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 tropic 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 Blot t 1 was produced in *Escherichia coli* and tested against sera from 54 allergic individuals by the dot blot technique.**

Results. **IgE response to Blot 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 *Dermathophagoides 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 hyperresposivenes 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 paramyosin (9); rBlot 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).

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Materials and Methods

Recombinant protein Blo t 1. Recombinant protein

rBlo t 1 expression and purification of recombinant Blo 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 Blo 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 1mM. 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 NaH_2PO_4, 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-polycrylamide gel electrophoresis (SDS-PAGE).

Serum samples. For the purpose of assessing the antigenic role and specificity of rBlo 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 radioallergosobent test (RAST) (>40units/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 rBlo 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 horseradish 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 rBlo t 1 was blotted in dots of 2 µl rBlo t 1 on a nitrocellulose membrane. Nonspecific sites were blocked with 5% nonfat dry milk. The blots 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 horseradish peroxidase anti-human IgE monoclonal antibody (1/200,000 dilution in 10% nonfat dry milk) for 1h at room temperature. The membranes were stringently washed as before and were subsequently incubated with the Supersignal West Femto Maximun 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.

Results of skin test	Positive results of dot blot	Negative results of dot blot	Total
Positive results	A	B	A+B
Negative results	C	D	C+D
Total	A+C	B+D	N

A=Samples with positive results in skin test and positive in the dot blot.

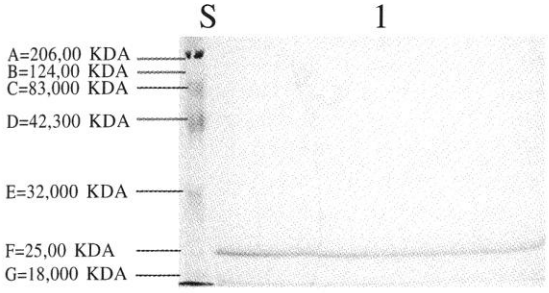
B=Samples with positive results in skin test but negative in dot blot.

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 t 1 showed a single band with a molecular weight of about 25 KDa (Figure 1). This protein



S = Molecular weight standards
1 = rBlo t 1

Figure 1. Shows electrophoresis of rBlo t 1

was transferred to nitrocellulose and Western blot were performed using sera sample of allergic and non allergic 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 t 1 by dot blot along TBS as negative control. Figure 2 shows the immunoblotting of rBlo t 1 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 r Blo t 1. All 5 sera from individuals that were reactive to Dp

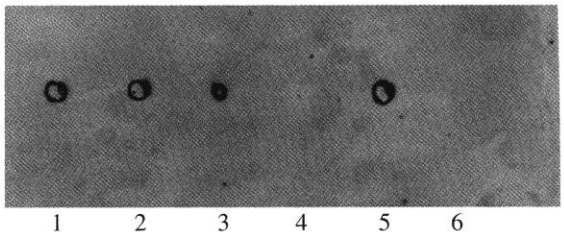


Figure 2. Shows the immunoblotting of rBLO t 1 (Dot Blot), 1-3,5: Bt - positive skin test sera; 4 Dp - positive skin test serum, 6: negative control

(but no Bt) did not demonstrate any IgE antibodies binding to rBlo t 1; also the 3 negative controls failed to react with rBlo t 1. Table 1 shows a summary these results.

Statistical analyses of results are as follows:

- Sensitivity of dot blot test: 71.74%
- Specificity of dot blot test: 100%
- Positive predictive value of dot blot test: 100%
- Negative predictive value of dot blot test: 38.10%
- False positive results: 0%
- False negative results: 28.26%.

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 t 1, has been cloned and expressed by Mora and collaborators (10). Blo t 1 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 t 1 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 t 1 to the sera tested

Table 1. Results of dot blot assays

Results of skin test	Positive results of dot blot	Negative results of dot blot	Total
Positive results	33	13	46
Negative results	0	8	8
Total	33	21	54

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 1 allergens from pyroglyphid 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 rBlo 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, 27, 28, 29) and suggest that highly specific reagents are necessary for precise diagnosis and immunotherapeutic treatment of sensitization to group 1 mite allergens.

