

Short communication

Quantification of Blo t 5 in mite and dust extracts by two-site ELISA

Background: *Blomia tropicalis* is an important mite species in the tropical and sub-tropical regions of the world. Blo t 5 is the major allergen with up to 70% sensitization rates in *B. tropicalis* allergic populations.

Methods: Mice were immunized intramuscularly with naked plasmid DNA encoding Blo t 5 gene with *in vivo* electroporation. Blo t 5 monoclonal antibodies were generated using methylcellulose-based hybridoma kit. Monoclonal antibody (mAb) 4A7 was characterized by two-dimensional electrophoresis immunoblotting. A specific quantitative two-site enzyme-linked immunosorbent assay (ELISA) was developed with mAb 4A7 and guinea pigs Blo t 5 polyclonal antibody as capture and detection antibodies, respectively. This system was tested with Blo t 5 in crude extracts and dust samples.

Results: A high-affinity mAb 4A7 recognizing several isoforms of Blo t 5 has been generated. Monoclonal antibody 4A7 is useful for immunoblotting and two-site ELISA. The two-site ELISA developed has a high sensitivity, with a detection limit of 10 pg/ml. The assay is species-specific and recognized the same epitopes on both native and recombinant Blo t 5. The assay developed is able to detect Blo t 5 in commercial diagnostic and therapeutic *B. tropicalis* extract. Blo t 5 quantification in dust samples showed that Blo t 5 is present in a high quantity in Singapore dust.

Conclusions: A highly sensitive and specific two-site ELISA has been developed. The assay system developed is useful for the quantification of Blo t 5 in mite and environmental dust extracts.

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House dust mites are a major risk factor for allergic diseases worldwide (1). Antigens generated by house dust mites elicit allergic inflammation from individuals who are genetically predisposed to produce large amount of allergen specific immunoglobulin (Ig)E.

Epidemiological studies clearly indicated that *Dermatophagoides pteronyssinus* (Dp) from the Pyroglyphidae is the most common mite species worldwide, while *Blomia tropicalis* (Bt) from the Glycyphagidae (2) is an important mite species in the tropical and sub-tropical regions of the world (3, 4). Among Bt allergens cloned and characterized to date, 14-kDa Blo t 5 is a major allergen with reactivity in at least 70% of allergic subjects (5, 6) and is reported to be species-specific (6, 7).

Mite extracts are commonly used for diagnosis and treatment of mite allergy. Thus, accurate standardization of allergen extract is essential to guarantee the quality of extracts. These extracts contain complex mixtures of proteins of which only a few have allergenic activity. The trend in allergen standardization is moving toward the

measurement of major allergens (8), but the evaluation of the total allergenic potency of an extract should rely on the quantification of most representative allergens (9).

Monoclonal antibodies (mAbs) have become very much a part of biology and medicine that a catalog of their applications seems superfluous. Their uses include the quantification of environmental allergens (9), allergens purification (10, 11), epitopes mapping of allergens (12), immunocytochemistry (13) and crude extract standardization. Allergen-specific mAbs have some advantages as they show specificity for a unique epitope and can be produced in unlimited amount *in vitro*. Two-site enzyme-linked immunosorbent assay (ELISA) for groups 1 and 2 dust mite allergens (9, 11) have been widely used for environmental allergens quantification, and the monoclonal antibodies are available commercially. However, development of more sensitive assays is essential to enable the quantification of the minute amount of specific allergen present in the environment and crude extract. In this study, we report the development

of a sensitive Blo t 5 two-site ELISA that has high specificity and its use in the standardization of Bt extracts for diagnosis and therapeutic, and quantification of environmental Blo t 5.

