

# Identification of Immunoglobulin E Binding Components of the Two-Spotted Spider Mite *Tetranychus urticae*: Allergenic Relationships with the Citrus Red Mite and House-Dust Mite

Hae-Sim Park, Ph.D.,\* Young-Koo Jee, M.D.,# Youn-Keun Kim, M.D., Ph.D.,§  
Soo-Keol Lee, M.D., Ph.D.,\* Myung-Hyun Lee, M.D., Ph.D.,¶ and You-Young Kim, Ph.D.¶ (Korea)

## ABSTRACT

The two-spotted mite (TSM) is commonly found on fruit trees, herbaceous plants, and greenhouse flowers. However, a recent investigation indicated that the sensitization rate to TSM was as high as that of house-dust mites (HDMs) in nonfarmers as well as in farmers working in orchards in this country. The aim of this study was to identify immunoglobulin E (IgE)-binding components within TSM and to evaluate the allergenic relationship with the citrus red mite (CRM) and HDM. Sera were collected from eight patients who were not farmers and who had asthma with high serum-specific IgE to the

TSM and from unexposed controls showing negative responses to the TSM on skin-prick test. Twelve percent sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis were applied. To evaluate allergenic relationships with HDMs and CRMs, two kinds of sera pools were used: one (A) showing positive responses to both TSMs and HDMs and the other (B) showing an isolated positive response to TSMs. ELISA inhibition tests using A and B pooled sera were conducted. The TSM-ELISA inhibition test using sera A showed significant inhibition with addition of TSMs and CRMs, partial inhibition with HDMs, and minimal inhibition with other inhalant allergens. The ELISA inhibition test using sera B showed significant inhibition with TSMs and CRMs and minimal inhibition was noted with HDMs as well as other inhalant allergens. Immunoblot analysis using individual sera showed seven IgE-binding components (75, 56, 47, 41, 37, 28, and 14 kDa) and two (75 and 14 kDa) of them were bound to IgE in >50% of the sera tested. Seven IgE-binding components were identified within TSM extract and two (75 and 14 kDa) could be considered major allergens. It is suggested that the TSM contains species-specific allergen in addition to shared allergens with CRMs and HDMs. (Allergy and Asthma Proc 23:199–204, 2002)

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From the \*Department of Allergy and Clinical Immunology, Ajou University School of Medicine, Suwon, Korea; #Department of Medicine, Dankook University College of Medicine, Cheonan, Korea; §Department of Internal Medicine, Seoul National University College of Medicine, Seoul, Korea; and ¶Department of Pediatrics, Cheju University, College of Medicine, Cheju, Korea  
Supported by a Research Grant (Kosef, 1999–2-212–003-3) from the Ministry of Health and Welfare, Republic of Korea  
Address correspondence and reprint requests to Hae-Sim Park, Associate Professor, Department of Allergy and Clinical Immunology, Ajou University School of Medicine, Paldalgu Wonchondong San-5, Suwon, Korea 442–749

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**T***tetranychus urticae* (TU) known as the two-spotted spider mite (TSM), is a common pest of cultivated plants including fruit trees and herbaceous plants.<sup>1–8</sup> They are

TABLE I

## Clinical Features of the Study Subjects

| Patient | Age/Sex | PC20<br>Methacholine<br>(mg/ml) | Duration<br>(yr) | Total<br>IgE<br>(IU/mL) | Skin Reactivity |     |      | Occupation          |
|---------|---------|---------------------------------|------------------|-------------------------|-----------------|-----|------|---------------------|
|         |         |                                 |                  |                         | Dfa             | Dpt | TSMs |                     |
| LJ      | 19/M    | 1.34                            | 20               | 651                     | 2+              | 2+  | 3+   | Student             |
| CY      | 60/M    | ND                              | 8                | >1000                   | 2+              | 3+  | 3+   | Farmer              |
| SD      | 32/M    | 1.88                            | 1                | >1000                   | 3+              | 3+  | 3+   | Office worker       |
| KI      | 55/M    | 2.5                             | 30               | 530                     | 2+              | 2+  | 2+   | Construction worker |
| MD      | 17/M    | 1.5                             | 5                | 799                     | 0               | 0   | 3+   | Chicken breeder     |
| SY      | 17/M    | 9.47                            | 2                | 510                     | 5+              | 5+  | 3+   | Student             |
| KK      | 36/M    | ND                              | 10               | 516                     | 0               | 0   | 3+   | Taxi driver         |
| LH      | 51/M    | ND                              | 3                | >1000                   | 0               | 0   | 2+   | Industry worker     |