The dot blot technique was used to detect the IgE reactivity to Blo 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 explication 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 rBlo 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 alergenos recombinantes de este organismo podrían ser útiles para el desarrollo de diagnóstico y terapia efectivos.

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 rBlo t 1 de Bt. También se analizó la reactividad cruzada entre Bt y *Dermatophagoides pteronyssinus* (Dp).

La proteína recombinante rBlo 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 rBlo 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 alergenos 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

1. Flaum M, Lum CL, Tinkelman D. Take control of high-cost asthma. J Asthma 1997;34:5-14.
2. Barnes P J. Anti-inflammatory therapy for asthma. Annu Rev Med 1993;44:229-242.
3. Abbas A K., Lichtman A H. Cellular and molecular immunology. 5th ed. Philadelphia: Saunders, 2003.
4. Platts-Mills TAE, Chapman MD. Dust mites: immunology, allergic disease, and environmental control. J Allergy Clin Immunol 1987;80:755-775.
5. Van Bronswjik JEMH, De Cock AWAM, Oshima A. The genus *Blomia oudemans* (Acari: Glycyphagidae). Description of *Blomia tropicalis* sp. n. from house dust in tropical and sub-tropical regions. Acarologia 1973;15:477-489.
6. Fernández Caldas E, Puerta L, Mercado D, Lockey RF, Caraballo LR. Mite fauna, Der p 1, Der f 1 and *Blomia tropicalis* allergen levels in a tropical environment. Clin Exp Allergy 1993;23:292-297.

