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# Two-spotted spider mite allergy: immunoglobulin E sensitization and characterization of allergenic components

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**Background and Objective:** Previous investigations demonstrated that two-spotted spider mite (TSM) inhalation causes allergic asthma in agricultural workers. This work investigates whether TSM causes similar problems in the urban population. We determined the sensitization rate to TSM. We also identified immunoglobulin (Ig)E-binding components and evaluated their relationship with house-dust mite (HDM) allergens.

**Methods:** We carried out skin prick test (SPT) with TSM in 1,806 respiratory allergy patients over 1 year. TSM-IgE was detected by enzyme-linked immunoadsorbent assay (ELISA). TSM-sensitized patients were classified into two groups: patients who were skin test-positive to both TSM and HDM were included in group A and patients who were skin test-positive to TSM only were included in group B. ELISA inhibition test using sera from group A and B were conducted. IgE-immunoblotting was used to identify major allergens. These were purified by 2-dimensional electrophoresis and blotted onto polyvinylidene difluoride, and N-terminal sequences were identified.

**Results:** SPT ( $\geq 2+$  of allergen/histamine) was positive in 358 (19.8%) patients. Twelve (6.6%) showed positive response to TSM only, and 54.5% had positive specific IgE. ELISA inhibition test using sera from two groups showed significant inhibition by TSM with minimal inhibition by HDM. Amino acid sequence of three major allergens was not homologous with any previously characterized allergens.

**Conclusion:** IgE-sensitization rate to TSM was 19.8% in respiratory allergy patients. Eleven IgE-binding components and three major allergens were identified. The pIs and amino acid sequences of the major allergens were determined.

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## INTRODUCTION

*Tetranychus urticae*, known as two-spotted spider mite (TSM), is a common pest of cultivated plants, including fruit trees and herbaceous plants.<sup>1–8</sup> They are classified in the order Prostigmata, family Tetranychidae, subfamily Tetranychinae. The other spider mite, citrus red mite (*Panonychus citri*), is the most important inhalant allergen related to occupational asthma found in citrus farm farmers.<sup>9,10</sup> All previous TSM-related reports have been confined to occupational exposures.<sup>2–8</sup>

Although house-dust mite (HDM) has been known as the most important allergen in Korea,<sup>11</sup> a recent investigation demonstrated that TSM could be one of the prevalent inhalant allergens in nonfarmers as well as farmers in pear orchards.<sup>12</sup>

Skin prick test (SPT) results of adult asthmatic patients complaining of summer-type asthma indicated that TSM was second in prevalence of sensitivity to HDM.<sup>12</sup> Two studies on immunoglobulin (Ig)E-binding components of TSM have been reported.<sup>3,4</sup> The sera used in that study were derived from farmers working in fruit orchards. To the best of our knowledge, this is the first study to observe the prevalence of skin reactivity, serum specific IgE (sIgE) and IgE-binding components of TSM in urban residents not engaged in agriculture. There has been one study<sup>6</sup> suggesting the presence of significant cross-reactivity with *Dermatophagoides pteronyssinus*. However, only one kind of farmers' sera sensitized to both TSM and HDM was used in that study.

In this study, to evaluate the clinical significance of TSM in nonfarmer inhabitants in urban areas in this country, SPT and sIgE determination by enzyme-linked immunoadsorbent assay (ELISA) were performed. To characterize IgE-binding components and major allergens, 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), iso-electric focusing (IEF), and IgE-immunoblot analysis were performed using sera from two groups of TSM-sensitized patients. Further, to clarify the allergenic relationship with two HDMs, ELISA inhibition tests were conducted using two kinds of sera pools.

