

Production of monoclonal antibodies for immunoaffinity purification and quantitation of Blo t 1 allergen in mite and dust extracts

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Summary

Background Blo t 1 is a cysteine protease-like allergen from *Blomia tropicalis*. Recombinant Blo t 1 binds up to 90% of IgE from allergic patients and shows limited cross-reactivity to Der p 1. The generation of monoclonal antibodies (mAbs) against Blo t 1 is important for the detection, isolation and characterization of the native form of the allergen.

Methods Mice were immunized intramuscularly with naked plasmid DNA encoding Blo t 1 gene with *in vivo* electroporation and boosted intraperitoneally with recombinant Blo t 1. mAbs against Blo t 1 were generated using a methylcellulose-based hybridoma cloning kit. The native Blo t 1 was isolated by mAb affinity purification and its allergenicity was determined by ELISA. A two-site ELISA for Blo t 1 was developed using the mAbs generated.

Results A DNA-based immunization protocol induced high titre Blo t 1-specific antibodies in mice. Six stable hybridoma clones secreting mAbs recognizing the native and recombinant Blo t 1 were generated. The native Blo t 1 was affinity-purified from a *B. tropicalis* extract and its allergenicity was determined at 63% using a panel of Singaporean and Malaysian mite allergic patients' sera. A two-site ELISA was developed, which showed a detection limit of 10 ng/mL of Blo t 1.

Conclusion Six Blo t 1 mAbs were successfully generated by DNA immunization. These mAbs are useful for nBlo t 1 immunoaffinity isolation and quantitative immunoassays for Blo t 1 in mite and environmental dust extracts.

Keywords Blo t 1, *Blomia tropicalis*, cysteine protease, DNA immunization, house dust mite, monoclonal antibody, sandwich ELISA

Submitted 8 July 2003; revised 28 November 2003; accepted 19 December 2003

Introduction

House dust mites (HDM) are the most important sources of indoor allergens responsible for the development of sensitization and allergic diseases [1, 2]. Allergens from HDM species *Dermatophagoides pteronyssinus* (Dp) and *Blomia tropicalis* (Bt) are important triggering factors of allergic asthma, allergic rhinitis and atopic dermatitis in tropical and subtropical regions of the world [3–6]. Despite the fact that a large panel of allergens have been identified and characterized in Dp [7], only few Bt allergens have been cloned and their allergenicity established [8–14].

We previously reported a 1104 base pair cDNA gene from Bt with an open reading frame coding for a 330 amino acid protein homologous to cysteine proteases and identified as Blo t 1 [15]. Recombinant Blo t 1 binds up to 90% of IgE from allergic patients [13, 15] and shows limited cross-reactivity to Der p 1 [15], a major allergen from Dp. A three-dimensional image modeling analysis of Blo t 1 revealed the

presence of conserved secondary and tertiary structures with Der p 1 indicating a potential similarity in terms of allergenicity and biological activity between the two allergens [15].

Monoclonal antibodies (mAbs) are indispensable reagents in the identification, quantitation and purification of proteins; identifying antigenic sites; and in assessing the antigenic similarity between recombinant and native proteins. In our laboratory, various mAbs had been extensively used in HDM allergy for the development of immunoassays such as sandwich ELISA to monitor and determine environmental allergens; quantitation and standardization of allergen extracts; epitope mapping of allergens; comparison of IgE-binding determinants; immunolocalization of allergens; assessment of cross-reactivity; and in the isolation of native allergens.

The isolation and characterization of the native forms of the HDM allergens are important steps in determining their actual sensitization profiles and their inherent allergenic properties. While the utilization of recombinant allergens are potentially beneficial as alternative reagents for diagnostic and therapeutic purposes, their use as the sole basis in determining the sensitization profile of an allergen might lead to underestimation of the percentage of allergenicity. Recombinant allergens with improper protein folding, under-

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or over-glycosylation and the lack of other post-translational modifications could lead a potential alteration or masking effect of IgE epitopes thereby affecting the allergen-antibody recognition. It has been shown that the native Blo t 11 registered higher percentage of IgE reactivity compared with its recombinant form [16]. Likewise, recombinant Der p 1 expressed in *Escherichia coli* showed a reduction of IgE-binding activity compared with the natural allergen [17].

Sandwich ELISAs for HDM allergens are important to facilitate the quantitation of crude extracts used for diagnosis and therapy of mite allergy. In addition, the assay could be used to monitor environmental mite allergen concentrations; thus it is useful for allergen avoidance and HDM control measures. The development of sandwich ELISA using recombinant allergens as standard for quantitating allergen concentrations is in line with recommendations of the WHO/IUIS Allergen Standardization Committee [18].