Methods

Generation of Blo t 5 mAb

Eight-week old female Balb/cJ mice were immunized intra-muscularly with electroporation with pcDNA3-Blo t 5 plasmid on three occasions with 10 days interval as previously described (14). The hybridomas were produced by the fusion of myeloma cells (X63-Ag 8.653) and splenocytes from Blo t 5 immunized mice using the ClonaCell-HY system (StemCell Technologies Inc., Vancouver, Canada), according to manufacturer instructions. Hybridoma clones were screened with both Blo t 5 and whole mite extract using ELISA. Ascites fluid was produced for the highly Blo t 5 specific clone. Immunoglobulins were enriched by protein G affinity chromatography. Animal experiments were performed according to Institutional Guidelines for Animal Care and Handling, National University of Singapore.

Production of anti-Blo t 5 serum

Mouse and guinea pig anti-Blo t 5 polyclonal sera were raised using recombinant Blo t 5 with complete Freund adjuvant (CFA; Life Technologies, Grand Island, NE), and boosted on days 10 and 20 with incomplete Freund adjuvant (IFA; Life Technologies). Guinea pig immunoglobulins were enriched by Protein A and biotin hydroxysuccinimide ester (Sigma, St Louis, MO) was used for biotinylation, as described by the manufacturer.

Two-dimensional electrophoresis and immunoblotting

The Bt extract was prepared in a cocktail of 9 M urea (Fluka, Milwaukee, WI), 4% 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (Sigma), 40 mM Tris (JT Baker, Phillipsburg, NJ), and 1 mM phenylmethylsulfonyl fluoride (Sigma). Two-dimensional electrophoresis, silver-stained and immunoblotting were conducted according to protocol described previously (14, 15).

Preparation of *B. tropicalis* and dust extracts

The diagnostic and therapeutic Bt extracts were kindly provided by Strallergenes (Antony, France). Dust was collected from the floor of 10 urban homes. Fine dust was obtained by separating the large particles with a domestic sieve. Every 50 mg of dust was extracted with 1 ml of PBS-T with rotating at 4°C overnight.

Purification of native Blo t 5

Immuno-affinity purification column was produced as described previously (14) with Blo t 5 mAb (4A7). Bt extract were prepared from cultured mites (16) and loaded to the column. The column was then washed thoroughly with TBS, pH7.5. Native Blo t 5 was eluted with acidic buffer 0.1 M glycine, 0.15 M NaCl, pH2.6 and neutralized with Tris-HCl, pH8.0. The purified native Blo t 5 was dialyzed against deionized water and kept at -80°C until further analysis.

Two-site ELISA

Microtiter plates were coated overnight at 4°C with 100 ng/well of purified mAb in 0.1 M sodium bicarbonate buffer, pH 8.3. After blocking, 50 µl of twofold dilutions of recombinant Blo t 5 was added to the wells as duplicate and incubated at 4°C, overnight. Biotinylated guinea pig's serum was used as detection antibodies (250 ng/ml), followed by incubating with ExtrAvidin alkaline phosphatase (1 : 2000; Sigma). Signal was developed by addition of p-nitro-phenylphosphate (Sigma).

Results

Recognition of Blo t 5 isoforms by mAb 4A7

A high titer Blo t 5 mAb 4A7 of IgG1 isotype was obtained. The reactivity of mAb 4A7 to Blo t 5 isoforms was shown in Fig. 1. Results revealed that mAb recognized all the full-length isoforms of Blo t 5, ranging from isoelectric point 4.5–5.5. However, the mAb was unable to bind degraded (Fig. 1C) Blo t 5 as compared with polyclonal antibody (Fig. 1B).

Development of two-site ELISA for Blo t 5

A Blo t 5 two-site ELISA was developed using mAb and polyclonal antibody, giving rise to a more sensitive two-site ELISA than commercially available ELISA kit (Fig. 2A). The detection limit of this assay was determined to be approximately 10 pg/ml by doubling dilution of recombinant Blo t 5 (Fig. 2A). Figure 2B showed that the two-site ELISA developed poses high specificity to