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## METHODS

### *Patients*

Patients were selected from the Allergy Clinic of Ajou University Hospital in Suwon, Korea (40 km south of Seoul). Between January and December of 1999, 1,806 patients admitted for treatment of asthma and/or rhinitis were skin tested with common inhalant allergens as well as TSM. Routine SPTs using 26-gauge needles were performed on the backs of the patients simultaneously using 50 common inhalant allergens and 20 food allergens. The results were read 15 minutes later. The size of the wheal produced by each antigen and histamine was expressed as maximum diameter and vertical length at midportion of maximal length. Skin reactivity was expressed as the ratio of wheal size of allergen to histamine (A/H). If A/H was from 0.1 to 1 and erythema had a diameter <21 mm, it was read as 1+. If A/H was from 0.1 to 1, but erythema was >21 mm, it was read as 2+. If A/H ratio was from 1 to 2, it was read as 3+. If A/H ratio was from 2 to 3, it was read as 4+. If A/H was greater than 3, it was read as 5+. A positive responder was defined as a responder who demonstrated a response greater than 2+ on SPT. TSM-sensitized patients were classified into two groups: group A included positive responses to both TSM and HDM and group B included patients with positive response to TSM, but not to HDM. This study was reviewed by the institutional review board regulated by Ajou University Medical Center, Suwon, Korea.

### *Preparation of TSM Extract*

TSMs were provided by the Apple Research Center in Korea. They were extracted with phosphate-buffered saline (PBS, pH 7.5, 1:5 wt/vol) kept at 4° C overnight followed by centrifugation at 3,500 rpm for 40 minutes. The supernatant was dialyzed (the cut-off molecular weight was 6,000 Da; Spectrum Medical Industries, Houston, TX) against 4 L of PBS at 4° C for 48 hours, and stored at -20° C until testing by ELISA, ELISA inhibition, and immunoblot analysis. The protein content by Bradford method was 2.53 mg/mL. For SPT, it was mixed with sterile glycerin in the ratio of 5:1.

### *ELISA for sIgE Antibodies to TSM Extract*

The presence of sIgE to TSM was determined by ELISA as described previously.<sup>12</sup> Microtiter plates (Costar, New York, NY) were coated with 100 µL/well of TSM (10 µg/well) extract and left at 4° C overnight. Each well was washed three times with 0.05% PBS-Tween (PBST), and the remaining binding sites were blocked by incubation with 3% BSA-PBST for 1 hour at room temperature (RT). The wells were then incubated for 3 hours at RT with either 50 µL of the patient or control sera (all sera was diluted 1 to 5). Control sera was from 20 asthmatic patients with a negative SPT to common inhaled allergens as well as TSM. After washing three times with PBST, biotin-labeled goat anti-human IgE antibody (Vector Co, Burlingame, CA) was added to the each well and incubated for 1 hour. After washing, the wells were incubated with 50 µL of 1:1,000 vol/vol streptavidin-perox-

idase (Sigma Co, St. Louis, MO) for 30 minutes before another washing step. Colorimetric reaction was developed with B (3,3', 5,5'-tetramethylbenzidine) substrate solution for 10 minutes at RT. The reaction was stopped by the addition of 50 µL of 2 N sulfuric acid and the absorbance was read at 450 nm by an automated microplate reader (Benchmark, Bio-Rad, Hercules, CA). All assays were performed in duplicate. Amounts of specific antibodies in the samples were calculated from control curves made by optical densities from serial dilutions of positive control samples which showed high titer antibodies using a spline fit program (Microplate Manager, Bio-Rad).

### *ELISA Inhibition Test*

Competitive ELISA inhibition was performed to determine the specificity of IgE-binding to the TSM and to confirm crossallergenicity with two HDMs. Two kinds of sera pools were used (A and B). The sera from five patients who had high sIgE-binding were pooled and preincubated with four concentrations (0.1 to 100 µg) of *D. pteronyssinus*, *D. farinariae*, and TSM extracts, respectively, overnight at 4° C. Then, they were incubated with the TSM-coated microtiter plate for 3 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 sIgE-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 Analysis*