This study reports the generation of mAbs against Blo t 1 by DNA immunization; immunoaffinity purification of the native Blo t 1 (nBlo t 1) and determination of its allergenicity; and development of a two-site ELISA for Blo t 1 detection and quantitation.

Materials and methods

DNA construct

The open reading frame coding for Blo t 1 was amplified by PCR and ligated in-frame with the Igk leader sequence of the pSecTag-A mammalian expression vector (Invitrogen, Carlsbad, CA, USA). The construct was transformed into *E. coli* DH5 α (Gibco BRL, Rockville, MD, USA) and plasmids were isolated and purified using QIAGEN Plasmid Giga Kit (Qiagen, Hilden, Germany). Plasmids were sequenced using the ABI PrismTM 377 Automated DNA Sequencer (PE Applied Biosystems, Foster City, CA, USA).

Animals

Five 6-week old female BALB/c mice used for DNA immunization and 30 8-week old female BALB/c mice used for the ascites production were purchased from the Animal Holding Unit of the National University Singapore. All mice were housed under conventional conditions and animal experiments were performed according to Institutional Guidelines for Animal Care and Handling, National University of Singapore, Singapore.

Human sera

A panel of 85 Singaporean and 103 Malaysian Bt extract-positive sera from asthmatic and/or atopic dermatitis patients was used for the IgE ELISA. Ten IgE-negative sera to recombinant and native Blo t 1 but IgE-positive to other Bt allergens and 32 cord blood sera were used as negative controls. All sera were stored at -80°C until use.

Preparation of recombinant Blo t 1

Recombinant Blo t 1 was expressed in *E. coli* BL21 (Promega, Madison, WI, USA) and *Pichia pastoris* GS115 (Invitrogen,

San Diego, CA, USA) expression systems as previously described [15]. In brief, PCR-amplified Blo t 1 was cloned into pGEX-4T-1 (Amersham Biosciences, Buckinghamshire, UK) for *E. coli* expression or into pPIC9 (Invitrogen, San Diego, CA, USA) for *P. pastoris* expression. Recombinant glutathione S-transferase (GST)-Blo t 1 was expressed by isopropyl- β -D-thiogalactopyranoside, IPTG (BioRad, Hercules, CA, USA) induction for 3 h at 37°C and purified by affinity chromatography using the glutathione-agarose beads (Sigma-Aldrich, Saint Louis, MO, USA). Recombinant yeast Blo t 1 (yBlo t 1) was expressed as a secretory product by methanol induction for 2 days at 30°C and partially purified by dialysis against phosphate-buffered saline (PBS). Recombinant Blo t 1 samples were analysed by SDS-PAGE and quantitated by DC protein assay (BioRad, Hercules, CA, USA).

Preparation of Bt mite, spent-mite medium and dust extracts

Ten grams (wet weight) of Bt mites were frozen in liquid nitrogen and ground using a pre-cooled mortar and pestle for 2 h. Mechanically ground Bt mites or 10 g of spent-mite medium (SMM) or dust samples were extracted in 50 mL of $\times 1$ Tris-buffered saline (TBS), pH 7.4, with 2 mM phenyl-methylsulphonyl fluoride (Sigma-Aldrich, Saint Louis, MO, USA) and 1 mM EDTA (BioRad, Hercules, CA, USA) for 16–20 h at 4°C with constant shaking. Supernatants were separated by centrifugation at $17\,000g$ at 4°C for 30 min. Dust extract was concentrated using the 5 kD CO Millipore protein concentrator (Millipore, Bedford, MA, USA). Crude extracts were analysed by SDS-PAGE and quantitated by DC protein assay (BioRad, Hercules, CA, USA).

