

Original article

DNA immunization for the production of monoclonal antibodies to Blo t 11, a paramyosin homolog from *Blomia tropicalis*

Background: Blo t 11 is a high molecular weight allergen from *Blomia tropicalis* with significant immunoglobulin (Ig)E binding frequency. Native and recombinant Blo t 11 are susceptible to degradation and the isolation and expression of the allergen is problematic thus obtaining sufficient amounts of purified Blo t 11 for antibody production is limiting. DNA-based immunization is an attractive alternative strategy that bypasses antigen purification for antibody production. **Objectives:** To use a DNA-based immunization protocol for the production and characterization of Blo t 11 monoclonal antibodies (mAbs).

Methods: The 2625 bp cDNA coding for Blo t 11 was cloned into a mammalian expression vector and immunized intramuscularly with electroporation into mice. Monoclonal antibodies to Blo t 11 were generated using a methylcellulose-based hybridoma cloning kit. These mAbs were utilized for native Blo t 11 isolation and the development of sandwich enzyme-linked immunosorbent assay (ELISA).

Results: Six mAbs recognizing the native and recombinant Blo t 11 were generated and characterized. Native Blo t 11 was affinity purified from Bt extract and its identity was confirmed by matrix assisted laser desorption/ionization – time of flight mass spectrometry. The native Blo t 11 showed IgE reactivity with 67% of mite allergic sera. A two-site ELISA developed showed a detection limit of 100 pg/ml of Blo t 11.

Conclusion: A DNA-based immunization protocol was successfully used to generate Blo t 11 mAbs with a spectrum of distinct epitopes located throughout the whole molecule, and they are useful for immunoaffinity purification and immunoassays.

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Key words: Blo t 11; *Blomia tropicalis*; DNA immunization; house dust mite; monoclonal antibody; paramyosin; sandwich enzyme-linked immunosorbent assay.

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House dust mite (HDM) allergen exposure has been recognized as an important risk factor for the development of sensitization and allergic diseases (1–3). *Blomia tropicalis* (Bt) (4) is a clinically important HDM species in tropical and subtropical regions causing allergic sensitization among susceptible individuals (5–7). Several recombinant Bt allergens with complete cDNA sequences have been cloned and their allergenicity established (8–14).

Blo t 11, a 2625 bp cDNA clone coding for an 875 amino acid protein homologous to paramyosin, was isolated from a Bt cDNA library (11). Recombinant and native Blo t 11 bind up to 59% of IgE from Bt allergic patients tested (15). The full-length (FL) Blo t 11 allergen contains multiple IgE and IgG epitopes scattered throughout the molecule and the major IgE epitopes were located in an immunodominant peptide, fD (16). Native and recombinant Blo t 11 are susceptible to degradation and the isolation and expression of the allergen is problematic (11, 15, 16).

Generation of monoclonal antibodies (mAbs) is important in the identification, quantitation and purification of proteins, identifying antigenic sites, and in assessing the antigenic similarity between recombinant and native proteins. In HDM allergy, mAbs had been extensively used in the development of immunoassays such as sandwich enzyme-linked immunosorbent assay (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 conventional method of antibody production requires significant amount of purified proteins for immunization (17). In the case of low abundance proteins or recombinant proteins that lack post-translational modifications, mAb production may be problematic and challenging. DNA-based immunization is a promising new strategy for the production of mAbs bypassing the

step of antigen purification (18, 19). Intramuscular immunization of naked DNA constructs in mice results in the *in situ* expression of the encoded proteins (18) thereby generating antibody responses in the immunized organism (19, 20). In recent years, the use of electroporation in conjunction with intramuscular immunization has attracted considerable attention as it does not only increase the expression of recombinant proteins encoded by the immunized gene, but also elevates the immune responses of animals against the expressed proteins (21, 22).

In this study, we report the generation of Blo t 11 mAbs by DNA immunization. The Blo t 11 mAbs were characterized and utilized for the identification and isolation of native Blo t 11 (nBlo t 11) and the development of sandwich ELISA.

