Sensitization to *Blomia tropicalis* in Patients with Asthma and Identification of Allergen Blo t 5

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In tropical and subtropical regions of the world, allergens produced by *Blomia tropicalis* are an important cause of IgE-mediated sensitization among patients with asthma. We compared the relative importance of sensitization to the two mite species among asthma patients from Florida, Puerto Rico, and Brazil (n = 83), who were concurrently exposed to *B. tropicalis* and *D. pteronyssinus*, with patients from the United States and from the United Kingdom (n = 56) exposed to *D. pteronyssinus*. In addition, molecular cloning techniques were used to clone and express a major *B. tropicalis* allergen. There were significant differences between IgE antibody responses to *B. tropicalis* and *D. pteronyssinus* that were related to exposure: only 22% of patients exposed to both species had a high ratio (>10) of IgE to *D. pteronyssinus*: *B. tropicalis*, whereas 68% of patients exposed only to *D. pteronyssinus* had a ratio of >10 (p < 0.001). A major 14-kD allergen (Blo t 5), cloned from a *B. tropicalis* cDNA library, showed 43% sequence homology to *D. pteronyssinus* Der p 5. Recombinant Blo t 5 produced in E. coli reactivated with 43% of sera from *B. tropicalis*-allergic asthatics and induced positive immediate skin tests at 10⁻³ to 1 µg/ml. In vivo and in vitro comparisons of IgE responses to *B. tropicalis*, *D. pteronyssinus*, rBlo t 5, and rDer p 5, showed that *B. tropicalis* has unique allergens that cause specific IgE responses.

The results suggest that *B. tropicalis* is an independent cause of sensitization and that use of recombinant Blo t 5 should lead to a better understanding of the role of *B. tropicalis* in causing asthma in tropical environments. A recent survey in the United States revealed that, along with *D. pteronyssinus* and *D. farinae*, the common mite species in the house dust fauna were *Blomia tropicalis* and *Euryglyphus maynei* (9).

*Blomia tropicalis* (family Glycyphagidae) and *D. pteronyssinus* occur with high frequency and at high levels of infestation in houses from tropical and subtropical areas of the world. These regions include the southern states of the United States, Central and South America, Hong Kong, Taiwan, India, and Egypt (9-12). Epidemiologic studies in São Paulo, Brazil, have shown that sensitization to *Blomia* is strongly associated with childhood asthma (13, 14). No significant evidence of sensitization or exposure to cockroaches, grass pollens, or *Aspergillus* was found among children in São Paulo, and dust mites appear to be by far the most important allergens associated with asthma in Brazil. A recent multicenter skin testing study in several Latin American countries, including Brazil, Venezuela, and Colombia, confirmed the high prevalence of sensitization to mites, notably *B. tropicalis*, in patients with asthma (15).
Evaluation of the role of B. tropicalis allergens in asthma has been complicated by the fact that concurrent sensitization occurs in houses where both B. tropicalis and D. pteronyssinus are present. In contrast to D. pteronyssinus, allergens from B. tropicalis are poorly defined, and no major allergens have yet been identified. Previous immunological studies showed that B. tropicalis does not produce allergens homologous to the Group I and Group 2 Dermatophagoides pteronyssinus allergens. Inhibition studies suggested that B. tropicalis allergens were species-specific, and the extent of cross-reactivity with D. pteronyssinus was estimated at ~30% (13, 16–19). We have investigated sensitization to B. tropicalis and D. pteronyssinus among patients with asthma exposed to both mite species or exposed primarily to D. pteronyssinus. We have also cloned and expressed an important allergen from B. tropicalis (Bla t 5), which shows homology to D. pteronyssinus allergen Der p 5. The recombinant Bla t 5 will facilitate clinical and immunologic studies of the role of B. tropicalis in asthma, and it offers the prospect of developing new strategies for the diagnosis and treatment of asthma in tropical and subtropical areas of the world.