Skin reactivity was presented as allergen-to-histamine ratio.

M, male; ND, Not done; Dfa, *D. farinae*; Dpt, *D. pteronyssinus*.

\*Patients showing an isolated positive response to TSMs on skin-prick test.

classified in the order Prostigmata, family Tetranychidae, and subfamily Tetranychinae and are macroscopic in size (0.3–1 mm). The other spider mite, citrus red mite (*Panonychus citri*), is the most important inhalant allergen of occupational asthma found in farmers working on citrus farms in this country.<sup>9,10</sup> These spider mites are known as a source of occupational asthma, rhinoconjunctivitis, and urticaria, but all TSM-related reports are confined to the occupational basis only.<sup>2–8</sup>

Although the house-dust mite (HDM) is known to be the most important allergen in this country,<sup>11</sup> a recent investigation indicated that TSM could be one of the prevalent causes of inhalant allergens in nonfarmers as well as farmers in pear orchards.<sup>12</sup> Skin-prick test results of adult patients with asthma complaining of summer-type asthma in this area showed that the positive rate to TSM was the second most common inhalant allergen after HDMs.<sup>11</sup> There have been a few studies reporting on immunoglobulin E (IgE)–binding components within TSMs.<sup>3,4</sup> The sera used in that study were derived from farmers working in fruit orchards. Regarding allergenic relationships with HDMs, there has been only one study<sup>6</sup> suggesting the presence of significant cross-reactivity with *Dermatophagoides pteronyssinus*. However, they used only one kind of farmers' sera sensitized to both TSMs and *D. pteronyssinus*.

In this study, to identify the IgE-binding components within TSMs, both sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and IgE-immunoblot analysis were applied using sera from nonfarmers. Furthermore, to evaluate the allergenic relationships with HDMs and citrus red mites (CRMs), ELISA inhibition tests were conducted using two kinds of sera pools: one showing positive responses to both TSMs and HDMs, and the other sera showing an isolated positive response to TSMs on skin-prick test.

## SUBJECTS AND METHODS

Eight subjects with asthma with high serum-specific IgE to TSMs and 20 unexposed healthy controls were enrolled. All the subjects with asthma showed positive responses during a TSM-bronchoprovocation test. Their clinical features are summarized in Table I.

### Preparation of TSM Extract

TSMs were provided by the Apple Research Center in Taegu, Korea. They were extracted with phosphate-buffered saline (PBS, pH 7.5, 1:5 wt/vol) at 4°C for 40 minutes followed by centrifugation at 3500 rpm. The supernatant was dialyzed (the cutoff molecular weight was 6000 Da) against 4 L of distilled water at 4°C for 48 hours and lyophilized at –70°C for the preparation of antigens used in ELISA, ELISA inhibition, and immunoblot analysis. The supernatants were passed through a syringe filter (MSI, Sartorius, Goettingen, Germany), and protein content was 2.795 µg/mL. For skin-prick test, the extract was mixed with an equal amount of sterile glycerin.

### Allergen Skin-Prick Test

Skin-prick testing with 55 common inhalant allergens, TSMs, and histamine (1 mg/mL; Allergopharma Co., Hamburg, Germany) were performed on the back. After 15 minutes, the mean diameter of a wheal formed by the allergen was compared with that formed by histamine. The reaction was considered positive if the induced wheal size was >3 mm with concurrent flare.