SDS-PAGE and immunoblot analysis was performed under reducing conditions according to methods described previously.<sup>13</sup> TSM antigen was mixed with sample buffer (63 mmol/L tris-HCl, 10% glycerol, 2% SDS, 0.0025% bromophenol blue, 2.5% 2-mercaptoethanol, pH 6.8) and heated in boiling water for 5 minutes. Standard marker (4 to 250 kDa, Novex, San Diego, CA) and TSM antigen sample were loaded to 12% SDS-PAGE gel for the separation of antigens. Electrophoresis was performed with Bio-Rad Mini Protein II cell for 90 minutes at 125 V. The gel was fixed and stained with Coomassie brilliant blue. For immunoblotting, transfer of the proteins onto polyvinylidene difluoride membrane (PVDF; pore size-0.3 µm; MSI, Westborough, MA) was performed with Bio-Rad transfer apparatus in transfer buffer (25 mmol/L trisbase, 192 mmol/L glycine, and 20% methanol) at 200 mmol/L for 90 minutes. The blotted PVDF membrane was sliced into 4-mm widths. The sliced membranes were blocked by 5% BSA-Tris-buffered saline (TBS)-Tween (TBST) for 1 hour to block nonspecific binding. Each membrane was then incubated overnight at RT with patient or control sera diluted 1:5 vol/vol with 3% BSA-TBST. It was washed with 0.1% TBST for 10 minutes. After washing, goat anti-human IgE conjugated with alkaline phosphatase (Sigma) was diluted 1:1,000 in 3% BSA-TBST before incubation with the blots. After washing with TBST, the membrane was

Table 1. Skin Reactivity and sIgE to TSM in Allergy Patients Visiting Allergy Clinic during 1999

|                              | Skin reactivity to TSM (A/H) |              |              |              |            |
|------------------------------|------------------------------|--------------|--------------|--------------|------------|
|                              | 2                            | 3            | 4            | 5            | 6          |
| Positive reactor (%)         | 68 (3.8)                     | 178 (9.8)    | 63 (3.5)     | 26 (1.4)     | 23 (1.3)   |
| Isolate positive case (%)    | 4 (5.9)                      | 5 (2.9)      | 2 (3.2)      | 0 (0)        | 1 (4.3)    |
| Specific IgE prevalence* (%) | 6/22 (27.3)                  | 16/22 (72.7) | 14/22 (63.6) | 13/22 (59.1) | 11/22 (50) |

\*  $P < 0.05$ .

Total patients no.: 1,806.

$\geq 2+$ : 358/1806 = 19.8%.

$\geq 3+$ : 290/1806 = 16.1%.

developed by a Western blue (Promega, Madison, WI) substrate solution.

#### Two-dimensional (2-D) PAGE

Forty micrograms of TSM extract mixed with the IEF sample buffer (20 mol/L arginine, 20 mol/L lysine, 15% glycerol) was applied on precasted native IEF gel (pH 4.45 ~ 9.6, Bio-Rad). The gel was run at 100 V for 60 minutes, 200 V for 60 minutes, and 500 V for 30 minutes. After running, 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% SDS-PAGE with a 4% stacking gel. Then, SDS-PAGE was performed using the aforementioned method. For 2-D PAGE-immunoblot, transfer of the allergens onto PVDF membrane was performed in transfer buffer at 300 mA for 3 hours. The membrane was blocked by 5% BSA-TBST for 1 hour, incubated with the reactive pooled sera diluted with 1:5 dilution overnight at 4° C, and washed with TBST. Goat anti-human IgE conjugated with alkaline phosphatase was diluted 1:1,000 in 3% BSA-TBST before incubation with the blots. After washing with TBST, the membrane was developed by a Western blue substrate solution (Promega).

#### N-Terminal Amino Acid (AA) Sequencing Analysis

For N-terminal sequencing, 2-D PAGE was blotted to a PVDF membrane. The membrane was stained with 0.1% Coomassie blue in 50% methanol, de-stained in 50% methanol, and air-dried. The protein spot was excised and micro sequencing was performed by using Procise 476 A protein sequencer (Applied Biosystems, Foster, CA).

