IgE Reactivity from Serum of Blomia tropicalis Allergic Patients to the Recombinant Protein Blo t 1

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Objective. To determine the IgE reactivity against recombinant protein Blo t 1 from the dust mite Blomia tropicalis (Bt) using serum from patients with positive skin test to this mite and to investigate the cross-reactivity between Bt and Dermatophagoides pteronyssinus.

Background. Dust mites have an important role as inducers of allergic asthma and rhinitis. Particularly, Bt is an important mite specie in the tropical and subtropical regions of the world. Therefore, recombinant allergens of this organism could be an important feature for development of effective diagnostic and therapeutic measures.

Materials and Methods. Purified recombinant Blo t 1 was produced in Escherichia coli and tested against sera from 54 allergic individuals by the dot blot technique.

Results. IgE response to Blo t 1 was 72% for sera from patients with positive skin test to Bt. Cross-reactivity with Dermatophagoides pteronyssinus was not detected. Statistical analyses of the dot blot test results show 71.74% of sensitivity and 100% of specificity.

Conclusion. By using a panel of allergic sera and an in vitro assay, the allergen rBlo t 1 exhibits no IgE cross-reactivity with Dermatophagoides pteronyssinus allergens. This finding suggests that specific clinical reagents are necessary for precise diagnosis and treatment of sensitization to Bt.

Keywords: Allergen, rBlo t 1, Blomia tropicalis, Dust mite, Dot blot

Asthma, a chronic inflammatory disease of the respiratory tract, affects one of ten children in the United States. This condition is one of the major causes for Emergency Room treatment, hospitalization and school absenteeism (1). One of characteristics of asthma is the hyperresponsiveness to a variety of environmental factors, especially inhaled agents in concentrations than do not affect the majority of people (2). In these conditions, allergens may cause an IgE mediated hypersensitivity reactions. The magnitude of the IgE response to allergen depends on the dosage, the route of exposure and the genetic composition of the host (3).

Allergens, especially those derived from house dust mite, appear to have a role for primary stimulus in development of asthma (4). The dust mite Blomia tropicalis (Bt) is a common living organism mostly founded on the habitat of houses in the tropical and subtropical regions and it is important as inducers of allergic asthma and allergic rhinitis (5-6). The characterization of individual allergens in Bt is an important factor for proper diagnosis of mite induced allergy reaction, the choice of immunotherapy and the development of assays for determination of Bt allergen levels in dust. The use of recombinant allergens of Bt may suggest a possibility for the treatment of allergies and asthma episodes induced by them. Various allergens of Bt have been cloned and expressed such as rBlo t 5 which shares 43% homology with rDer p 5, its counterpart from Dermatophagoides pteronyssinus (7); rBlo t 13 has homology to fatty acid-binding proteins (8); rBlo t 11 is homologous to panmyosin (9); rBlo t 1 has 35% identity and 50% similarity with Der p 1 (10).

Here, we report the analysis of the IgE reactivity from sera of allergic patients against rBlo t 1 to determine the importance of this protein as allergen. In addition, we tested the cross-reactivity with Dermatophagoides pteronyssinus (Dp).

Materials and Methods

Recombinant protein Blo t 1. Recombinant protein
rBto t 1 expression and purification of recombinant Bto t 1 as previously described by Mora et al (10). Briefly, _Escherichia coli_ XL1 Blue cells harboring a pDS56 expression vector containing the cDNA for Bto t 1 was cultured overnight in 300 ml of LB medium (10 g Bacto-tryptone, 5 g Bacto-yeast extract, 5 g NaCl/l, pH 7.5) containing ampicillin (50 µg/ml) and tetracycline (10 µg/ml) at 37 °C. Two hundred sixty five ml of the overnight culture was inoculated in 5 liters of prewarmed LB medium with the appropriate antibiotics and growth at 37 °C until the optical density at 600 nm will be 0.6. Expression was induced by adding isopropylthiobeta-D-galactoside (IPTG) to a final concentration of 1 mM. The culture was grown for an additional 2 hours and centrifuged for cell harvesting (10,000 rpm/10 min). The supernatant was discarded and the cell pellets was resuspended in 50 ml of lysis buffer B (8 M urea, 100 mM NaHPO₄, 10 mM Tris-HCl 20 mM imidazole, pH 8.0) and incubated two hours at room temperature with mixing. The lysate was centrifuged for 25 min at 4,000 rpm to remove cellular debris and the supernatant transferred to a 25 ml equilibrated Ni-NTA Superflow resin. The mixture was mixed by shaking for 1 hour at room temperature. Then, it was loaded into an empty column (23 cm L x 3 cm W) and the flow-through will be collected. After three washes with 80 ml of buffer C (same composition buffer B except for the pH is 6.3), the recombinant protein was eluted 5 times (10 ml each one) with buffer D (same composition as buffer B except for the 20 mM imidazole and the pH is 5.9). The recombinant protein expression and molecular weight was confirmed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Serum samples.** For the purpose of assessing the antigenic role and specificity of rBto t 1 it was reacted against sera from non-immunotherapy treated Bt positive allergic individuals. These patients (n=46) had tested positive in previous skin prick test with Bt extracts/or positive in the radioallergosorbent test (RAST) (>40 units/ml). Sera of non-allergic individuals from Puerto Rico were used as negative control (n=3). These individuals had no history of atopy and negative skin prick test or RAST to dust mite allergens. Also, sera from individuals who had positive skin test to Dp extracts were used (n=5). Sample sera were properly maintained in our laboratory. These samples (n=54) have been used in previous studies from our laboratory and informed consent forms have been signed by participant subjects (11).