METHODS

Subjects

A total of 139 patients were enrolled in the present study. Sixty-two were children 3 to 14 yr of age with moderate to severe asthma who attended the Pediatric Allergy Clinic of Paulista School of Medicine, São Paulo, Brazil. Sixteen were asthma children from Poole, United Kingdom, who had participated in a previous study (1). Thirty-six were adult patients with asthma: 14 from Ponce, Puerto Rico; 7 from Tampa, Florida; 15 from Charlottesville, Virginia. Twenty-five were children or adult patients with atopic dermatitis seen at the University of Virginia, Charlottesville, VA. The patients from Brazil, Puerto Rico, and Florida (n = 83) were selected on the basis of a positive immediate skin test to B. tropicalis and/or D. pteronyssinus, and a positive radioallergosorbent test (RAST) (>40 units/ml) to B. tropicalis extract. The group of patients with asthma or atopic dermatitis from Charlottesville and Poole (n = 55) were selected on the basis of positive immediate skin tests and RAST (>40 units/ml) to D. pteronyssinus. Serum samples from patients in Brazil, Puerto Rico, and Florida were collected in 1993; the serum samples from Charlottesville and Poole were collected between 1989 and 1994. Serum samples were stored at −20 °C. Nine children 7 to 12 yr of age with rhinitis and/or asthma from Brazil, and five nonallergic adult subjects from Manchester, UK (20), underwent skin testing with recombinant Bla t 5.

IgE Antibodies to D. pteronyssinus and B. tropicalis

Serum IgE antibodies (ab) to D. pteronyssinus and B. tropicalis were measured by quantitative RAST, as described previously (22). The assay was calibrated using a control curve of D. farinae discs and serial 2-fold dilutions of a mite allergic serum pool (UVA 87/01), containing 1,000 units/ml of IgE ab. The UVA serum pool was standardized against an international reference serum pool (National Institute of Biological Standards and Control; Code No. 83/S2B), and one RAST unit is equivalent to 0.01 μg/ml of IgE (21).

Molecular Cloning of B. tropicalis Allergens

Total RNA was extracted from 3.8 g of B. tropicalis whole bodies, using 5 M guanidium isothiocyanate (0.1 M EDTA, 5 M LiCl, 0.1 M Tris HCl at pH 7.5) (22). Messenger RNA was obtained using a FastTrack kit (Invitrogen, San Diego, CA). A B. tropicalis cDNA library was prepared in the UniZAP XR expression vector (Stratagene, La Jolla, CA), using 12 μg B. tropicalis mRNA. The cDNA library (amplified titer, 7×10^8 pfu/ml) was screened with IgE antibodies obtained from eight asthmatic children with a high B. tropicalis RAST (600 to 4,700 units/ml), i.e., 60 to 470 ng/ml IgE ab. The B. tropicalis library (400,000 pfu) was screened as described previously, and eight positive plaques were identified and cloned (22). Partial nucleotide sequencing revealed that 7/8 cDNA clones were identical. One of the cDNA clones, bla 2B (357 bp), was further screened against individual sera from 159 patients by plaque immunoscreening. Double stranded sequencing of bla 2B DNA was carried out by dideoxyribonucleotide chain termination using a Sequenase kit (USB Biologicals, Cleveland, OH). The FASTA program was used to search the NBRF, GenBank, and Swiss-Prot databases for sequence homologies (23). The protein encoded by the bla 2B cDNA clone was designated Bla t 5, according to the WHO/IUIS allergen nomenclature (24).

Expression of Recombinant Group 5 Mite Allergens

Polymerase chain reaction (PCR) was used to generate a 366 bp Bla t 5 DNA fragment containing BamHI and XhoI cloning sites, which allowed subcloning into the pGEX-4T1 expression vector (Phar- macia Biotech, Piscataway, NJ). Fifty nanograms of Bla t 5 plasmid DNA were amplified by PCR using Pfu DNA polymerase (Stratagene). Reactions were carried out in 50 μl volume, with denaturation at 94 °C for 1 min, annealing at 37 °C for 1 min, and extension at 72 °C for 3 min for 30 cycles. An initial 5-min incubation step at 94 °C was performed, and each reaction was terminated for 15 min at 72 °C. Primers for PCR were as follows: 5′ CGG GCA TCC TCA GAG CAC AAG CCA AAG Y (sense); and 5′ CGG CTC GAG GTA TTC GGT TGG ATT ATC T (antisense). The PCR amplified Bla t 5 DNA did not contain the 5′-coding sequence and the predicted signal peptide sequence (nucleotides t 1 to 83). Expression of the 366 bp Bla t 5 DNA into BamHI/XhoI digested pGEX-4T1 and transformation of competent Escherichia coli strain Top10F (Biotraining) were performed as described (25).