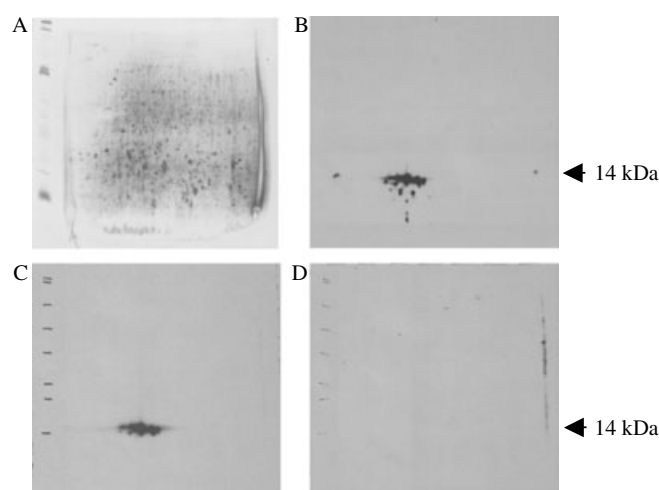


Figure 1. Two dimensional electrophoresis and immunoblotting of *Blomia tropicalis* extract. Fifty micrograms of mite extracts were focused on 13 cm, pH 4–7 linear IPG strips and separated on 15% polyarylamide gel. (A) Two-dimensional protein profile of mite extract. Immunoblots of mite extract incubate with mouse anti-Blo t 5 polyclonal serum (B), Blo t 5 specific monoclonal antibody (C), and naïve mouse serum (D).

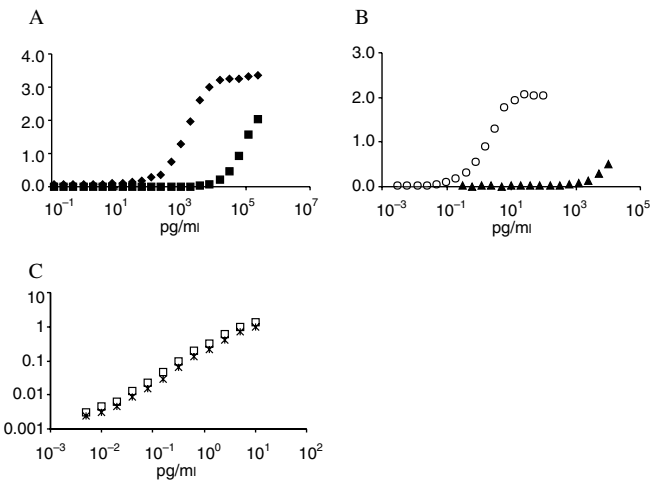


Figure 2. Development of two-site enzyme-linked immunosorbent assay (ELISA) using Blo t 5 mAb and Blo t 5 specific guinea pig immunoglobulins as capture and detection antibody, respectively. The Blo t 5 two-site ELISA developed has high sensitivity and specificity. (A) Comparison of rBlo t 5 standard curve by two-site ELISA developed (◆), commercial available ELISA kit (■). (B) Comparison of rBlo t 5 (○) to rDer p 5 (▲) as standard curve for the developed two-site ELISA. (C) Comparison of nBlo t 5 (*) and rBlo t 5 (□).

Blo t 5 but not Der p 5. Native and recombinant Blo t 5 showed superimposed curve in two-site ELISA (Fig. 2C), indicating that the recombinant protein has comparable structure as native protein. Therefore, the possibility of underestimation by using recombinant Blo t 5 as a standard for the assay has been ruled out.

Blo t 5 in commercial *B. tropicalis* extracts

Comparison of Blo t 5 two-site ELISA developed and commercial ELISA kit (Table 1) showed that the commercial kit was not able to detect any Blo t 5 in all of the commercially available extracts. While the two-site ELISA developed described in this study was able to detect Blo t 5 even in the lowest concentration (1IR) of the extracts.

Table 1. Comparison of two detection assays (ELISA) for Blo t 5 in the evaluation of commercially available extracts

Concentration	Two-site ELISA (pg/ml)	
	4A7/GP	Indoor Biotechnologies
100 IR*	6453.9	nd
1 IR†	16.62	nd
100 IR†	114.00	nd
300 IR†	1315.73	nd

* Diagnostic extract for skin prick test.
† Therapeutic extract for sublingual administration.
ELISA, enzyme-linked immunosorbent assay; nd, not detected; IR, index of reactivity.