**Immunoblotting.** SDS-PAGE and immunoblotting were performed according to standard methods (12-13). One hundred fifty µl of rBto t 1 were loaded per track in polyacrylamide ready gel at 15% (Bio-Rad Laboratories, Hercules, CA) and stacking gel was prepared at 5%. Protein concentration was determined by the Bradford method (Bio-Rad). Protein band was visualized by staining with Silver Stain Plus (Bio-Rad). For immunoblotting, proteins were transferred to nitrocellulose membrane in the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). Each membrane was incubated overnight with 1:5 diluted serum samples to obtain a total volume 600 µl sufficient for the Multi-Screen Apparatus (Bio-Rad). IgE binding was visualized by incubating the membrane with horse-dermal peroxidase anti-human IgE monoclonal antibody (1/200,000 dilution in 10% nonfat dry milk) and the Supersignal West Femto Maximum Sensitivity Substrate (PIERCE, Rockford, IL). Chemiluminescence detection was performed by autoradiography.

**Dot blotting.** The dot blotting technique was performed according to Chew and collaborators (14). Purified undiluted rBto t 1 was spotted in dots of 2 µl rBto t 1 on a nitrocellulose membrane. Nonspecific sites were blocked with 5% nonfat dry milk. The blanks were incubated overnight with 2 µl of sera at 4°C and followed by washing with TBS-Tween 20 (0.05%). Serum samples of non-allergic individuals and TBS were used as negative control. For IgE detection, the blots were then incubated with horse-dermal peroxidase anti-human IgE monoclonal antibody (1/200,000 dilution in 10% nonfat dry milk) for 1 h at room temperature. The membranes were stringently washed as before and were subsequently incubated with the Supersignal West Femto Maximum Sensitivity Substrate (PIERCE). Chemiluminescence detection was performed by autoradiography.

**Statistics.** Sensitivity, specificity and predictive values of the assays were calculated according to Wayne (15). The sensitivity of the test is the probability that the person with skin test positive was correctly identified by dot blot test. The specificity of the test is the probability that non-allergic patients were correctly identified by the dot blot test. The positive and negative predictive values express how often the test is correct or incorrect, respectively, when it is positive or negative, respectively. These values were determined as follows using information provided in the table.

<table>
<thead>
<tr>
<th>Results of skin test</th>
<th>Positive results of dot blot</th>
<th>Negative results of dot blot</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive results</td>
<td>A</td>
<td>B</td>
<td>A+B</td>
</tr>
<tr>
<td>Negative results</td>
<td>C</td>
<td>D</td>
<td>C+D</td>
</tr>
<tr>
<td>Total</td>
<td>A+C</td>
<td>B+D</td>
<td>N</td>
</tr>
</tbody>
</table>

A=Samples with positive results in skin test and positive in the dot blot.
B=Samples with positive results in skin test but negative in dot blot.
C = Samples with negative results in skin test and negative in dot blot.
D = Samples with negative results in skin test and negative in dot blot.
N = Number of subjects
Sensitivity = A/(A+B)
Specificity = D/(C+D)
Positive predictive value = A/(A+C)
Negative predictive value = D/(B+D)

Other statistic tests used in this study were false positive and false negatives. False positive results arise when a test indicates a positive status but a true status is negative and false negative results arise when a test indicates a negative status but a true status is positive.

**Results**

SDS-PAGE of rBlo t1 showed a single band with a molecular weight of about 25 KDa (Figure 1). This protein was transferred to nitrocellulose and Western blot were performed using sera sample of allergic and nonallergic subjects, but we face many troubleshooting to interpret the IgE reaction; by this reason, we decided to change to the dot blot technique that give better results. A total of 54 samples of allergic individuals were tested; 46 of them were allergic to Bt according to the results of skin test and had no reaction to Dp. Five individuals had positive skin test reaction to Dp but negative to Bt and three individuals have not reaction to Bt and Dp. All samples were tested against rBlo t1 by dot blot along TBS as negative control. Figure 2 shows the immunoblotting of rBlo t1 with representative serum samples from Bt positive and negative skin test individuals.

Thirty-three out 46 sample (71.74%) skin test-positive to Bt extracts demonstrated strong IgE binding to rBlo t1. All 5 sera from individuals that were reactive to Dp

**Discussion**

Recombinant allergens are an exciting alternative for improved diagnostics and for therapy of allergy, since they provide pure and well-characterized allergens in contrast to the crude allergen extracts in use today. In addition, modified recombinant allergens with reduced IgE reactivity, are believed to be potential tools for improved specific immunotherapy. The use of isoforms of allergens (16), mutated allergens (17) and fragments of allergens with reduced IgE binding capacity (18) would increase the safety of immunotherapy by reducing the risk of anaphylactic reactions and give the possibility of applying higher doses, which may be of benefit for the clinical outcome of the treatment (19).