### ELISA for Specific IgE Antibodies to TSM Extract

The presence of specific IgE antibody to TSMs was determined by ELISA as described previously.<sup>12</sup> Microtiter plates (Costar, Cambridge, MA) were coated with 100 µL/well of TU (10 µg/well) extracts and left at 4°C

overnight. Each well was washed three times with 0.05% tween PBS (PBST), and the remaining binding sites were blocked by incubation with 350  $\mu$ L of 3% bovine serum albumin (BSA)-PBST for 1 hour at room temperature. Then, the wells were incubated for 3 hours at room temperature with 50  $\mu$ L of either the patient or the control sera ( $\frac{1}{5}$  diluted) from 20 patients with asthma showing a negative skin-prick test response to common inhaled allergens as well as TU. After washing three times with PBST, 50  $\mu$ L of the 1:500 vol/vol biotin-labeled goat anti-human IgE antibody (Vector Laboratories, Burlingame, CA) was added to the wells and incubated with 1:1000 vol/vol streptavidin-peroxidase (Sigma Co., St. Louis, MO) for 30 minutes before another washing step, which was followed by incubation with 100  $\mu$ L of 3,3',5,5'-tetramethylbenzidine for 10 minutes at room temperature. The reaction was stopped by the addition of 100  $\mu$ L of 2.5 N sulfuric acid azide and the absorbance was read at 450 nm by an automated microplate reader. All assays were performed in duplicate. The cutoff value (0.12) of the positive IgE binding was determined as the mean plus twofold SD of the absorbance value from 30 control subjects.

#### ELISA Inhibition Test

Competitive ELISA inhibition was performed to determine the specificity of the IgE binding to the TSM antigen and to confirm cross-allergenicity with *D. pteronyssinus*. Two kinds of sera pools were used (A, sera showing positive responses to both TSMs and HDMs; B, sera showing isolated positive response to TSMs). The sera of the patients who had high specific IgE binding were pooled. Fifty microliters of this pooled serum was preincubated with four concentrations (0.1–100  $\mu$ g) of *D. pteronyssinus*, TSMs, Japanese Hop (Hop J) pollen, and diluent as the control. Then, they were incubated with the TSM-coated microtiter plate for 2 hours. The same steps of the experiments were followed as in the ELISA. As a control, samples were preincubated with equal volumes of PBS (pH 7.5) instead of inhibitors, and the inhibition percent of the specific IgE binding was expressed as  $(100 - [\text{absorbance of samples preincubated with allergens} / \text{absorbance of samples preincubated with PBS}] \times 100\%)$ .

#### SDS-PAGE and Immunoblot

SDS-PAGE and immunoblot analysis were conducted according to the method described previously.<sup>13</sup> CRM antigen was mixed with sample buffer (0.5 M of Tris-HCl [pH 6.8], 1 mL; glycerol, 1.6 mL; 10% (wt/vol) SDS, 1.6 mL;  $\beta$ -mercaptoethanol, 0.4 mL; 0.1% bromophenol blue, 0.4 mL; and distilled water, 3 mL), which then was adjusted to 1 mg/mL and heated in boiling water for 5 minutes. Ten microliters of standard marker (4–250 kDa; Novex, San Diego, CA) and TSM antigen solutions were added to this glycine gel (12%) for the separation of antigens. Electrophoresis was performed with the Bio-Rad Mini Protein II