## RESULTS

### SPT

The results of SPT throughout the year revealed that 358 (19.8%) of 1,806 patients showed more than 2+ of A/H to this antigen as shown in Table 1. Among them, 290 (16.1%) showed more than a 3+ A/H response. Twelve (0.66%) of them showed isolated positive responses to TSM with negative responses to other common inhalant allergens.

### SigE to TSM

ELISA on sera of 110 positive reactors as determined by SPTs (22 from each group) showed that prevalence of sIgE was 27.3% in 2+ reactors, 72.7% in 3+ reactors, 63.6% in 4+ reactors, 59.1% in 5+ reactors, and 50% in 6+ reactors (Table 1).

### ELISA Inhibition Test

ELISA inhibition test using sera pool from group A showed significant dose-dependent inhibitions with TSM. Minimal inhibitions were noted with *D. pteronyssinus* and *D. farinae* antigen as shown in Figure 1a. Figure 1b shows TSM-ELISA inhibition test using sera pool from group B. Significant inhibitions were noted with TSM extract in a dose-dependent manner, whereas no inhibitions were noted with *D. pteronyssinus* and *D. farinae* antigen.

### SDS-PAGE and Immunoblot Analysis

Figure 2 shows the patterns of IgE-binding components in 9 individuals from group A, 7 individuals from group B, one pooled sera from controls and buffer control. Control sera pool was derived from five patients' sera showing negative responses to TSM on SPT. There were 11 bands (77-, 74-,

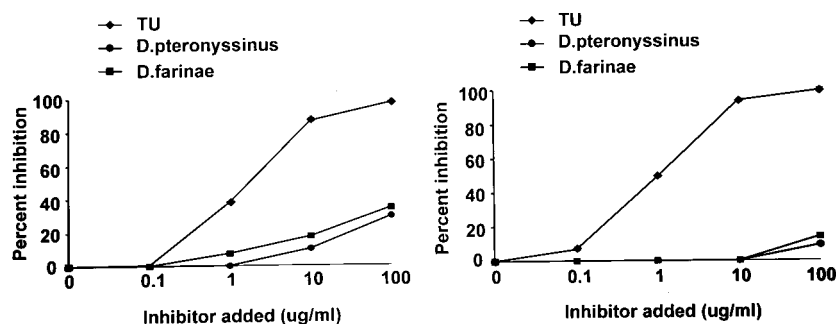


Figure 1. Percent inhibition of specific IgE-bindings to *T. urticae* (TU) with serial addition of TU, *D. pteronyssinus* (DP), and *D. farinae* (DF) using two kinds of pooled sera: one showing positive responses to both TSM and HDMs (Fig 1a) and the other showing an isolated positive response to TSM (Fig 1b) on SPT. Significant inhibitions were noted with TSM in a dose-dependent manner, and minimal inhibitions were noted with two HDM extracts.

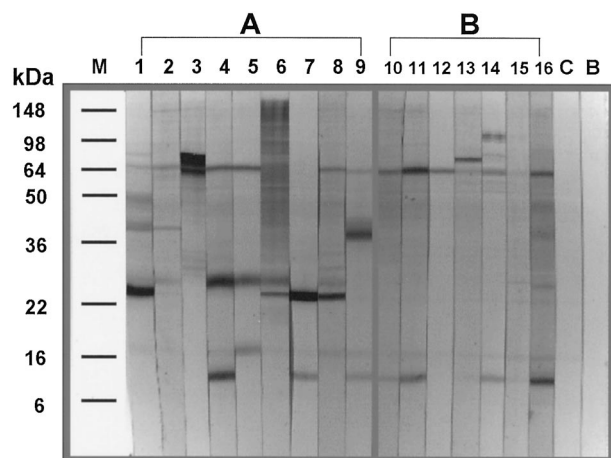


Figure 2. SDS-PAGE and IgE-immunoblotting of TSM antigen in sera of the individual patients,<sup>1-9</sup> from group A (A) and 7 group B (B), control sera (C) and buffer (B). M, marker protein.