Immunization

Mice were immunized intramuscularly with $50\mu\text{g}$ pSecTag-A-Blo t 1 plasmid DNA on the quadriceps muscle of the hindleg followed by electroporation using the ECM 830 apparatus (BTX, Genetronics Inc., San Diego, CA, USA). Electroporation was performed with a 0.5 cm two-needle array inserted 2 mm deep into muscles, which delivered four pulses of 20 ms, 82 V each, at 200 ms interval. Each mouse received two DNA booster doses at days 14 and 28 following the same protocols mentioned above and two protein booster doses at 7 days interval using $50\mu\text{g}$ of yBlo t 1 in 200 mg alum, Amphojel antacid suspension (Whitehall Laboratories, Punchbowl, Australia) suspended in $200\mu\text{L}$ PBS administered intraperitoneally. Blood was extracted infra-orbitally every week to monitor the titre of Blo t 1-specific antibody production by ELISA.

mAb production

Cell fusion and cloning of hybridomas was performed using the ClonaCellTM-HY Hybridoma Cloning Kit (StemCell Technologies Inc., Vancouver, Canada). In brief, 2×10^7 viable myeloma cells, a subclone of P3X63Ag8.653 (ATCC, Manassas, VA, USA), were resuspended in 30 mL of Medium A. Simultaneously, splenocytes from two mice with high titre of Blo t 1 antibody production were separated into single cell suspension by mechanical digestion and were resuspended in Medium B. A total of 1×10^8 viable splenocytes were fused

with the previously prepared myeloma cells using the supplied polyethylene glycol (PEG) solution and Medium B. Hybridomas were incubated overnight at 37 °C in Medium C. Hybridoma clones were plated using methylcellulose-based Medium D and were incubated for 14 days at 37 °C. Individual colonies were picked and cultured in 96-well Falcon[®] tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) containing 200 µL Medium E. Media A, B, C, D and E; and PEG solution were supplied as part of the ClonaCell[™]-HY Hybridoma Cloning Kit. Hybridoma clone supernatants were collected and assayed for Blo t 1-specific antibody by ELISA. Isotyping was performed by ELISA using biotinylated anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, IgE and IgD (Serotec Ltd, Oxford, UK).

Ascites production

Six stable Blo t 1 hybridoma clones were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone, Logan, UT, USA) with 10% fetal calf serum (Hyclone, Logan, UT, USA) at log phase-growth, were harvested, washed $\times 3$ in PBS, and assayed for viability using the Trypan Blue (Sigma-Aldrich, Saint Louis, MO, USA) exclusion assay. Five million viable cells in 1 mL PBS were injected intraperitoneally into each mouse previously administered with 1 mL Pristane, 2, 6, 10, 14-tetra-methylpentadecane (Sigma-Aldrich, Saint Louis, MO, USA). Ascitic fluid was harvested 1–2 weeks after cell inoculation. The antibody titre and specificity were determined by ELISA and Western blot analysis using recombinant Blo t 1 and crude Bt and SMM extracts. Ascites were stored at -80°C until use.

ELISA

ELISA was used to determine the titre of Blo t 1-specific antibody in the culture supernatant or ascitic fluid, and to evaluate the profile of sensitization of a panel of asthmatic and/or atopic dermatitis patients' sera to the isolated native Blo t 1 as described previously [19]. Antigens (5 µg/mL of rBlo t 1 or native Blo t 1 or 100 µg/mL of Bt or SMM extract) were coated onto ELISA in duplicates by incubation for 14–16 h at 4 °C. Plates were blocked with 1% bovine serum albumin (BSA) (Sigma-Aldrich, Saint Louis, MO, USA) in PBS-T (PBS+0.05% Tween 20) for 1 h at 22 °C. The plates were incubated with culture supernatant or serial dilutions of the ascites or human sera; followed by biotinylated anti-mouse Ig (Sigma-Aldrich, Saint Louis, MO, USA), or biotinylated anti-isotype antibodies (Serotec Ltd, Oxford, UK), or biotinylated anti-human IgE (BD Pharmingen, San Diego, CA, USA); and finally with ExtrAvidin-alkaline phosphatase conjugate (Sigma-Aldrich, Saint Louis, MO, USA), for 1 h each at 22 °C. Plates were washed with PBS-T between incubation steps. Colorimetric reaction was performed using *p*-nitrophenyl phosphate, pNPP (Sigma-Aldrich, Saint Louis, MO, USA) substrate solution and absorbance at 405 nm was determined using the Spectra & Rainbow ELISA reader (Tecan, Austria).

Western blot analysis

The specificity of the Blo t 1 mAbs was determined by Western blot analysis as described previously [16]. In brief,

rGST-Blo t 1 (0.4 µg), yBlo t 1 (0.4 µg), Bt extract (20 µg), SMM extract (20 µg) and BSA (0.4 µg) were electrophoresed on a 12% Tris-glycine gel and electroblotted onto Hybond C nitrocellulose membrane (Amersham Life Sciences, Buckinghamshire, UK) using the MiniProtein 3 cell (BioRad, Hercules, CA, USA) at 110 V for 1 h. The membrane was incubated for 1 h at 22 °C each in blocking buffer (5% skimmed milk in PBS-T), $\times 2000$ dilution of Blo t 1 mAbs (ascites), and $\times 10\,000$ dilution of HRP-conjugated anti-mouse Ig (Amersham Life Science, Piscataway, NJ, USA). The membrane was finally incubated in SuperSignal[®] WestPico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) for 5 min at 22 °C. The membrane was washed $\times 6$ with PBS-T between incubation steps. Results were detected by autoradiography using the Kodak Biomax Light ML film (Eastman Kodak Co., Rochester, NY, USA).

