) for 30 minutes at RT and applied onto 12% polyacrylamide with a 4% polyacrylamide stacking gel. Then SDS-PAGE was carried out by using the above method. For 2-dimensional PAGE-immunoblot, transfer of the allergens onto polyvinylidene difluoride (pore size, 0.45 μ m; Bio-rad) membrane was performed in transfer buffer at 300 mA for 3 hours. The membrane was blocked by 5% BSA-TBST for 1 hour and then incubat-

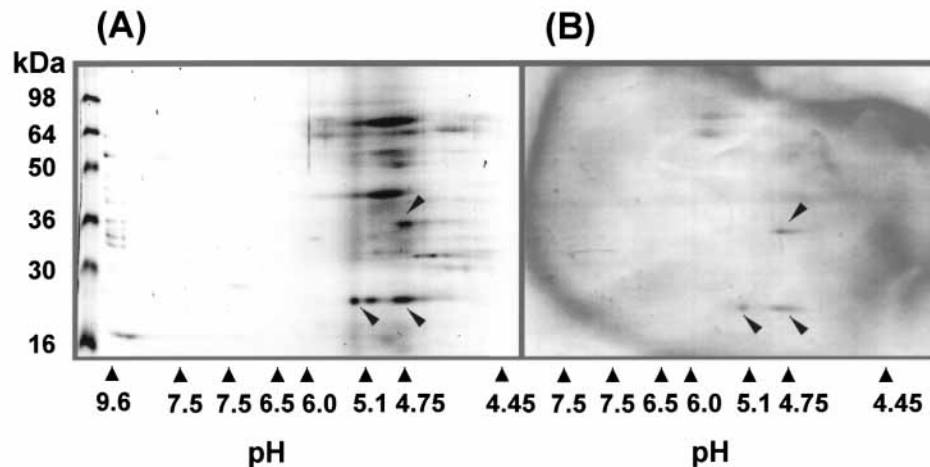


FIG 3. Two-dimensional PAGE analysis of CRM extract. **A**, Coomassie blue staining of 2-dimensional PAGE; **B**, IgE immunoblotting of 2-dimensional PAGE with the reactive pooled sera.

ed with the reactive pooled sera (1:5 diluted in 3% BSA-TBST) at 4°C overnight and washed with TBST. Goat anti-human IgE conjugated with alkaline phosphatase (Sigma Co) was diluted 1:1000 in 3% BSA-TBST before incubation with the blots. After washing with TBST, bound goat antibodies were detected with a Western blue (Promega) substrate solution.

N-terminal sequencing analysis

For N-terminal sequencing, 2-dimensional PAGE was blotted to a polyvinylidene difluoride membrane. The membrane was stained with 0.1% Coomassie blue in 50% methanol, destained in 50% methanol, and air-dried. The protein spot was excised, and microsequencing was performed by using Procise 476 A protein sequencer (Applied Biosystems).

RESULTS

Clinical characteristics

Table I shows the clinical characteristics of 10 patients with CRM-induced asthma. The mean duration of latency between the start of citrus farm work and the onset of asthmatic symptoms was 12.9 years (range, 7-20 years). The period of the development of symptoms from their onset to the diagnosis of occupational asthma ranged from 8 to 20 years. The patients' asthmatic symptoms were aggravated in late spring and fall, when populations of CRMs increased. Nine (90%) patients had a history of recurrent nasal symptoms that had been aggravated during work on citrus farms and developed at an earlier time than asthmatic symptoms.

Allergy skin prick testing and specific serum IgE to CRM antigen

All subjects had strong positive responses on skin prick tests. High total IgE levels were noted in all but one patient, as shown in Table I. Specific IgE to CRM antigen was considered positive if the absorbance value was higher than the cut-off value, which was derived from mean $\pm$ 2-fold SD absorbance values from unexposed healthy control subjects; all the subjects had high serum-specific IgE antibody.

