MICROBIOLOGY

Monoclonal Antibodies Against Whole Body Extract of the Dust Mite *Blomia tropicalis*

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Objective. **To produce and characterize monoclonal antibodies (mAbs) against whole body extract of the dust mite *Blomia tropicalis* (Bt).**

Background. **Bt is an important source of allergens causing allergic diseases in tropical and subtropical regions. mAbs are excellent tools for delineating cross-reactivity between Bt and other mites.**

Materials and Methods. **Mice were immunized with extracts of Bt. mAbs were produced by standard techniques. Hybridoma screening was performed by ELISA. Ascitic fluids were produced and partially purified by adsorption chromatography. Reactivity of mAbs against extracts of Bt and *Dermatophagoides pteronyssinus* (Dp) was analyzed by ELISA, immunoblots and ELISA inhibition. Also, reactivity of mAbs against the putative cysteine protease rBlo t 1 from Bt was tested.**

Results. **Three IgG mAbs were obtained and partially purified. The mAbs reacted with Bt by ELISA. In immunoblots, mAbs recognized two protein components of 29 and 35 KD. Also, the mAbs reacted with Dp extracts by ELISA, and the same sized components were detected in immunoblots. In competitive ELISA, Dp extract reduced in 62% the reactivity with Bt antigens, and Bt extract produced a maximum reduction of reactivity against Dp antigens of 83%. The mAbs recognized rBlo t1. The homology between Blo t 1 and the proteins recognized by the mAbs was high (90%) as the inhibition assays demonstrated.**

Conclusions. **The cross-reactivity between Dp and Bp could be related with the presence of proteins as their respective cysteine proteases. The produced mAbs have proven to facilitate the identification of antigens of Bt and to determine the possible cross-reactivity between Bt and other common mites of the acarofauna of tropical and subtropical countries where Bt is commonly found.**

Key words: Monoclonal antibodies, Mite allergens, Cysteine protease, Blomia tropicalis, Blo t 1, Dermatophagoides pteronyssinus

H ouse dust mites have proven to be involved in the development of allergic diseases such as asthma, allergic rhinitis and atopic dermatitis (1). Mites from the family Pyroglyphidae, genus *Dermatophagoides*, especially *Dermatophagoides pteronyssinus* (Dp) and *Dermatophagoides farinae* (Df) are the main source of house dust allergens worldwide (2). *Blomia tropicalis* (Bt), a storage mite of the family Echymiopodidae, has been found among the most prevalent mites in dust samples in tropical and subtropical countries, including the Southern part of the US and Puerto Rico (3-6). Despite the importance of Bt, the majority of its antigenic components remain uncharacterized. The whole body extract of Bt has, at least, 25 allergens ranging between 11 and 85 KD among which the 11, 13, 14 and 16 KD components represent clinically important allergens as defined by their IgE binding frequencies (7). According to Caraballo et al. (8), Bt has one major allergen (11-33 KD) and three important allergens of 64, 36 and 33 KD with about 50% of IgE binding.

Various allergens of Bt have been cloned such as Blo t 5, a 14 KD protein which showed low to moderate cross-reactivity with allergens from Dp even with its counterpart, Der p 5, which shares 43% homology with Blo t 5 (9). Another recombinant protein, rBlo t 13, has been described (10). rBlo t 13 has high structural homology to fatty acid-binding protein (FABPs) from different sources. rBlo t 11 is homologous to paramyosin (11). Blo t 3 is highly homologous to Group 3 dust mite allergens (6). Recently, a 25 KD recombinant protein with homology to a cysteine protease, rBlo t 1, has been cloned in our laboratory. rBlo

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t 1 is a mature recombinant protein that has a 35% identity and 50% similarity with Der p 1, with 60% IgE-binding frequency to sera from allergic patients with a positive skin test to Bt extracts (12).

Monoclonal antibodies (mAbs) have been produced against antigens from Dp, Df and *D. siboney* (13-15) but Bt has low to moderate reactivity with these mites (16), and these mAbs cannot be used to identify Bt antigens. Labrada et al. produced mAbs to rBlo t 13 which cross-reacted with extracts of *D. siboney* (17). In this study, we report the production and characterization of a group of mAbs against whole body extracts of Bt. These mAbs recognized two sized protein components of 29 and 35 KD, and they showed considerable reactivity with Dp extracts. Also, they recognized rBlo t 1 being a useful tool for the study of antigenicity and other biological properties of this recombinant protein.