Table 2. Quantification of environmental Blo t 5 in 10 Singapore house dust samples

Dust samples	Blo t 5 (ng/g of dust)
D1	450.52
D2	1385.89
D3	9070.44
D4	4489.75
D5	6154.59
D6	421.80
D7	762.91
D8	3905.84
D9	1298.08
D10	1022.83

Quantification of Blo t 5 in dust samples

Quantification of allergens in the environment is one of the main applications for two-site ELISA. Results revealed that Blo t 5 presents in high quantity in Singapore dust with 0.4 µg (lowest) to 9 µg (highest) per gram of dust (Table 2).

Discussion

One of the drawbacks of the mAbs is that they are too specific and recognize only certain isoforms. It has been reported that isoforms are recognized differently by Der p 2- and Bet v 1-specific mAbs (17, 18). Studies have shown that antibodies derived from DNA-immunized animals are able to recognize the recombinant and the native forms of the protein encoded by the immunized DNA (14). In this study, DNA immunization via intramuscular administration was used to generate the mAbs with the attempt to obtain mAbs that recognize a full spectrum of native epitopes of Blo t 5 as a better conformation or protein presentation can be achieved with *in vivo* expression of antigen. Two-dimensional immunoblotting of Bt extract showed that Blo t 5 consists of several isoforms that have different isoelectric point (Fig. 1). The 4A7 mAb is able to bind all the isoforms of Blo t 5 in full length, but not the degraded Blo t 5, suggesting that the 4A7 mAb is recognizing a conformational epitopes.

Two-site immunoassays present several advantages over direct or inhibition ELISA procedures as nonrelevant proteins do not interfere with target detection and the antigens are detected as native proteins in the liquid phase. However, the possible occurrence of conformational changes produced by mAb binding has been reported for the Bet v 1 specific mAb Bip1 (19). The use of a specific mAb as capture antibody in this study avoids over estimation of allergens by reducing the cross-reactive epitopes recognition, while use of specific polyclonal serum would avoid the loss of detection produced by conformational changes affecting one or more epitopes. Hence, a specific anti-Blo t 5 polyclonal serum has

been used as the secondary antibody in our assay, unlike most of the other immunoassays described (9, 11).

The main criteria of a good immunoassay are high sensitivity and specificity. A comparison of ELISA sensitivity to Blo t 5 allowed us to conclude that the two-site ELISA using 4A7 and guinea pig polyclonal serum as capture and detection antibodies, respectively, resulting in a more sensitive assay (Fig. 2A). This was further confirmed with the results shown in Table 1. Furthermore, the quantification of major allergens has become an important goal for the standardization of allergen products intended for clinical use. Therefore, a more sensitive two-site ELISA assay is essential. Group 5 allergens of these mites shared 77% of sequence homology, with similar predicted structure (20). Therefore, high specificity of assay system for Blo t 5 is important because of the widely used of two-site ELISA in environmental allergens quantifications. High species-specificity of 4A7 produced in this study was validated by two-site ELISA, where the concentration of natural form rDer p 5 was detected as 10^4 times less than the actual concentration (Fig. 2B). These phenomena confirmed the species specificity of the system developed.

It has been proposed that exposure at levels of more than 2 μg of Der p1 per gram of dust is a risk factor for sensitization in mite-allergic subjects, while the comparable level of nonatopic children was approximately 50 $\mu\text{g/g}$ dust (21). It is worth noting that the level of Blo t 5 present in Singapore dust samples (Table 2) has reached this proposed sensitization risk. The high reactivity of allergic population to Blo t 5 ($> 70\%$) might be due to the abundance of the allergen in the environment.

In conclusion, we have developed a highly sensitive and species-specific Blo t 5 two-site ELISA assay. The assay system described here could be very useful for the quantification of environmental Blo t 5 and the standardization of extracts for clinical use.

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