67-, 46-, 39-, 35-, 32-, 29-, 27-, 15-, 10-kDa) recognized by IgE from group A. Two of these (67-, 29-kDa) bound IgE from  $\geq 50\%$  of the sera tested. IgE-immunoblot using group B sera pool showed seven bands (77-, 67-, 46-, 39-, 29-, 15-, 10-kDa) ranging from 10 to 77 kDa. Two of these (67-, 10-kDa) bound IgE from  $\geq 50\%$  of the sera tested (Fig 2).

#### 2-D PAGE and Immunoblot

Figure 3 indicates the iso-electric point (pI) of each of the three major allergens. Major allergens are defined as those allergens which were recognized by IgE from  $>50\%$  of the

sera tested. These IgE immunoblots used pooled sera containing individuals from both groups A and B. The 67-kDa had a pI of 5.1, the 29-kDa had pI of 5.6, and the 10-kDa had a pI of 5.5. All of the major allergens were bound to IgE on 2-D PAGE and IgE-immunoblot.

#### N-Terminal AA Sequencing

Table 2 shows the results of AA sequencing for the three major allergens with molecular weights 67, 29, and 10 kDa.

## DISCUSSION

This study demonstrated that the prevalence of positive responses on SPT was 19.8% in respiratory allergy patients, most of whom lived in urban areas not related with the orchard industry. The prevalence of sIgE binding as determined by ELISA increased according to skin reactivity. When the results of ELISA were compared with skin reactivity, sensitivity was found in 54.5% of the reactors with scores of 2+ or greater and in 61.3% of the reactors with scores of 3+ or greater. This high rate of sensitization to TSM could be explained by the observation that more people have been exposed to this predominantly fruit-related pest since pesticides were introduced approximately 40 years ago to eliminate other fruit pests. These pesticides eliminate other fruit pests, allowing increases in the resistant TSM population.<sup>14,15</sup> Our recent study demonstrated that TSM inhalation induced bronchial asthma in nonfarmers complaining of summer-type asthma.<sup>12</sup> These findings suggest that we have to consider TSM to be one of the most important outdoor allergens in respiratory allergy patients in urban as well as rural area.

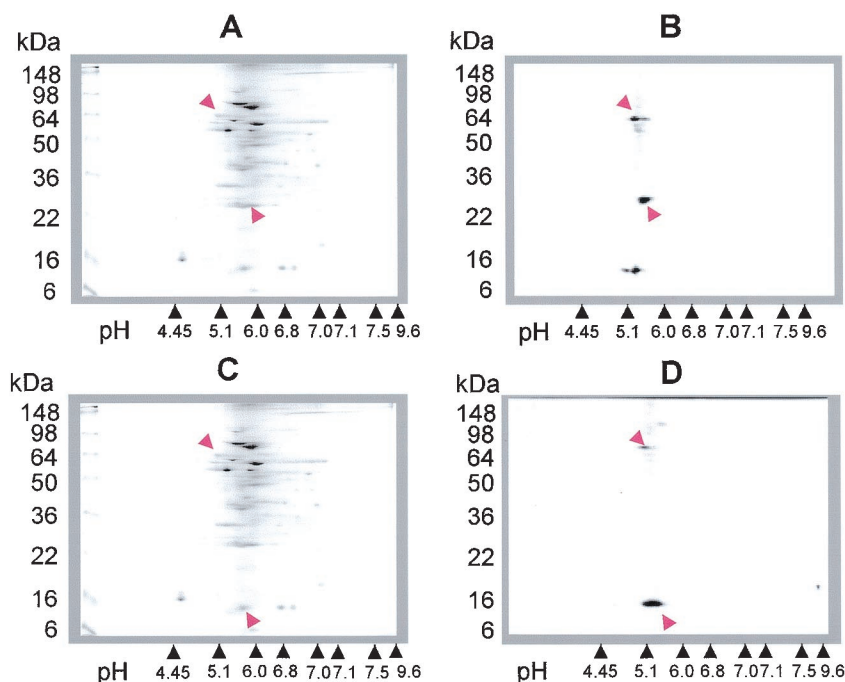


Figure 3. 2-D PAGE and IgE immunoblot analysis of TSM using two kinds of sera pool. (A and C), Coomassie blue staining of 2-D PAGE. (B and D), 2-D PAGE and IgE-immunoblot using sera pool from group A (B) and group B (D).