Materials and Methods

Whole mite body extracts. Bt and Dp mites were gently provided by Dr. Federico Montealegre (Ponce Medicine School). Mites were separated from mature culture of mites grown *in vitro* at 18-25°C, 70-80% relative humidity using a Sieve Shaker RX-86 (WS Tyler, OH, USA). Whole mite bodies were defatted for 4 hrs with anhydrous ether in a Soxhlet extractor and dried at room temperature (RT). Defatted mites were extracted (1:20 w/v) in 0.1M ammonium bicarbonate for 24 hrs at 4°C and clarified by centrifugation (6,600 x g for 10 min). Then, the extracts were dialyzed using a 6,000 to 8,000 MW cut off membrane overnight at 4 °C against deionized water, centrifuged and kept frozen at -20 °C.

Production of mAbs. Balb/c mice were immunized intraperitoneally (i.p.) with 200 µl of Bt body extracts diluted at 0.5 mg/ml in phosphate buffer saline (PBS) pH 7.2, and emulsified 1:1 with Hunter's Titer Max adjuvant (Cyt Rx, Atlanta, GA). Twenty-eight days after the first injection, mice received a second dose of 50 µg of antigen in 100 µl of PBS. Blood were obtained from the retroorbital plexus to determine the presence of antibodies (Ab) against Bt antigens using enzyme-linked immunosorbent assay (ELISA). If the concentration of Ab was high, the mice were sacrificed by cervical dislocation 3 to 4 days after the second booster. In some experiments, a third and fourth boosters were administered to ensure a secondary response.

Fusion processes were done using the technique developed by Kohler and Milstein (18). Non-secreting Sp2O-Ag14 murine myeloma cells were fused in the presence of polyethylene glycol 1500 (pH 7.8) with the mononuclear cells from the spleens of immunized mice in a ratio of 3:1. Cells were resuspended in H-Y medium (Sigma, St. Louis, MO) with 10% of fetal bovine serum (FBS), 10% of origen hybridoma cloning factor (OHCF) (IGEN, Gaithersburg, MD). The suspension were seeded (100µl/well) into flat bottom 96 wells plates (Costar, Cambridge, MA) and incubated at 37°C and 5% CO_2. Twenty-four hours later, 100 µl of H-Y medium, containing 10% of FBS, 10% OHCF and 4% of HAT (10^{-4}M hypoxanthine, 4 X 10^{-7}M aminopterin and 1.6 X 10^{-5}M thymidine) (Sigma) were added to each well. Five days later, the cells were fed (50% volume) with HT medium (10^{-4} M hypoxanthine and 1.6 X 10^{-5}M thymidine) (Sigma). The plates were screened daily for the microscopic detection of the hybrids using a cell culture microscope. Wells with growth were transferred to 48 and 24 well plates (Costar) to obtain larger volumes of each sample. Those hybridomas found to be positive against Bt extracts by ELISA were cloned twice by limiting dilution and the selected clones were expanded. The chosen positive clones were injected into pristine-primed mice to induce ascitic fluid production. Antibodies from ascitic fluid were purified by thiophillic adsorption chromatography (Pierce).

Hybridoma screening. Supernatant culture fluids (100 µl) were evaluated by indirect ELISA tests using 96 well polystyrene disposable microplates (Costar) as the solid phase. Briefly, 200 µl of Bt whole body extract (4µg/well) were disposed in each well and incubated overnight at 4°C. Microplates were washed three times with Tris buffer saline with 0.5% Tween 20 (TTBS) and blocked with 1% bovine serum albumin (BSA) (Sigma) for 45 min at RT. After 3X washing, 100 µl of culture supernatants were placed in each well and incubated for 1hr at RT. Microplates were washed 3 times with TTBS and incubated with 100 µl of goat anti-mouse polyvalent immunoglobulin (Ig) conjugated to alkaline phophatase (Sigma) diluted 1:4500 in 1% BSA for 2 hr at RT. Plates were washed 5 times with buffer and 100 µl of freshly prepared alkaline phosphatase substrate were added to each well. Plates were incubated for 30 min in dark at RT and the reaction was stopped with 50 µl/well of 3N NaOH. Finally, the reaction absorbance was read a 410 nm in a MR 700 Microplate Reader (Dynatech Laboratories). Positive control (immune serum from mice diluted 1:1000) and negative control (TBS) were included. Readings with a mean optical density (OD) greater than 0.2 units above the mean OD of the negative control were considered as positive.