Recently, a group 1 recombinant allergen from Bt, designated Blo t1, has been cloned and expressed by Mora and collaborators (10). Blo t1 encodes a 221 amino acid mature protein with an estimate molecular weight of 25,000 Daltons (see Figure 1). This recombinant protein is 35% identical to the cysteine proteases from Dp, D. farinae and Euroglyphus maynei previously characterized (10, 20).

Here, the ability of recombinant Blo t1 to react with IgE present in serum from Bt and Dp highly positive skin test patients was examined. It was found to react strongly with 33 out 46 from Bt skin positive patient sera (see Table 1 and Fig. 2). The high reactivity of Blo t1 to the sera tested
Table 1. Results of dot blot assays

<table>
<thead>
<tr>
<th>Results of skin test</th>
<th>Positive results of dot blot</th>
<th>Negative results of dot blot</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive results</td>
<td>33</td>
<td>13</td>
<td>46</td>
</tr>
<tr>
<td>Negative results</td>
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<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
<td>21</td>
<td>54</td>
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</table>

represents a high incidence of sensitization to this cysteine protease-like protein and suggests its possible role as a major allergen of Bt reinforcing the clinical importance of this protein. The frequency of recognition (71.24 %) contrasts with > 90 % frequencies reported for native group I allergens from pyrolyphid mites (20). Although similar to that seen with other recombinant Bt allergens (7, 8, 21, 22, 23, 24), the true frequency may be higher because reactivities of recombinant proteins may be relatively low (25, 26). Actually, studies using native material are in progress at our laboratory. Interestingly, Dp skin test positive serum (Bt negative) did not react with the protein, indicating the possible presence of unique IgE-binding epitopes on the rB10 t 1 molecule, which do not cross-react with Dp extract antigens. These results are consistent with previous studies which show low cross-reactivity between Bt and Dp (7, 10, 14, 17, 27, 28, 29) and suggest that highly specific reagents are necessary for precise diagnosis and immunotherapeutic treatment of sensitization to group I mite allergens.

The dot blot technique was used to detect the IgE reactivity to B10 t 1. This technique has been successfully used by other investigators for diagnosis of *Onchocerca volvulus* (30), *Mycobacterium tuberculosis* (31), *Neisseria meningitides* (32), influenza A and B virus (33), and periodontopathic bacteria (34) infections; also, it was used for detection of IgE reactivity and cross-reactivity studies using recombinant allergens from mites (8, 14, 23). Our results show that the specificity and positive predictive value were 100 % and no false positive reaction was detected. The false negative reaction was 28.26 %, a possible explanation is that a false negative in the dot blot test could really be due to a false positive in the skin test, but we have no way of determining this. The dot blot technique results a simple, cheap, reproducible and rapid as well sensitive (71.74 %) and specific (100 %) means of detection of IgE serum antibodies to allergens from mites.

The residents of the tropical and sub-tropical regions are continuously exposed to *Blomia tropicalis*. This explains the high levels of sensitization to this allergen among Puerto Ricans and the high prevalence of asthma in this population. The recombinant product rB10 t 1 therefore constitutes an important tool to study the mechanisms of allergy and for diagnosing events of Bt exposures.

Resumen

Los ácaros tienen un considerable rol en la etiología del asma alérgico y rinitis. Particularmente, en las regiones tropicales y subtropicales del mundo, *Blomia tropicalis* (Bt) representa un importante factor de riesgo para estas condiciones, por lo tanto el uso de alérgenos recombinantes de este organismo podrían ser útiles para el desarrollo de diagnóstico y terapia efectivas.

El objetivo de este trabajo fue determinar la reactividad de IgE presente en el suero de pacientes con prueba de piel positiva para extracto de Bt contra la proteína recombinante rB10 t 1 de Bt. También se analizó la reactividad cruzada entre Bt y *Dermatophagoides pteronyssinus* (Dp).

La proteína recombinante rB10 t 1, producida en *Escherichia coli*, fue probada por la técnica de dot blot con suero de 46 pacientes alérgicos a Bt, 5 pacientes alérgicos a Dp y 3 controles normales. Los pacientes no habían recibido inmunoterapia.

El 72 % de los sueros de pacientes con prueba de piel positiva a Bt reaccionaron con rB10 t 1 detectando la presencia de IgE para esta proteína. Por otro lado, no se detectó reactividad cruzada con Dp. De acuerdo a los análisis estadísticos, la técnica de dot blot tuvo un 71.74 % de sensibilidad y 100 % de especificidad.

Estos resultados sugieren que la respuesta a alérgenos de Bt es específica y que, por lo tanto, es necesario utilizar reactivos clínicos específicos para el correcto diagnóstico y tratamiento de alergias y asma debidos a Bt.

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References


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