cell for 90 minutes at a constant voltage of 125 V. The gel was fixed and stained with colloidal brilliant blue. For immunoblotting, the membrane was soaked in a transfer buffer (25 mM of Tris base and 192 mM of glycine in 10% methanol) for 10 minutes. Electroblothing was carried out for 120 minutes at 200 mA using a Bio-Rad transfer apparatus. The blotted nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) solution was blocked by 5% BSA in TBS-tween (TBST Sigma Chemical Co., St. Louis, MO) for 1 hour to block nonspecific binding. Each membrane then was incubated with patient and control sera diluted with 1:5 vol/vol 3% BSA-TBST, overnight at room temperature. It was washed with 0.1% TBST for 10 minutes. Affinity-purified biotinylated goat anti-human IgE antibody (Vector Laboratories) was diluted to 1:1000 with TBST and used as a secondary antibody solution. The membrane was incubated with secondary antibody for 90 minutes at room temperature and washed again. Streptavidin-alkaline phosphatase (Sigma Chemical Co.) was diluted to 1:1000 with TBST and added to the membrane for 30 minutes. As a substrate solution, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma Chemical Co.) was applied to the membrane. After the reaction, the membrane was washed with distilled water and observed.

## RESULTS

#### Clinical Characteristics

Table I shows the clinical characteristics of eight TSM-induced patients with asthma. The mean duration of asthmatic symptoms was  $90.2 \pm 88.2$  months. Their asthmatic symptoms were aggravated between May and October, especially in summer and early fall. Seven subjects had rhinitis symptoms such as watery rhinorrhea, paroxysmal sneezing, and nasal obstruction. None of them had worked in orchards, although all of them had lived around the pear orchard area since childhood.

#### Allergy Skin-Prick Test and Specific Serum IgE to TSMs

All the subjects had strong positive responses on skin-prick test to TSMs. Bronchoprovocation test with TSM extract showed early, late, and dual asthmatic responses. Specific IgE antibody to CRMs was considered positive if the absorbance value was higher than the cutoff value, which was derived from the mean + twofold SD of the absorbance values from unexposed healthy controls; all the subjects had high serum-specific IgE antibody. Five of them had concurrent positive responses to the two HDMs on skin-prick test. Three had an isolated positive response to TSMs.

#### ELISA Inhibition Test

ELISA inhibition test using sera pool A showed significant inhibition with addition of TSMs and CRMs in a dose-dependent manner. A dose-dependent inhibition pat-