Isotype determination. Isotyping of mAbs in culture supernatants and ascitic fluids (1:100) were performed by indirect ELISA using the same procedure described above. Goat-antimouse IgG (1:10,000) or IgM (1:8,000) conjugated to alkaline phosphatase (Sigma) were used instead of just anti-Ig polyvalent antibodies.

Reactivity of purified mAbs. The purified recombinant protein Blo t 1 (rBlot 1) was kindly provided by Dr. Carlos Mora. To determine the reactivity of the purified mAbs (20 µg/ml) indirect ELISA was performed as previously described using Bt and Dp extracts (4µg/well) and rBlo t 1 (20 µg/well).

Determination of mAbs specificity by competitive assays. Competitive assays were done using ELISA as previously described. Purified mAbs were diluted in TBS (pH 7.5) to 20 µg/ml and mixed 1:1 with dilutions of Bt or Dp extracts (20, 15, 10, 5 and 1 µg/ml). The reaction was incubated overnight at 4°C. The mixture of each mAb with the dilution of Bt extract was applied to the plates already coated with Dp antigens. The mixture of each mAb with the dilution of Dp extracts was applied to the plates already coated with Bt.

To determine if the presence of rBlo t 1 reduced the reactivity of the purified mAbs, competitive assays were performed as described above. Dilutions of mAbs (20µg/ml) were mixed with dilutions of rBlo t 1 (20, 10, and 5 µg/ml) and incubated overnight at 4°C. These mixtures were applied to plates coated with Bt extracts. Percentage of inhibition was calculated as follows:

$$\% \text{ inhibition} = 1 - \frac{\text{OD with inhibitor}}{\text{OD without inhibitor}} \times 100$$

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Non-denaturing and denaturing SDS-PAGE and immunoblotting were performed according to standard methods (19, 20). One hundred µg of Bt, Dp extracts or 50µg of rBlo t 1 were loaded per track in a 12 or 15 % polyacrylamide gel. Protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Richmond, CA). Proteins were visualized by Coomassie blue staining. For immunoblotting, proteins were transferred to a nitrocellulose membrane (Bio-Rad). Each nitrocellulose strip was incubated overnight with the mAbs. IgG binding were visualized by incubation with ABC Vectastain Peroxidase System (Vector, Burlingame, CA).

Results

Reactivity of mAbs. Mice immunized with Bt whole body extracts followed by fusion process produced 3 hybrids (C1B, C6 and D3) against Bt extracts. After two cloning out of the C1B and C6 hybrids, fourteen IgM and thirteen IgG secreting mAbs clones were identified by ELISA (data not shown). Six IgM clones and three IgG clones were concentrated *in vivo* by production of ascitic fluids. The clones were chosen for their high reactivity to

Bt and their growth conditions at the time of the experiment. In order to determine the reactivity of the selected mAbs against Bt extract, ELISA tests were performed. Results are shown in Table 1.

Table 1. Reactivity of ascitic fluids against Bt extract determined by ELISA

Subclone	Reactivity to Bt (Absorbance at 410 nm)	Isotype
C1BE2C9	1.882	IgM
C1BE2F3	>2.000	IgM
C1BE2F9/2	1.007	IgM
C1BE2F10	1.493	IgM
C1BE2H3/2	0.816	IgM
C1BE2H9	0.454	IgM
C6B6D7	0.943	IgG
C6B6F2	1.331	IgG
C6E11C2	1.296	IgG
Negative Control IgG	0.142	—
Negative Control IgM	0.492	—
Positive Control IgG	>2.000	—
Positive Control IgM	>2.000	—

Negative control: TBS, pH 7.5
Positive control: Immune serum against Bt extract (1:1,000)
Ascitic fluids (1:20) were tested by ELISA against Bt whole body mite extract (4 µg/well). Goat anti mouse IgG (1:10,000) or IgM (1:8,000) antibodies conjugated to alkaline phosphatase were used as secondary antibodies

The IgG mAbs-producer clones C6B6D7, C6B6F2 and C6E11C2 were purified by thiophillic adsorption chromatography. The purification process was corroborated by SDS-PAGE under denaturing conditions. The protein bands of 50 and 25 KD corresponding to the heavy and light chains of the IgG were detected (data not shown).