7. Arruda LK, Vailes LD, Platts-Mills TAE, et al. Sensitization to *Blomia tropicalis* in patients with asthma and identification of allergen Blo t 5. Am J Respir Crit Care Med 1997;155:343-350.
8. Caraballo L, Puerta L, Jiménez S, et al. Cloning and IgE binding of recombinant allergen from the mite *Blomia tropicalis,* homologous with fatty acid-binding proteins. Int Arch Allergy Immunol 1997;112:341-347.
9. Downs SH, Marks GB, Sporik R, Belosouva EG, Car NG, Peat JK. Continued increase in the prevalence of asthma and atopy. Arch Dis Child 2001;84:20-23.
10. Mora C, Flores I, Montealegre F, Díaz A. Cloning and expression of Blo t 1, a novel allergen from the dust mite *Blomia tropicalis*, homologous to cysteine proteases. Clin Exp Allergy 2003;33:28-34.
11. Diaz AM, López Malpica F, Delgado R, Rosario G, Montealegre F. Relation among skin test reactivity, serum IgE levels and IgG and IgE reactivity to Dp and Bt in atopic patients. Abst 97th General Meeting ASM: 575(V-6),1997.
12. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-685.
13. Towbin H, Stachelin T, Gordon J. Electrophoretic transfer of protein from acrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci 1979;76:4350-4354.
14. Chew FT, Yi FC, Chua KY. Allergenic differences between the domestic mite *Blomia tropicalis* and *Dermatophagoides pteronyssinus*. Clin Exp Allergy 1999;29:982-984.
15. Wayne W D. Biostatistics: a foundation for analysis in the health sciences. 7th ed. New York: John Wiley & Sons, Inc 1999:71-74.
16. Ferreira F, Hirtenlehener K, Jilek A, et al. Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: Potential use of hypoallergenic isoforms for immunotherapy. J Exp Med 1996;183:599-609.
17. Smith AM, Chapman MD. Reduction in IgE binding to allergens variants generated by site directed mutagenesis: contribution of disulfide bonds to the antigenic structure of the major house dust mite allergen Der p 2. Mol Immunol 1996;33:399-405.
18. Vrtala S, Hirtenlehener k, Vangalista L, et al. Division of major birch pollen allergen, Bet v 1, into two non-anaphylactic fragments. Int Arch Allergy Immunol 1997;113:246-248.
19. Chapman MD, Smith AM, Vailes LD, Arruda LK, Dhanaraj V, Pomes A. Recombinant allergens for diagnosis and therapy of allergic disease. J Allergy Clin Immunol 2000;106:4409-418.
20. Stewart GA, Robinson C. The immunobiology of allergic peptidases. Clin Exp Allergy 2003;33:3-6.
21. Tovey ER, Johnson MC, Roche AL, Cobon GS, Baldo BA. Cloning and sequencing of a cDNA expressing a recombinant house dust mite protein that binds human IgE and corresponds to an important low molecular weight allergen. J Exp Med 1989;170:1457-1462.
22. Yi FC, Cheong N, Shek PC, Wang DY, Chua KY, Lee BW. Identification of shared and unique immunoglobulin E epitopes of the highly conserved tropomyosins in *Blomia tropicalis* and *Dermatophagoides pteronyssinus*. Clin Exp Allergy 2002;32:1203-10.
23. Ramos JD, Cheong N, Lee BW et al. cDNA cloning and expression of Blo t 11, the *Blomia tropicalis allergen* homologous to paramyosin. Int Arch Allergy Immunol 2001;126:286-293.
24. Puerta L, Caraballo L, Fernandez-Caldas E, et al. Nucleotide sequence analysis of complementary DNA coding for a *Blomia tropicalis* allergen. J Allergy Clin Immunol 1996;98:932-937.
25. Greene WK, Cyster JG, Chua KY, O'Brien RM, Thomas WR. IgE and IgG binding of peptides expressed from fragments of cDNA encoding the major house dust mite allergen Der p I. J Immunol 1991;147:3768-3773.
26. Greene WK, Thomas WR. IgE binding structures of the major house dust mite allergen Der p I. Mol Immunol 1992;29:257-262.
27. Smith A, Green R, Custovic A, Woodcock A, Chapman M. *In vivo* cross-reactivity between IgE of *Blomia* and *Dermatophagoides* spp and to the recombinant group 5 allergens among mite sensitive allergic patients in the UK. J Allergy Clin Immunol 1996;97:420 (Abstract).
28. Simpson A, Green R, Custovic A, Woodstock A, Arruda LK, Chapman MD. Skin test reactivity to natural and recombinant Blomia and Dermatophagoides spp. Allergens among mite allergic patients in UK. Allergy 2003;58:53-56.
29. Kuo IC, Cheong N, Trakultivakorn M, Lee BW, Chua KY. An extensive study of human IgE cross-reactivity of Blo t 5 and Der p 5. J Allergy Clin Immunol 2003;111:603-609.
30. Guzman GE, Awadzi K, Opoku N, Narayanan RB, Akuffo HO. Comparison between the skin snip test and simple dot blot assay as potencial rapid assessment tools for Onchocerciasis in postcontrol era in Ghana. Clin Diagn Lab Immunol 2003;9:1014-1020.
31. Attallah AM, Abdel Malak CA, Ismail H, El-Saggan A, Ommran MM, Tabll AA. Rapid and simple detection of a *Mycobacterium tuberculosis* circulating antigen in serum using dot-ELISA for field diagnosis of pulmonary tuberculosis. J Immunoassay Immunochem 2003;24:73-78.
32. Fukasawa LO, Gorla MC, Lemos AP, et al. Immune response to native NadA from *Neisseria meningitidis* and its expression in clinical isolates in Brazil. J Med Microbiol 2003;52:121-125.
33. Reina J, Padilla E, Alonso F, Ruiz De Gopegui E, Mun M, Mari M. Evaluation of new dot blot enzyme immunoassay (directigen flu A+B) for simultaneous and differential detection of influenza A and B virus antigens from respiratory samples. J Clin Microbiol 2002;40:3515-3517.
34. Kawashime Y, Ishikawa I. Simple and rapid detection of serum antibody to periodontopathic bacteria by dot blotting. J Periodontal Res 2002;37:223-229.