Expression of Bla t 5 as a fusion protein with glutathione-S-transferase (GST) was induced with 1 mM IPTG, and purification from bacterial lysates was performed over glutathione sepharose (Pharmacia Biotech) followed by elution with 10 mM reduced glutathione. Digestion with thrombin (10 units/mg protein for 18 h at room temperature) released the recombinant 14 KD Bla t 5 protein (Bla t 5), which was collected in the flow through after further purification over glutathione sepharose. Recombinant Der p 5 (Der p 5) was prepared as for Bla t 5. Briefly, E. coli strain TG1 transformed with a pGEX-2T Der p 5 construct was grown at 37 °C, and expression of Der p 5 as a fusion protein with GST was induced with 1 mM IPTG. Purification of Der p 5 from bacterial lysates was carried out over glutathione sepharose, followed by elution with 10 mM reduced glutathione and digestion with thrombin, releasing the 14 kDa Der p 5 protein (26). The purity of Bla t 5 and Der p 5 was assessed by silver-stained SDS-PAGE using an 8-25% gel on a PhastSystem (Pharmacia), and the N-terminal amino acid se- quence was determined by Edman degradation. The yield of recombinant Group 5 allergens was 1.0 to 1.5 mg of culture.

IgE Antibodies to Recombinant Group 5 Allergens

IgE ab to Bla t 5 and Der p 5 were measured in sera from 138 or 136 mite-allergic patients, respectively, using an antigen-binding RIA (27). Two serum samples were not available for the Der p 5 assay. Briefly, 20 μg Bla t 5 and Der p 5 were radiolabled with 0.5 μCi 125I, using the chloramine-T technique (specific activity, 2.53 Ci/mmol and 16 Ci/mg, respectively). Sera diluted 1/2 to 1/10 were incubated with 2 ng/ml 125I-Bl105 or 125I-Der p 5 (100-200 cpm added for 4 h at room temperature. IgE antibodies were precipitated overnight at 4 °C with 50 μl sheep antihuman IgE (The Binding Site, San Diego, CA), washed, and counted in a y counter. The assays were quanitized using control curves constructed with serial 2-fold dilutions of patient sera No. 37 (Brazil) or Y.P. (Charlottesville), for the Bla t 5 and Der p 5 assays, respectively. Each serum was assigned 1,000 units/ml of IgE antibody.

Immediate Skin Testing

Skin prick tests were performed using commercial Der p 5 (0.000 0, 0.000 AU/ml; Bayer Corp., Sparks, WA). B. tropicalis extract was prepared from mites cultured on a medium of autoclaved nude mouse food and Brine shrimp (E), and separated using a Talisman apparatus. In this sys- tem, the phoresbic live mites were induced to pass through six layers of cheese cloth to escape the light and heat produced by a 60-W light bulb, allowing collection of >95% pure mite bodies. Whole mite bodies were defatted and extracted in 100 mM ammonium bicarbonate. Af- ter centrifugation, the supernatant was dialyzed against distilled water and filtered (12). The protein concentration of the B. tropicalis extract was 1,900 μg/ml. Purified Bla t 5 and Der p 5 were used for prick tests at 1 μg/ml. A wheal of 3 × 4 × 2 mm diameter 15 to 20 min after
TABLE 1

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<th>Serum from Patients in</th>
<th>Dp 6 RAST</th>
<th>Bl 2 RAST</th>
</tr>
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<td>Brazil</td>
<td>9 (14%)</td>
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</tr>
<tr>
<td>Puerto Rico</td>
<td>16 (45%)</td>
<td>8 (35%)</td>
</tr>
<tr>
<td>Florida</td>
<td>3 (12%)</td>
<td>4 (17%)</td>
</tr>
<tr>
<td>All</td>
<td>32 (40%)</td>
<td>20 (22%)</td>
</tr>
<tr>
<td>Charlestown, VA, and Poole, UK</td>
<td>35 (42%)</td>
<td>15 (27%)</td>
</tr>
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</table>

* IgE antibodies to D. pteronyssinus and B. tropicalis were measured by quantitative RAST and the ratio IgE ab to D. pteronyssinus IgE ab was calculated. The number of sera from different patient groups with a ratio > 10 are shown, with percentage values shown in parentheses.

p < 0.001 (chi-square test).