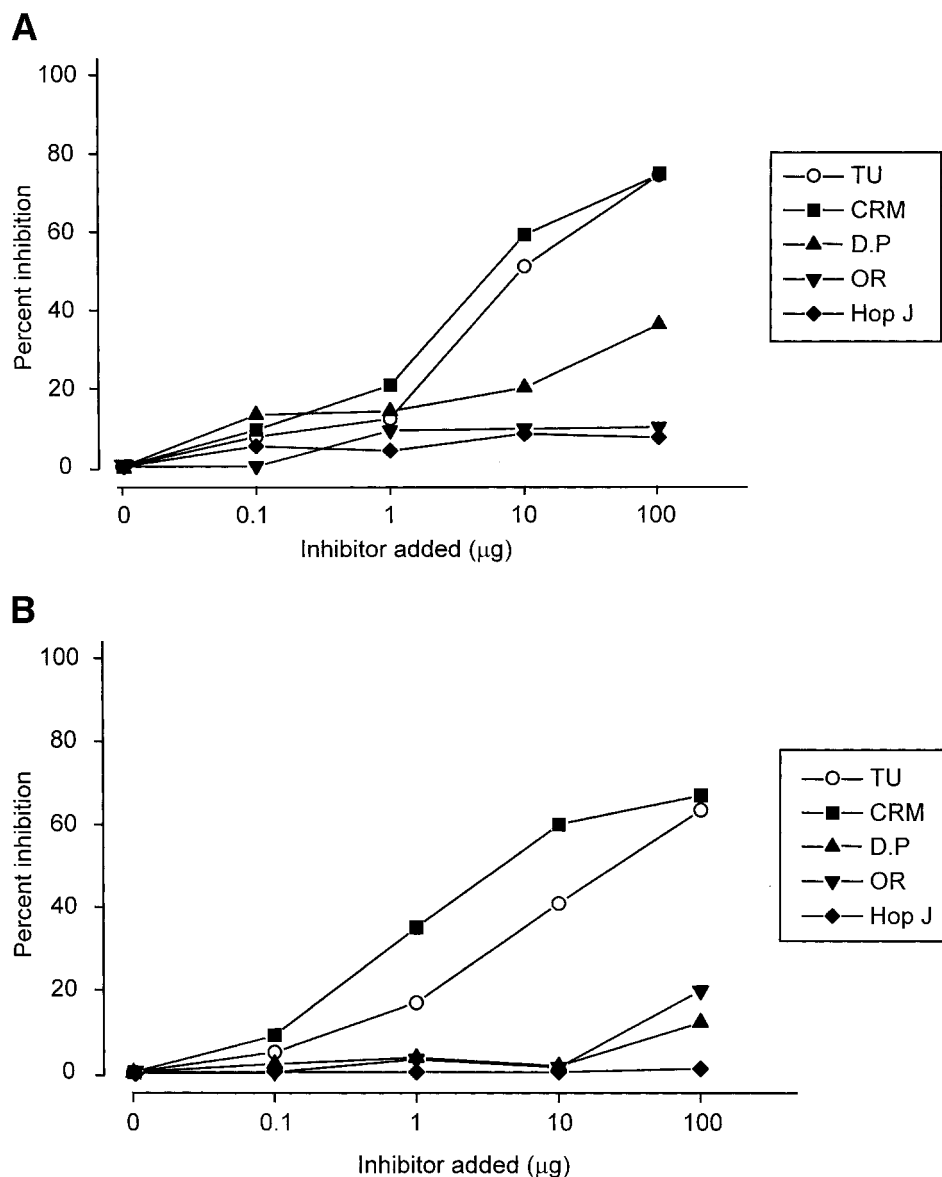


**Figure 1.** Percent inhibition of specific IgE bindings to TU with serial addition of TU, CRMs, *D. pteronyssinus* (DP), oilseed rape, Hop J antigens using two kinds of pooled sera, (A) one showing positive responses to both TSMs and HDMs and (B) the other showing an isolated positive response to TSMs on skin-prick test.

tern of up to 38% was noted with addition of *D. pteronyssinus* antigen. Minimal inhibition was noted with addition of oilseed rape, Hop J pollen, and *D. pteronyssinus* as shown in Fig 1 A. Figure 1 B shows TSM-ELISA inhibition test using sera pool B. Significant inhibition was noted with TU and CRMs in a dose-dependent manner, and no inhibition was present with the addition of oilseed rape, Hop J, or *D. pteronyssinus*.

#### SDS-PAGE and Immunoblot Analysis

**T**wo percent SDS-PAGE analysis showed that TSM extract was resolved into bands ranging from 16 to 65 kDa. Figure 2 shows IgE-binding components in sera from eight individual patients and two pooled sera from control

patients and buffer control. Each control sera pool was derived from five patients' sera showing negative responses to TSM on skin-prick test. There were seven bands (75, 56, 47, 41, 37, 28, and 14 kDa) bound to the IgE and three (75 and 14 kDa) of them were bound to the IgE in  $\geq 50\%$  of the sera tested.

#### DISCUSSION

**S**kin prick test data obtained from 1806 respiratory patients who had allergies and who were examined in an allergy clinic in this country showed that the sensitization rate ( $>2+$  A/H ratio) to TSMs was 19.8% and 6.6% showed isolated positive responses to TSMs.<sup>14</sup> This high sensitization rate to TSM could be explained by the fact that