In order to determine the reactivity and specificity of the purified mAbs, ELISA and Western blot analysis against Bt or Dp whole body extracts were done. By ELISA, the purified IgG mAbs showed a high reactivity against both mites with OD's between 1.717 and over 2.000. Western blotting demonstrated that the mAbs react with two protein components of approximately 29 and 35 KD showing similar reactivity to Bt and Dp extracts. No reactivity was observed when mice pre-immune serum (1:1,000) was used (data not shown).

The cross-reactivity was corroborated by measuring the percentage of inhibition of reactivity of the mAbs after been absorbed by either Bt or Dp extracts. Table 2 shows that absorption of mAbs with Dp extracts reduced the reactivity of the mAbs against Bt extracts following a dose-response behavior. The percentage of inhibition of reactivity against Bt extracts ranged between 15% and 62% when the mAbs C6B6D7, C6B6F2 and C6E11C2 were

Table 2. Inhibition of reactivity by competition determined by ELISA

Purified mAb Concentration of inhibitor	Percentage of inhibition Inhibitor: Dp extract Antigen on solid phase: Bt extract	Percentage of inhibition Inhibitor: Bt extract Antigen on solid phase: Dp extract
C6B6D7		
5 µg/ml	15.86	51.97
10 µg/ml	22.38	51.32
15 µg/ml	61.17	51.32
20 µg/ml	61.76	69.74
C6B6F2		
1 µg/ml	8.27	47.30
5 µg/ml	34.50	54.05
10 µg/ml	55.47	62.16
20 µg/ml	52.07	64.86
C6E11C2		
1 µg/ml	35.09	73.98
5 µg/ml	47.15	74.27
10 µg/ml	47.18	78.95
15 µg/ml	50.66	82.75

Purified mAbs (20 µg/ml) were absorbed 1:1 with either Bt or Dp extract. The absorbed mAbs were tested by ELISA against Dp or Bt extract respectively. Goat anti mouse IgG antibodies conjugated to alkaline phosphatase (1:10,000) were used as secondary antibodies

absorbed with Dp extracts. mAb C6B6D7 showed the maximum reactivity inhibition. When mAbs were absorbed with Bt extract, the reduction in reactivity to Dp extract was higher, ranging among 26% and 83%. The higher inhibition was found with mAb C6E11C2

Reactivity of mAbs with rBlo t 1. Fifty mg of mature rBlo t1 were electrophoresed in a 15% gel under denaturing conditions and transferred to nitrocellulose. The MW of the protein was determined as 25 KD. Purified IgG mAbs detected the protein in immunoblots (data not shown). Purified mAbs of subclones C6B6F2 and C6E11C2 had positive reactions to rBlo t1 in ELISA, besides the subclone C6B6D7 who did not recognize the protein (Table 3).

Table 3. Reactivity of purified mAbs with rBlo t 1 determined by ELISA

mAb	Absorbance at 405 nm
C6B6D7	0.187
C6B6F2	1.050
C6E11C2	1.075
Negative Control	0.237
Positive Control	1.282

Negative control: TBS, pH 7.5
Positive control: Immune serum against Bt extract (1:1000)
Purified mAbs (20 µg/well) were tested by ELISA against rBlo t 1 (20 µg/well).
Goat anti mouse IgG (1:10,000) antibodies conjugated to alkaline phosphatase were used as secondary antibodies

Since C6B6F2 and C6E11C2 detected the protein by ELISA and Western blot, they were chosen for the assays to corroborate the cross-reactivity between Bt extract and rBlo t1. As shown in Table 4, the reactivity of the mAbs after been absorbed with rBlo t 1 had a range of reduction in reactivity of 45% to 90%. The maximum inhibition was found with the mAb C6E11C2.