RESULTS

Analysis of IgE ab to B. tropicalis and D. pteronyssinus

Sera for these studies were obtained from patients with asthma and/or atopic dermatitis living in selected geographic areas where exposure to B. tropicalis and D. pteronyssinus is known to differ. Patients living in Brazil, Puerto Rico, and Florida were exposed to both B. tropicalis and D. pteronyssinus (11). In contrast, patients from Charlottesville and Poole were exposed to D. pteronyssinus but not to B. tropicalis. Extensive mite counts carried out in the 1980s (in more than 200 homes) failed to detect B. tropicalis in Charlottesville or Poole (T. A. E. Platt-Mills and S. Wilkins, unpublished data). Indeed, B. tropicalis has never been reported in the UK. The 139 sera were analyzed for IgE ab to B. tropicalis and D. pteronyssinus by RAST to assess the degree of cross-reactivity between both species in exposed and unexposed populations.

Among patients exposed to both B. tropicalis and D. pteronyssinus who had detectable IgE ab to B. tropicalis (RAST > 40 units/ml), 88% also had IgE ab to D. pteronyssinus. Conversely, among patients exposed to D. pteronyssinus but not to B. tropicalis, 50% had detectable IgE ab to B. tropicalis. Differences between B. tropicalis exposed and nonexposed patients were further assessed by comparing the ratio of IgE ab to D. pteronyssinus and B. tropicalis (Figure 1). Only 22% of the patients exposed to B. tropicalis had a ratio > 10, whereas in those not exposed to B. tropicalis, the ratio was > 10 in 68% of patients (p < 0.001) (Table 1 and Figure 1). These results showed that although as much as 50% of patients from Charlottesville or Poole had detectable IgE ab to B. tropicalis, the levels of IgE ab to D. pteronyssinus were 10 to 100-fold higher than to B. tropicalis. The Charlottesville/Poole patients included 25 with atopic dermatitis who had remarkably high levels of IgE ab to D. pteronyssinus (RAST, 240 to 34,770 units/ml), and 18 of 25 of these patients (72%) had IgE ab to B. tropicalis. In contrast, only 32% of mitoallergic asthmatic patients (D. pteronyssinus RAST, 78 to 10,000 units/ml) had IgE ab to B. tropicalis (p < 0.05, chi-square test).

Identification of Blomia tropicalis Allergen Blt 1.5

To further examine the antigenic relationship between B. tropicalis and D. pteronyssinus, molecular cloning was used to identify

B. tropicalis allergens. A B. tropicalis cDNA library was screened with IgE ab in pooled sera from mite-allergic children. Eight positive plaques were identified, and partial 5' end-sequencing revealed that seven of eight clones were identical. One cDNA clone (Blt 2B) was fully sequenced and further screened with 139 individual sera by IgE plaque immunosassay. Among patients from Brazil, Puerto Rico, and Florida, 57 of 83 (69%) had IgE ab to the protein encoded by clone Blt 2B. These results suggested that the protein encoded by cDNA clone blt 2B was an important allergen. In contrast, only 13 of 56 (23%) of the Charlottesville/Poole patients had IgE ab to this protein (p < 0.001). Another cDNA clone (Blt 8) coded for a protein with sequence homology to troponin-C and reacted with 49% of the patients' sera (sequence data on this allergen will be published elsewhere) (Table 2). Nucleotide-sequence data revealed that Blt 2B cDNA contained...
Figure 2. Nucleotide and deduced amino acid sequence of cDNA clone br2B, encoding B. tropicaus allergen Blt 5. The predicted translocation initiation codon ATG is boxed in bold. The putative signal peptide cleavage site is located between residues A12 and Q13 (in bold). The 17 N-terminal amino acid residues include 13 hydrophobic residues, forming a classic signal peptide sequence (29). A potential N-linked glycosylation site is indicated (bold dot) and the stop codon TAA (asterisks) is shown. A polyadenylation site (AATATAA) is underlined. An arrow indicates the nucleotide position. The sequence data presented here have been submitted to GenBank under the accession number US9102.

a 432 open reading frame encoding a 134 amino acid residue protein. The sequence included an ATG start codon at positions 33 to 35, and a 17-residue putative signal peptide (nucleotides 36 to 83) with a predicted cleavage site between alanine- and glutamine- residue (Figure 2) (29). The mature protein contains 117 amino acid residues, with a predicted molecular mass of 13,876 D.