Isolation of native Blo t 1

The Blo t 1 mAb 11F1 was purified from the pooled ascetic fluid by affinity chromatography using protein G-agarose column (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA), analysed under denaturing conditions by SDS-PAGE, dialysed against 0.1 M NaHCO₃, 0.5 M NaCl (pH 8.3), and quantitated using the DC protein assay (BioRad, Hercules, CA, USA). Purified 11F1 mAbs were coupled to cyanogen bromide-activated Sepharose beads (Pharmacia Biotech, Buckinghamshire, UK) and packed into a 10 mL polypropylene column (Pierce, Rockford, IL, USA) using $\times 1$ TBS (pH 7.5). After thorough washing with $\times 1$ TBS until the OD at 280 nm was zero, 500 mg of crude Bt protein extract was gently passed through the column followed by another wash as described. Bound nBlo t 1 was eluted in 1 mL fractions using 10 mL of 5 mM glycine, pH 2.6. Three hundred µL of each fraction were precipitated using trichloroacetic acid, TCA (Merck, Darmstadt, Germany) and analysed by denaturing SDS-PAGE followed by silver staining and Western blot analysis.

Sandwich ELISA

Protein G (11F1, 16H11 and 5G10) or (2B8, 14A12 and 1D9) A-purified Blo t 1 mAbs (5 µg/mL) were coated for 14–16 h at 4 °C on ELISA plates as capture antibodies. After blocking with 1% BSA (Sigma-Aldrich, Saint Louis, MO, USA) in PBS-T for 1 h at 22 °C, test antigens (recombinant yBlo t 1 or Bt extract) were added in duplicates into the ELISA plates and incubated for 14–16 h at 4 °C. ELISA plates were incubated for 1 h each at 22 °C with different dilutions of three Blo t 1 mAbs conjugated with biotinamidocaproate N-hydroxysuccinimide ester, C₂₀H₃₀N₄O₆S (Sigma-Aldrich, Saint Louis, MO, USA) followed by incubation with extravidin-alkaline phosphatase conjugate (Sigma-Aldrich, Saint Louis, MO, USA) for 1 h. ELISA plates were washed with PBS-T between incubation steps. Colorimetric reaction was performed using *p*-nitrophenyl phosphate (Sigma-Aldrich, Saint Louis, MO, USA) and absorbance at 405 nm was determined using the Spectra & Rainbow ELISA reader (Tecan, Austria). The detection range of antibody pairs was determined by sandwich ELISA as described above using a

serial dilution (as shown in Fig. 4) of the recombinant yBlo t 1 as test antigens. The amount of nBlo t 1 present in the crude Bt mite, SMM, Stallergenes Bt (Stallergenes, Antony, Cedex, France) and dust sample extracts, was determined using the developed sandwich ELISA.

Results

Antibody responses of immunized mice

High titres Blo t 1-specific antibodies were detected among the five immunized mice following the intramuscular immunization of the pSecTag-A/Blo t 1 naked DNA with electroporation followed by protein boosts with recombinant yBlo t 1 (data not shown). Polyclonal antibodies isolated from the five immunized-mice reacted strongly to the recombinant Blo t 1 expressed in *E. coli* and *P. pastoris*, as well as the native Blo t 1 present in the crude Bt extract as determined by ELISA. Analyses of the isotypes present in the anti-Blo t 1 polyclonal antibodies revealed that the majority of the antibodies were of IgG and IgM isotypes (data not shown). Two of the highest responder mice were euthanized after the second protein boost and splenocytes were isolated for fusion.