Table 4. Inhibition of reactivity to Bt extracts by competition with rBlo t 1 determined by ELISA

Purified mAb	Concentration of rBlo t 1	Percentage of inhibition
C6B6F2		
	5 µg/ml	28.78
	10 µg/ml	41.73
	20 µg/ml	44.96
C6E11C2		
	5 µg/ml	56.79
	10 µg/ml	68.31
	20 µg/ml	90.12

Purified mAbs (20 µg/ml) were absorbed 1:1 with rBlo t 1. The absorbed mAbs were tested by ELISA against extract.
Goat anti mouse IgG antibodies conjugated to alkaline phosphatase (1:10,000) were used as secondary antibodies

Discussion

The role of Bt antigens in allergenic reactions has been extensively studied (4, 5, 21-23). Bt occurs as part of the acarofauna of tropical and subtropical regions throughout the world where it is abundant in house dusts (7, 9, 23-26). Regarding its importance, little work has been done to identify the spectrum and relative importance of Bt allergens. Most studies characterized and identified allergens from Dp, Df, *Euroglyphus maynei* (Em).

In this study we report the production and preliminary characterization of three mAbs against whole body extract of Bt. Mice were primed with natural antigen that was a complex mixture that could vary in their components and allowed the mice to react with the components they find more reactive. After the production, concentration *in vivo* and partial purification of the mAbs, they were tested by ELISA and immunoblots using Bt and Dp extracts to determine their specificity. The obtained mAbs reacted with both mites in ELISA and recognized the same protein components of 29 and 35 KD in immunoblots. These results established a degree a homology between both mites and raised the question about the specificity of the mAbs to Bt. Competitive ELISA corroborated the existence of cross-reactivity among Bt and Dp mites. mAbs absorbed with Dp showed a maximum reduction in reactivity against Bt of 62%, and when the mAbs were absorbed with Bt had a maximum reactivity inhibition against Dp of 83% (Table

2). These results could be associated with the binding of the first antigen to the mAbs that accounted for a reduced accessibility to epitopes of the second antigen. According to this, mAbs had higher affinity to Bt epitotes than to Dp, but also, proposed that the degree of cross-reactivity is higher than expected.

Different, and even contradictory evidences has been published about the cross-reactivity between Dp and storage mites. At least, a 70% of identity is necessary to establish cross-reactivity. It has been reported that the cross-reactivity between these mites ranges from no significant (27, 28) and low to moderate (16) to considerable (29). In patients, a larger number (60%) exposed to Dp gave positive skin tests to Bt extracts (30). Some studies propose that 64% of Bt antigens are specie-specific (31) and that Bt has only three antigens and three allergens that cross-react with Dp and Df (32). The degree of cross-reactivity between antigens is variable and depends primarily on the induced immune response, but other factors as environmental controls, geographical distribution and climates differences could influence the number and diversity of allergen sources (31). In Puerto Rico, where Bt and Dp are concomitant, a higher cross-reactivity is expected according with the degree of cross-sensitization.

The protein components recognized by the mAbs have MW close to purified allergens of Dp and Df as cysteine protease (25 KD), serine protease (28-30 KD), chymotrypsin (Der p 5, 25 KD) and tropomyosin (36 KD). Some of these allergens have enzymatic activity that contributes to their allergenicity. Proteolytic enzymes as cysteine proteases Der p 1, Der f 1 and Eur m1, have proven to play an important role in the initiation and extension of atopic reaction (32).

In order to determine if the recognized protein components have any correlation with Blo t 1, an allergen from *Blomia tropicalis* homologous to cysteine proteases (12), ELISA and Western blots were done using rBlot t 1. All purified IgG mAbs were able to detect rBlo t 1 in immunoblots, but only C6B6F2 and C6E11C2 mAbs reacted against it by ELISA (Table 3). Generally, immunoblots are more sensitive than ELISA, so it can speculate that all mAbs are reactive with rBlo t 1. The results indicated that the proteins could be related and share common epitopes.