TABLE II. Amino acid sequence of the 2 major allergens from CRMs

Allergens	Sequences
24 kd	X-Arg-Leu-His-Asp-Ile-Asn-Gly-Asp-Asp-Val-Arg-Ala-Asp-Gln
35 kd	X-Asp-Thr-Pro-Ile-Asp-Phe-X-Phe-Pro-Leu-Phe

X, Unidentifiable.

SDS-PAGE and IgE-immunoblot analysis

Twelve percent SDS-PAGE analysis showed that CRM extract was resolved into bands ranging from 6 to 64 kd (Fig 1). Fig 2, A, shows the patterns of IgE-binding components in 10 individual patients, 2 pooled sera from control patients, and a buffer control. Each control sera pool was derived from 5 patient sera showing negative responses to CRM antigen on skin prick tests. There were 5 bands (64.3, 41, 35, 24, and 11 kd) bound to IgE reacting with molecular weights ranging from 11 to 64.3 kd, and two (35 and 24 kd) of them were bound to the IgE in 50% or more of the sera tested, as shown in Fig 2, B.

Two-dimensional PAGE and immunoblot analysis

Fig 3 shows that the major allergen at 24 kd had 2 bands with different isoelectric points of 4.75 and 5.1, whereas the 35-kd allergen had one band with an isoelectric point of 4.75. Each of the 2 major allergens was bound to IgE, which was derived from pooled sera of the study subjects as shown in Fig 3.

N-terminal sequencing

Table II shows the result of amino acid sequencing of the 2 major allergens of molecular weight 24 and 35 kd. The amino acid sequences of 2 individual bands composing 24 kd were the same for up to 15 amino acid sequences.

DISCUSSION

Previous studies revealed that CRMs are the source of the major causative allergen for asthmatic symptoms in citrus farmers, and IgE-mediated reaction was one of the major mechanisms because the prevalence of specific IgE to CRMs was significantly associated with the presence of asthmatic and rhinitis symptoms.^{4,6} This is the first study to identify IgE-binding components and amino acid sequencing of 2 major allergens within CRM antigens. In the present study all the patients had high CRM-specific IgE antibody levels determined by ELISA and ELISA inhibition tests, confirming the specificity of IgE binding to CRM antigen. Furthermore, immunoblot analysis revealed 5 IgE-binding components, and 2 of them may be considered major allergens because more than 50% of the subjects had positive binding on these 2 bands. N-terminal sequence and isoelectric points of the 24- and 35-kd allergens were identified. When the N-terminal sequence of these 2 allergens was compared with the National Center for Biological Information database, we could not find any allergens with a similar N-terminal sequence. These findings indicate that these 2 major allergens at 24 and 35 kd may be new allergenic materials derived from CRMs. These findings will be a basis from which to develop mAbs to major allergens, which might be essential to the development of diagnostic and therapeutic extracts.

There is agreement in the suggestion that the closer the taxonomic relationship between the mites, the greater the level of cross-allergenicity.^{10,11} CRMs belongs to the order of mites (Atari), to the suborder of Prostigmata, to the family of spider mites (Tetranychidae), and represents its own species. Bruches et al² reported that all subjects sensitized to spider mites were cross-reactive to house dust mites (HDMs). Our previous study⁴ revealed that serum-specific IgE levels to CRMs were higher in the subjects with positive skin responses to HDMs than to those without such responses. Most of the asthmatic patients had concurrent positive responses to HDMs, whereas some patients had an isolated positive response to CRMs with no response to HDMs. ELISA inhibition tests showed that minimal inhibitions were noted with additions of HDMs.

However, in subjects with positive skin responses to both CRMs and HDMs, significant inhibitions were noted with additions of HDM antigens. Although the 24-kd allergen of the CRM has the same molecular weight as the group II antigen of HDMs, amino acid sequencing results in this study showed that it is species specific and different from that of HDMs. Further investigations will be needed to clarify species-specific and common sharing allergens between CRMs and HDMs.

In conclusion, 5 IgE-binding components and 2 major allergens (24 and 35 kd) with amino acid sequencing were identified in CRM antigens.

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