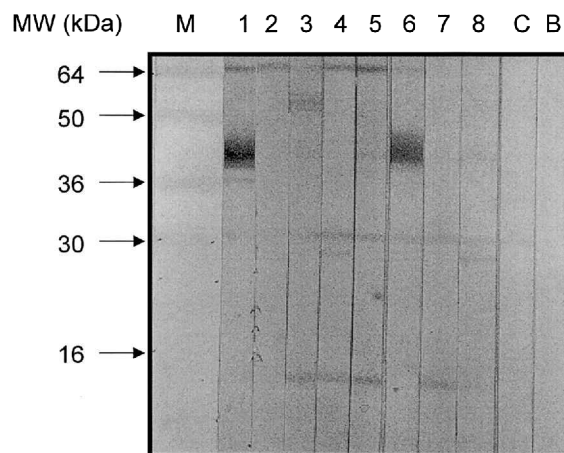


**Figure 2.** IgE immunoblotting of TU antigen in sera of the individual patient,<sup>1-8</sup> control sera (C) and buffer (B; M, marker protein).

an increasing number people have been exposed to this mite because of the dramatic increase in the densities of TSMs in outdoor environments. This mite has become one of the most important fruit-cultivating pests since pesticides were introduced ~40 years ago to eliminate other fruit pests.<sup>14,15</sup> Our previous study indicated that TSM inhalation induced bronchial asthma in nonfarmers complaining of summer-type asthma.<sup>21</sup> We consider TSM to be one of the most important outdoor allergens in rural areas of this country.

In regard to the pathogenic mechanism of TSM-induced asthma, the IgE-mediated reaction is the major mechanism responsible for their asthmatic symptoms. In this study, all the patients had strong positive responses to TSM on skin-prick test, which was shown also by ELISA. Furthermore, the ELISA inhibition test confirmed the specificity of specific IgE binding. Immunoblot analysis showed seven IgE-binding components and two (75 and 14 kDa) of them could be considered major allergens since >50% of the study subjects had positive bindings on these two bands. These findings confirmed that TSM inhalation induced bronchoconstriction by IgE-mediated reaction in nonfarmers. Seven IgE-binding components and three major allergens were identified.

There is an agreement in the suggestion that the level of cross-allergenicity is greater if the taxonomic relationship between the mites is closer.<sup>16-18</sup> TSM belongs to the order of mites (Acari), to the suborder Prostigmata, and to the family of spider mites (Tetranychidae). The CRM belongs to the same family but is classified as *Panonychus* spp. Burches *et al.*<sup>6</sup> reported that all subjects sensitized to spider mites (Tetranychinae family) were cross-reactive to HDMs. Our previous study dealing with CRM asthma on citrus farmers<sup>10</sup> revealed that serum-specific IgE levels to CRMs were higher in the subjects with positive skin responses to HDM than in those without it. Most of our patients with asthma had concurrent positive responses to HDMs, and some patients had an isolated positive response to CRMs

with no response to HDM. ELISA inhibition test using sera pool, having an isolated positive response on CRMs, showed minimal inhibition with addition of *D. pteronyssinus*. However, in subjects with positive skin responses to both CRM and *D. pteronyssinus*, significant inhibition was noted with addition of *D. pteronyssinus* as well as CRM antigens. In this study, ELISA inhibition assays using pooled sera having positive responses to both TSMs and HDMs showed dose-inhibitory patterns with addition of HDMs. The same degree of significant inhibition was noted by CRMs as well as by TSMs. However, ELISA inhibition studies with the pooled sera having isolated positive responses showed no inhibition with HDMs; and significant inhibition was noted with addition of CRMs and TSMs. These findings suggest that extensive cross-reactivity exists between TSMs and CRMs, and that the TSM contains its own species-specific allergens and no evidence of cross-reactivity with HDM was noted. To the best of our knowledge, this is the first study to confirm the case of TSM-induced asthma occurring in nonfarmers with isolated positive responses to TSM and to identify the species-specific allergens different from HDMs. Extensive cross-reactivity between TSM and CRM also was confirmed.

In conclusion, seven IgE-binding components and three major allergens were identified within TSM antigens in nonfarmers' sera. Extensive cross-reactivity between CRMs and TSMs also was found. The TSM may contain species-specific allergens. Further studies will be needed to clarify the cross-allergenivity with HDMs.

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