Blo t 1 mAbs

Fusion of splenocytes from Blo t 1-immunized mice with the parental myeloma cells resulted in a total of 1632 hybridoma clones picked from the methylcellulose-based Medium D after 14 days of incubation. Primary screening of hybridoma clones for the secretion of antibodies specific to the recombinant and/or native Blo t 1 by ELISA resulted in the identification of 237 total Ig-positive hybridoma clones which was narrowed down further to 45 hybridoma clones after secondary screening. Isotyping of these clones by ELISA identified 16 IgG1-, 9 IgG2a-, 3 IgG2b-, 2 IgG3-, 14 IgM- and 1 IgA-secreting clones. The isotyping results of the antibodies secreted by the hybridoma clones are in accordance with the initial isotyping performed with the polyclonal antibody extracted from the immunized mice. Further screening of the Blo t 1 hybridoma clones identified six stable antibody-producing clones which were used for ascites production.

The reactivity of Blo t 1 mAbs present in the ascetic fluid extracted from inoculated mice using the six stable clones and

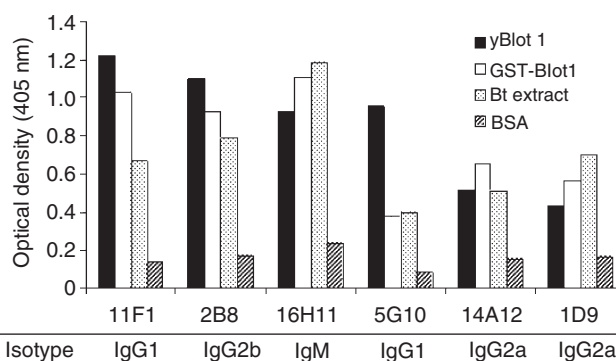


Fig. 1. Total Ig titres of the six Blo t 1 monoclonal antibodies and their corresponding isotypes as determined by ELISA. Recombinant yBlo t 1 (5 µg/mL), GST-Blo t 1 (5 µg/mL), Bt extract (100 µg/mL) and bovine albumin serum (5 µg/mL) were coated as antigens and reacted with $\times 1000$ dilution of ascites. Plotted values represent the optical density at 405 nm after 15 min incubation with pNPP substrate solution.

their corresponding isotypes/subclasses are summarized in Fig. 1. All six Blo t 1 mAbs reacted consistently with the recombinant Blo t 1 expressed in *P. pastoris* and *E. coli* as well as the native form present in the crude Bt extract. The observed differences in the reactivity of the six Blo t 1 mAbs to the different forms of the antigens indicate a potential difference in the epitopes recognized by the six mAbs (Fig. 1).

The reactivity of the Blo t 1 mAbs to the recombinant and native forms of the allergen was confirmed by Western blot analysis (Fig. 2). Blo t 1 mAbs 11F1 (IgG1) and 2B8 (IgG2b) reacted strongly to the recombinant Blo t 1-GST expressed in *E. coli* (lane 2, Figs 2a and b); and to the 40 kD recombinant Blo t 1 expressed in *P. pastoris* (lane 3, Figs 2a and b). Both mAbs likewise recognized a protein of approximately 26 kD present in both the crude Bt and the SMM extracts (lanes 4 and 5, Figs 2a and b). These bands are in good accordance with the predicted molecular weight of Blo t 1 deduced from its cDNA sequence [13]. The Blo t 1 mAbs reacted to a 50 kD band on the GST-Blo t 1 which could represent either a potential degraded product of the 60 kD intact fusion protein or a possible result of altered disulfide bridges. Both Blo t 1 mAbs showed no reactivity to BSA.

Native Blo t 1 and its allergenicity

Blo t 1 mAbs 11F1 (Fig. 2a) and 2B8 (Fig. 2b) were assessed for their suitability for the purification of the nBlo t 1 from

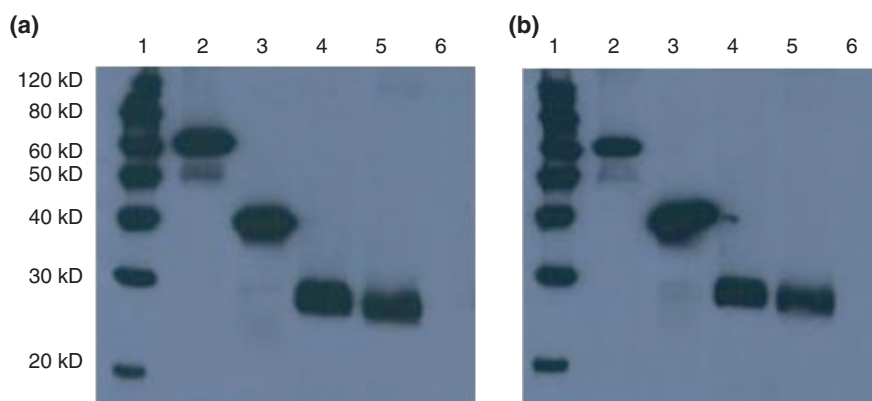


Fig. 2. Western blot analysis of recombinant GST-Blo t 1 (2), yBlo t 1 (3), Blo t extract (4), spent-mite medium extract (5), and bovine albumin serum (6) using $\times 2000$ dilution of Blo t 1 monoclonal antibodies 11F1 (a) and 2B8 (b). Recombinant antigens (0.4 µg) and crude extracts (20 µg) were separated on 12 % Tris-glycine gel followed by immunoblotting on nitrocellulose membrane.