The homology between the 29 and 35 KD protein components of the Bt extract recognized by the mAbs and rBlo t 1 (25 KD) were determined by competitive inhibition ELISA (Table 4). A maximum inhibition of reactivity of 90% against Bt antigens were found when the mAbs were absorbed with rBlo t 1. This result proposed a high degree of homology among the putative cysteine protease and the antigens recognized by the mAbs. The difference in

molecular size could be attributed to post-translational changes since the removal of the signal peptide, glycosylation, phosphorylation and formation of disulfide bridges could account for the production of the mature rBlo t 1. These proteins could represent glycosylated forms of the recombinant putative cysteine protease.

It can be proposed that the proteins recognized by the mAbs have a high homology to rBlo t 1 and may have an enzymatic role. Also, a considerable degree of cross-reactivity was detected between Dp and Bp. The cross-reactivity found could be related to the homology among their cysteine proteases. Blo t 1 had a 35% identity with other mite's cysteine proteases (Der p 1, Der f 1, Eur m1) with a 50% degree of divergence in the primary amino acid sequence (12). Five out of seven cysteine residues present in the mature protein of Der p 1, Der f 1 and Eur m 1 were conserved in Blo t 1. Also, sequence analyses indicate that in mite cysteine proteases the regions involved in secondary structure and enzymatic activity are highly conserved (33) and they share similar three-dimensional structures (12).

The role of the recognized antigens as allergen was not determined in this investigation, but rBlo t 1 has a 60% IgE-binding capacity that suggested its possible role as a major allergen of Bt (12), and, due to the demonstrated homology of these antigens with rBlo t 1, we can suppose that they are important allergens from Bt; nevertheless, additional studies are needed to confirm it. Also, studies must be done to further characterize the antigens found and to analyze the specificity against other mite allergens already isolated.

The mAbs produced represent a new tool to recognize the presence of antigens from Bt mites, to determine the antigenic components of Bt extracts and their relationship with other mites. The strong homology among the protein components recognized by the mAbs and Blo t 1 appointed to the possible use of these mAbs to purify this relevant protein from Bt extracts. They could be used to future studies on the allergenicity of Bt that could stand for the development of improved diagnostic and therapeutic strategies with allergen extracts that help in the management of atopic diseases.

Resumen

Los alergenos del ácaro de polvo doméstico *Blomia tropicalis* (Bt) representan un importante factor de riesgo para el desarrollo de enfermedades alérgicas y asma en zonas tropicales y subtropicales. Los anticuerpos monoclonales (mAbs) son reactivos útiles para estudiar estos alérgenos y delinear la reactividad cruzada entre Bt y otras especies de ácaros. El objetivo de este trabajo fue

producir y caracterizar mAbs contra extracto total de Bt. Para este fin, se inmunizaron ratones y se obtuvieron mAbs por técnicas standard. Se produjeron fluidos ascíticos ricos en mAbs, que fueron purificados por cromatografía de adsorción. Se probó la reactividad de los mAbs contra extractos de Bt y *Dermatophagoides pteronyssinus (*Dp), y la proteasa de cisteína putativa rBlo t 1 por ELISA, inmunoblots e inhibición de ELISA. Se obtuvieron y purificaron parcialmente tres mAbs de isotipo IgG que reaccionaron con extractos de Bt y Dp por ELISA. En inmunoblots reconocieron dos componentes proteicos de pesos moleculares 29 y 35 KD tanto en el extracto de Bt como en el de Dp. En ELISA competitivo, el extracto de Dp redujo en un 62% la reactividad con antígenos de Bt, y el extracto de Bt produjo una reducción máxima de reactividad contra extracto de Dp de 83%. Los mAbs también reconocieron a r Blo t 1. La homología entre Blo t 1 y las proteínas reconocidas por los mAbs es alta (90%) según demostraron los ensayos de inhibición.

La reactividad cruzada entre Bt y Dp puede estar relacionada con la presencia de proteínas tales como sus respectivas proteasas de cisteína. Los mAbs obtenidos han facilitado la identificación de antígenos de Bt y determinar su posible reactividad cruzada con otros organismos comunes en la ácarofauna de regiones tropicales y subtropicales donde Bt se encuentra comúnmente.

Acknowledgments

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