Table 2. AA Sequence Analysis of the Major Allergens Derived from TSM, *Tetranychus urticae*

| Major allergen | AA sequence                                                   |
|----------------|---------------------------------------------------------------|
| 67-kDa         | X-Lys-Glu-Ile-Lys-Phe-Gly-Ala-Asp-Val-Arg-Ala-Lys-Met-Leu-Ser |
| 29-kDa         | Val-Ile-Glu-Leu-Tyr-Gln-Leu-Pro-Phe-Ser-Ala-Pro-X-Arg-Gln     |
| 10-kDa         | Thr-Val-Gly-Ala-Leu-Lys-Pro-Thr-Phe-Ile                       |

In regard to the pathogenic mechanism of TSM-induced asthma, the IgE-mediated reaction is the major mechanism responsible for asthmatic symptoms, as all of the asthmatic patients with positive bronchial challenges had high sIgE binding.<sup>12</sup> In this study, ELISA inhibition results confirmed the specificity of the IgE binding in TSM-ELISA. Because a commercial kit is not available for TSM allergy, SPT using homemade allergen and sIgE determination by ELISA are valuable methods with which to identify patients allergic to TSM. Further, immunoblot analysis showed 11 IgE-binding components from group A patients and 7 components from group B patients. Three of these could be considered major allergens, as >50% of the study subjects produced IgE directed against on these proteins. These findings confirmed that TSM inhalation induced bronchoconstriction by IgE-mediated reaction in respiratory allergy patients.

This is the first study to identify IgE-binding components with 2-D PAGE and IgE-immunoblot, and to succeed in AA sequencing of three major allergens within TSM. The N-terminal sequence of these three major allergens was not similar to any found in National Center for Biologic Information database (BLAST search). These findings indicate that these three major allergens, 10-, 29-, and 67-kDa, may be new allergenic materials derived from TSM. These findings will be a basis to develop monoclonal antibodies to the three major allergens, which might be essential for developing diagnostic and therapeutic extracts.

Organisms close in the taxonomic scheme have more allergenic cross-reactivity.<sup>16–18</sup> TSM belongs to the order of mites (Acari), to the suborder Prostigmata, to the family of spider mites (Tetranychidae). Burches et al<sup>6</sup> reported that all subjects sensitized to spider mite (Tetranychinae family) were cross-reactive to HDM as evaluated by radioallergosorbent inhibition test. However, in this study, many patients had concurrent positive responses to HDM, whereas some patients had an isolated positive response to TSM with no response to HDM. Further, ELISA inhibition tests using sera pool from both group A and B showed minimal inhibitions with additions of *D. pteronyssinus* and *D. farinae*, the predominant HDMs in Korea. These results suggest that the possibility of crossreactivity between TSM and HDM is very low in nonfarmer patients living in urban areas.

This study reveals TSM to be capable of inducing both sensitization and respiratory symptoms in this country. Thus, this mite should be taken into account as a relevant source of

respiratory allergic disease in patients because there was a prevalence of 19.8% on SPTs and because 12 cases (0.66%) showed an isolated positive response to TSM only. These findings might encourage the research of hidden allergic sources in the etiology of asthma in this area.

## CONCLUSION

IgE sensitization rate to TSM was 19.8% in respiratory allergy patients living in urban area. Eleven IgE-binding components and three major allergens were identified in TSM antigen.

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