Table 1. Summary of the human IgE ELISA using the immuno-affinity purified nBlo t 1 in comparison with the recombinant yBlo t 1 and GST-Blo t 1

Sera	Antigens	Positive	Negative	Total	Percentage of reactivity (%)
Singaporean sera	nBlo t 1	44	26	70	63
	yBlo t 1	40	23	63	63
	Blo t 1-GST	23	62	85	27
	Bt extract	85	00	85	100
Malaysian sera	nBlo t 1	44	26	70	63
	yBlo t 1	72	31	103	70
	Blo t 1-GST	30	73	103	30
	Bt extract	103	00	103	100

A positive reaction is indicated by an IgE concentration above the cut-off values of 7.3, 3.3, 8.1 and 0.3 ng/mL for nBlo t 1, yBlo t 1, GST-Blo t 1 and Bt extract, respectively (computed as mean + 2SD of the negative controls).

the crude Bt extract by immunoaffinity purification. Both mAbs coupled to CNBr-activated beads bind the ~ 26 kD nBlo t 1, but the antigen was successfully eluted out only in the 11F1 column using 5 mM glycine, pH 2.6. An average of 50 µg of nBlo t 1 can be isolated from 1 g of Bt mites (wet weight). The isolated ~ 26 kD nBlo t 1 reacts with mAbs 11F1 and 2B8 in Western blot analyses and ELISA.

The isolated nBlo t 1 was analysed for its ability to bind IgE from Bt allergic patients by ELISA. The percentage of IgE reactivity of the isolated nBlo t 1 was 63% for both the Singaporean (44/70) and Malaysian (44/70) asthmatic and/or atopic dermatitis sera tested (Table 1). A significant number of the nBlo t 1-IgE positive sera registered an IgE concentration of more than 50 ng/mL (data not shown). Comparative analysis of the IgE reactivity of the recombinant and the native Blo t 1 revealed that the yBlo t 1 but not the GST-Blo t 1 exhibits comparable IgE binding with the isolated native allergen (Table 1). The data presented in Table 1 are in agreement with the IgE reactivity of the recombinant Blo t 1 described previously [13, 15].

Table 2. Summary of the results of Blo t 1 sandwich ELISA development. Protein G or A-purified monoclonal antibodies (5 µg/mL) were coated as capture antibodies and reacted with recombinant yBlo t 1 (10 µg/mL) or Blo t extract (100 µg/mL)

Capture antibody	Detection antibody			5G10	11F1	2B8
	5G10	11F1	2B8			
5G10		2.277	2.465		0.955	0.568
11F1	0.057		2.591	0.387		1.420
2B8	0.020	3.527		0.353	2.026	
16H11	0.075	0.079	0.059	0.311	0.452	0.680
14A12	0.006	0.001	0.005	0.171	0.643	0.481
1D9	0.004	0.011	0.006	0.241	0.374	0.457
	Recombinant yBlo t 1			Blo t Extract		

Biotinylated monoclonal antibodies ($\times 1000$ dilution) were used as detection reagents. Tabulated results represent the optical density at 405 nm after 30 min of incubation with pNPP substrate solution. Four antibody pairs (gray scale) were further used for the determination of Blo t 1 standard curves.

Blo t 1 sandwich ELISA

A total of 15 combinations with six different Blo t 1 mAbs were tested by sandwich ELISA using recombinant yBlo t 1 and the native allergen present in the crude Bt extract (Table 2). Results showed that the best mAb pair for Blo t 1 detection was obtained with 2B8 as capture antibody and biotinylated 11F1 as detection antibody. The ability of the pair to detect Blo t 1 is consistently high with the reverse reaction (11F1 as capture antibody and biotinylated 2B8 as detection antibody). Significant detection of recombinant and native Blo t 1 were likewise obtained with 5G10-11F1 and 5G10-2B8 pairs but the reverse reactions showed modest results.