Homology to D. pteronyssinus Allergen Der p 5
Sequence similarity searches of the NBRF, GenBank, and SwissProt databases revealed that the only protein with significant similarity to the deduced amino acid sequence of cDNA clone br2B was a 14 kD D. pteronyssinus allergen, Der p 5. This allergen was originally identified and cloned by Towe and colleagues (30) and further sequenced and expressed by Lin and colleagues (26). Comparison of the amino acid sequence showed 43% homology between the protein encoded by cDNA clone br2B and Der p 5 (Figure 3). The br 2B protein is the first R. tropicaus allergen that has been cloned and fully sequenced. In accordance with the WHO/IUIS nomenclature, the protein was designated Blomia tropicaus allergen 5 (Bla t 5) based on its sequence homology to Der p 5, rather than on the chronologic order of its discovery (24). This also distinguished Bla t 5 from the Group I allergens of Dermatophagoides spp., which are structurally unrelated (8).

IgE Antibody Responses to Recombinant Group 5 Allergens
Recombinant Blt 5, i.e., the protein encoded by cDNA clone br2B, was expressed in E. coli and gave a single 14-kD band on SDS-PAGE (Figure 4). In addition, the 8 N-terminal amino acid residues of rBla t 5 were identical to the predicted sequence from the cDNA. IgE ab to rBla t 5 was measured in sera from 138 mite-allergic patients by antigen-binding RIA. The prevalence of IgE to rBla t 5 among patients from Brazil, Puerto Rico, and Florida was 45% (37 of 83), and among patients from Charlottesville and Poole, it was 29% (11 of 38) (Figure 3B). The levels of IgE ab were also significantly lower in the group from Charlottesville and Poole when compared with the group from Brazil, Puerto Rico, and Florida (CM: 29.2 units/ml and 106 units/ml, respectively; p < 0.005) (Figure 5A). In contrast, there was no difference between IgE ab to rDer p 5 in the two groups of patients (p = 0.38) (Figure 5B). As much as 41% of radiolabeled rBla t 5 or rDer

Figure 3. Sequence alignment between Blomia tropicaus Blt 5 and Der p 5 (26, 30). Identical amino acid residues (.) or conserved substitutions (.) are indicated (overall homology, 43%).
Figure 5. Comparison of IgE antibodies to recombinant Group 5 allergens in sera from patients living in Brazil, Florida, and Puerto Rico exposed to both *D. pteronyssinus* and *B. tropicalis* (closed symbols), and patients from Charlottesville, VA, and Poole, UK exposed only to *D. pteronyssinus* (open symbols). IgE ab to Blo t 5 (A) or Der p 5 (B) were measured by antigen binding RIA, and sera from patients with asthma (circles) or atopic dermatitis (triangles) are indicated. Horizontal bars indicate the geometric mean values, and the dashed line is the minimal level of sensitivity of each assay. Student's t test was used to analyze differences between patient groups, and the p value is indicated above each figure.

IgE ab to Blo t 5 and Der p 5 could be bound by IgE ab from mite-allergic patients (maximum, 46,000 cpm bound), whereas sera from four nonallergic patients consistently gave background binding (< 0.5% radioactivity or 400 cpm bound). The results suggest that IgE ab responses to Blo t 5 in unexposed patients reflect antigenic cross-reactivity with *D. pteronyssinus* allergen Der p 5.

Linear regression plots were also used to compare IgE ab to the Group 5 allergens in *B. tropicalis*-exposed and -unexposed patients. A high proportion of sera from exposed patients had IgE ab to both Blo t 5 and Der p 5 (24 of 58, 41%), and in this group significant numbers of patients had IgE ab specific for both Blo t 5 (36%) or Der p 5 (23%) (Figure 6A). These results suggest that both Blo t 5 and Der p 5 are necessary to evaluate IgE ab responses in patients exposed to the two mite species. In contrast, among patients exposed to *D. pteronyssinus* but not to *B. tropicalis*, the majority (66%, 21 of 32) had IgE ab to Der p 5, but not to Blo t 5, and only eight sera had IgE ab to both allergens (Figure 6B).