The detection range of the Blo t 1 sandwich ELISA pairs was determined using a serial dilution of the recombinant yBlo t 1 as antigens. Figure 3 shows that the detection limit of the four best Blo t 1 antibody pairs is approximately 10 ng/mL yBlo t 1. The 2B8 and 11F1 antibodies pair was used in the computation of standard curves for the quantitation of nBlo t 1 in the crude Bt extract and SMM extract. The 2B8 and 11F1 antibody pair could detect the presence of approximately 500 and 300 ng of nBlo t 1 per mg total protein from the locally prepared crude Bt and SMM extracts, respectively. Native Blo t 1 was also detected and quantitated in two different concentrations of commercially available Bt extracts (Stallergenes, SA, Antony, France) as well as in dust sample extract (Fig. 4).

Discussion

An exclusive DNA-based immunization protocol [20] or in conjunction with protein boost [21] for the production of mAbs against HDM allergens has been reported by our laboratory previously. Here, we report the production, characterization and applications of Blo t 1 mAbs generated by DNA immunization with electroporation and protein boosts. This study supports our previous claim that DNA immunization of Bt allergen genes is an effective technique for inducing specific antibody response in mice for hybridoma production [20, 21]. In this study, all mice immunized with Blo t 1 DNA with electroporation produced high titre specific antibodies recognizing both the recombinant and native forms of the allergen. We and others have previously shown that monoclonal and polyclonal antibodies derived from DNA-immunized animals recognized the recombinant and the native forms of the protein encoded by the immunized DNA [20–25]. This study likewise supports the claim that electroporation enhances gene delivery *in vivo* resulting in increased expression and elevated immune responses [26, 27]. In the present study, the DNA-immunized mice were boosted with recombinant yBlo t 1 resulting in the further increase of the Blo t 1-specific antibody production (data not shown). Although we have previously shown that an exclusive DNA-based immunization protocol is sufficient to induce a strong immune response in mice useful for fusion experiments [20], a protein boost might be important in order to obtain a wide spectrum of matured, class-switched, and high affinity mAbs [21]. Furthermore, the type of immune response generated by DNA immunization is influenced by a wide array of factors

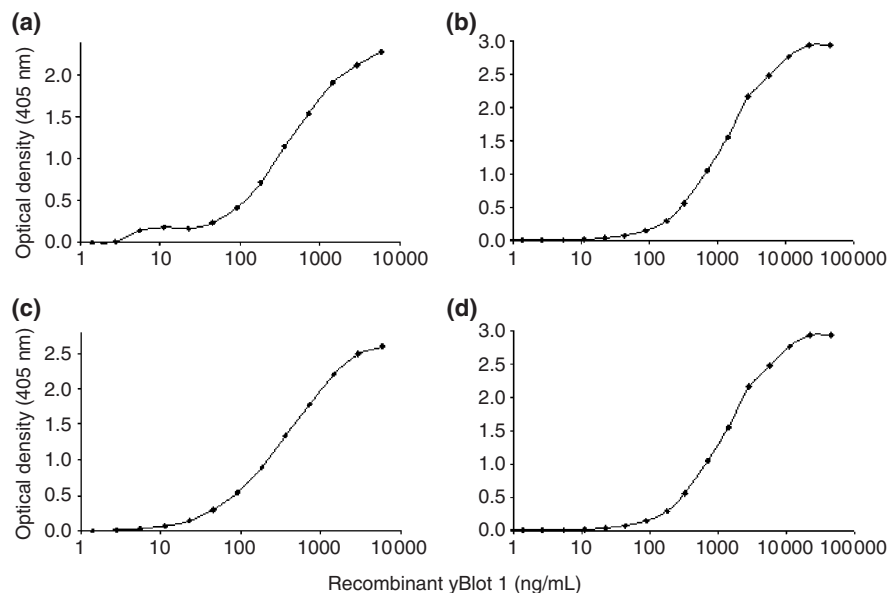


Fig. 3. Standard curves for the mAb-based Blo t 1 sandwich ELISA using the recombinant yBlo t 1. Protein G-purified 5G10 (a and b), 11F1 (c), and 2B8 (d) monoclonal antibodies (5 µg/mL) were coated as capture antibodies and reacted with a serial dilution of recombinant yBlo t 1 followed by $\times 1000$ dilution of biotinylated 11F1 (a and d) and 2B8 (b and c). Plotted results represent the optical density at 405 nm after 30 min of incubation with pNPP substrate solution.

including the nature of the antigen, the expression vector used, and the route of administration [28], thus we used protein boost to complement any potential deficiency generated by DNA immunization to ensure a successful hybridoma experiment.

The results presented in this study show the usefulness of the generated Blo t 1 mAbs for the detection, isolation and quantitation of the native allergen in crude Bt extracts. The Western blot analysis revealed that the native Blo t 1 was detected in both the whole mite body and SMM extracts (Fig. 2), indicating that Blo t 1, like the other group 1 HDM allergens, is a digestive enzyme associated with mite fecal pellets [29]. These results agree with the secondary structure predictions and crystal structure modeling of Blo t 1 being closely related to Der p 1 and other cysteine proteases such as actinidin and papain [13, 15].

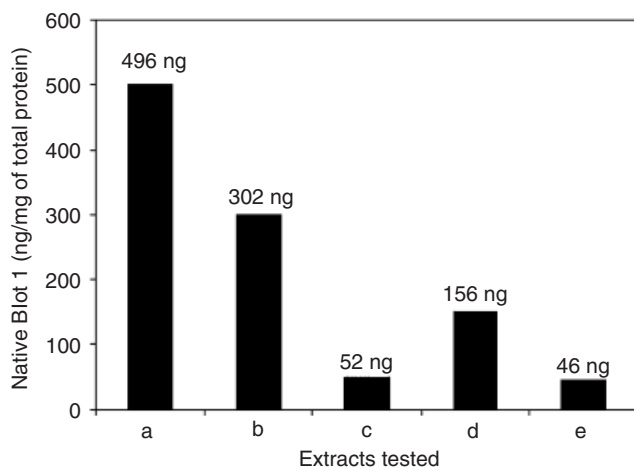


Fig. 4. Summary of the amount of native Blo t 1 present in different crude extract preparations. Native Blo t 1 was detected and quantitated in locally prepared Bt mite (a), spent-mite medium (b), 100 IR Stallergenes Bt (c), 300 IR Stallergenes Bt (d), and dust sample (e) extracts by sandwich ELISA using 2B8 and 11F1 antibody pair.

The isolation of the native Blo t 1 by immunoaffinity purification using the mAb 11F1 demonstrates the usefulness of the generated antibody. The ~ 26 kD native Blo t 1 is highly allergenic as it binds 63% of the sera from asthmatic and/or atopic dermatitis patients tested similar to the results previously presented by Mora et al. [13]. Given the high frequency of IgE reactivity of the native Blo t 1, and the significant number of patients exhibiting high titre of Blo t 1-specific IgE in the sera tested, the purified native Blo t 1 is a potentially important and major allergen in Bt mites. The detection of significant amounts of native Blo t 1 in both the crude whole body mite and SMM extracts further supports the potential allergenicity and its ability to induce sensitization among susceptible individuals. This study likewise showed that the allergenicity of the recombinant yBlo t 1 but not the GST-Blo t 1 is comparable with the native allergen, indicating that the recombinant yBlo t 1 is good potential candidate as a diagnostic and therapeutic reagent for mite allergy. Such results are expected as recombinant proteins expressed in *P. pastoris* exhibit post-translational modifications and better protein folding in contrast to that expressed in *E. coli*, which lacks post-translational modifications. This observation therefore potentially indicates that the IgE epitopes of Blo t 1 might be structure-dependent.

One of the main limitations of the present forms of crude extracts used for diagnosis and immunotherapy of HDM allergy is the unavailability of appropriate detection and quantitation assays to quantitate the exact allergen concentrations. The availability mAbs specific to allergens and development of sandwich ELISA are of utmost importance in this aspect. We demonstrated in this study the detection and quantitation of Blo t 1 in crude extracts by a two-site ELISA using the mAbs generated. The detection limit of this assay is about 10 ng/mL of Blo t 1, which is in the same order of magnitude as most immunoassays developed to measure indoor allergens [18, 30]. Using recombinant yBlo t 1 as standard, the developed sandwich ELISA was successfully

used to quantitate the amount of Blo t 1 in various crude extracts indicating the potential usefulness of this assay as a tool to facilitate the quantitation of commercial mite extracts for the diagnosis and therapy of mite allergy.

Acknowledgement

The authors thank Ms. Daphane Tan, Ms. Kang Chee Fen and Ms. Heng Hui for their technical assistance. This work was funded by grants from the Agency for Science, Technology and Research (A*STAR), Singapore (R-178-000-010-303), and Academic Research Fund, National University of Singapore, Singapore (R-178-000-010-112).

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