Figure 6. Correlation between IgE antibodies to Blo t 5 and Der p 5 in sera from mite-allergic patients. (A) Sera from patients in Brazil, Florida, and Puerto Rico with detectable serum IgE ab to Group 5 allergens (n = 58). (B) Sera from Charlottesville/Poole patients with IgE ab to Group 5 allergens (n = 32). Open symbols indicate sera from mite-allergic patients who had no detectable IgE ab to Group 5. Dashed lines indicate the minimal levels of assay sensitivity.
Biologic Activity of Recombinant Group 5 Allergens

To investigate the biologic activity of rBlO 5, selected mite-allergic patients with asthma and/or rhinitis underwent skin testing with the recombinant allergens. Positive immediate prick tests were obtained using rBlO 5 at a concentration of 5 μg/mL and intradermal tests at a concentration as low as 10 μg/mL, whereas no reaction was observed in nonallergic patients with rhinitis. These results confirmed that the B. tropicalis allergen BlO 5 produced in bacteria retains IgE binding capacity in vivo. Similarly, (Der p 5 induced positive skin testing in mite-allergic patients, as previously reported (26) (Table 3).

DISCUSSION

Comparison of IgE ab responses in sera from exposed and unexposed asthma patients, together with the cloning and expression of a major allergen, has made it possible to investigate the molecular basis for sensitization to B. tropicalis and D. pteronyssinus. Several lines of evidence from the present study suggest that B. tropicalis is an independent cause of sensitization in tropical and subtropical countries where patients may be exposed to this species as well as to D. pteronyssinus. A significant number of patients living in these areas have a higher level of IgE ab to B. tropicalis than to D. pteronyssinus; they are more likely to make IgE ab responses to both Group 5 allergens, and over 20% increase IgE ab responses to BlO 5, but no detectable responses to Der p 5. B. tropicalis appears to be a primary cause of sensitization in Brazil, Florida, and Puerto Rico, and the results would be expected to apply to other regions where Blomia spp constitute a large proportion of the mite fauna. In these regions, the proportion of mites that may be responsible for the sensitization is not unambiguous. The availability of rBlO 5 will enable quantitative comparisons of the levels of exposure of these homologous allergens in exposed and unexposed patients. The use of recombinant Group 5 allergens also has applications in cellular studies of asthma. These include studies of allergen accumulation in the airways after bronchial challenge, as has recently been demonstrated with Der p 1, and analysis of mediator release and cellular recruitment after allergen challenge (32). The sequence information is an essential prerequisite for studying T-cell proliferation in patients with asthma and defining the epitopes involved. Increasingly, T-cell-based peptide vaccines are being considered as a new therapeutic approach to allergen immunotherapy, and preliminary trials with T-cell peptides derived from Fd 1 have shown promising results in asthmatic patients allergic to cats (33). An alternative approach is the development of modified allergens with decreased IgE-binding capacity (also dependent on having expressed, recombinant allergens) that contain a broad repertoire of T-cell epitopes (34). These novel treatment strategies will now become applicable to Blomia spp using BlO 5 and other allergens identified using molecular cloning techniques (35).

In conclusion, the present study provides strong evidence for an important role of Blomia tropicalis allergens as an independent cause of sensitization among patients with asthma living in tropical areas of the world. Detailed comparisons of IgE reactivity to B. tropicalis and D. pteronyssinus supports the view that B. tropicalis contains unique allergens, and therefore should be included in the evaluation of immediate hypersensitivity in patients exposed to both mite species. The identification of BlO 1.5 as a major B. tropicalis allergen, and its production as a recom-
TABLE 3

<table>
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<th>Patient No.</th>
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*Skin tests are reported as either a wheel diameter (in mm) as peric test or as the concentration of antigen (in ug/ml) that caused a positive reaction (> 6 mm wheel diameter) 13 mm after intradermal injection.

1 Measured by antigen binding RIA.

2 These were negative with wheal and erythema > 50 mm in diameter. The mite allergens 5 and 7 were negative with wheal and erythema > 50 mm in diameter. The mite allergens 5 and 7 were negative with wheal and erythema > 50 mm in diameter.

References