Materials and methods

DNA Construct

The 2625 bp open reading frame coding for the FL-Blo t 11 was amplified by polymerase chain reaction (PCR) and ligated into pCI mammalian expression vector containing Der p 5 leader sequence (Dp5LS) as described previously (23). The ligated construct was transformed into *Escherichia coli* DH5 α (Gibco BRL, Rockville, MD) and plasmids were isolated and purified using Qiagen Plasmid Giga Kit (Qiagen, Hilden, Germany). Plasmids were sequenced using the ABI PrismTM Automated DNA Sequencer (PE Applied Biosystems, Foster City, CA).

Animals

Five 6-week-old female BALB/cJ 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 30 Bt extract-positive sera from asthmatic and/or atopic dermatitis patients was used for the IgE ELISA. Six IgE-negative sera to Blo t 11 but IgE-positive to other Bt allergens and 20 cord blood sera were used as negative controls. All sera were stored at -80°C until use.

DNA immunization

Mice were immunized intramuscularly with 50 μg pCI-Dp5LS-Blo t 11 plasmid DNA on the quadriceps muscle of the hindleg followed by electroporation using the ECM 830 apparatus (BTX, Genetronics Inc., San Diego, CA). Electroporation was performed with a 0.5 cm 2-needle array inserted 2 mm deep into muscles, that delivered four pulses of 20 ms, 82 V each, at 200-ms interval. Each mouse received two booster doses at days 14 and 28. Blood was extracted infra-orbitally every week to monitor the titer of Blo t 11-specific antibody production by ELISA. Antisera extracted from

each mouse were likewise tested for Blo t 11-specific IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, and IgE titers.

Cell fusion and cloning of hybridomas

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), were resuspended in 30 ml of medium A. Simultaneously, splenocytes from two mice with high titer of Blo t 11 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 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) containing 200 μl medium E. Media A, B, C, D, and E; and PEG solution were supplied as part of the ClonaCellTM-HY Hybridoma Cloning Kit. Hybridoma clone supernatants were collected and assayed for Blo t 11-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 11 hybridoma clones were cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT) with 10% Fetal Calf Serum (Hyclone) at log phase-growth, were harvested, washed $3\times$ in phosphate-buffered saline (PBS), and assayed for viability using the Trypan Blue (Sigma-Aldrich, Saint Louis, MO) 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-Methyl-Pentadecane) (Sigma-Aldrich). Ascitic fluid was harvested 1 to 2 weeks after cell inoculation and was assayed for antibody titer and specificity by ELISA and Western blot analysis using rFL-Blo t 11 and crude Bt extract. Ascites were stored at -80°C until use.

ELISA

Enzyme-linked immunosorbent assay was used to determine the titer of Blo t 11-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 11. Antigens (5 $\mu\text{g}/\text{ml}$ of rFL-Blo t 11 or native Blo t 11 or 100 $\mu\text{g}/\text{ml}$ of Bt 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) 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 IgG (Sigma-Aldrich), or biotinylated anti-idiotypic antibodies (Serotec Ltd.) or biotinylated anti-human IgE (Pharmingen, San Diego, CA); and finally with ExtrAvidin-alkaline phosphatase conjugate (Sigma-Aldrich), for 1 h each at 22°C . Plates were washed with PBS-T between incubation steps. Colorimetric reaction was performed using p-nitrophenyl phosphate (Sigma-Aldrich) and absorbance at 405 nm was determined using the Spectra & Rainbow ELISA reader (Tecan, Salzburg, Austria).

Western blot analysis

The specificity of the Blo t 11 mAbs and the reactivity of the purified nBlo t 11 were analyzed by Western blot. In brief, rFL-Blo t 11 (0.4 µg), Bt extract (20 µg), GST (0.4 µg), or nBlo t 11 (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 MiniProtean 3 cell (BioRad, Hercules, CA) at 110 V for 1 h. The membrane was blocked with 5% skimmed milk in PBS-T. After overnight incubation with Blo t 11 mAbs in blocking buffer at 4°C, the membrane was incubated with biotinylated anti-mouse IgG (Sigma-Aldrich). Prior to detection using the SuperSignal® WestPico Chemiluminescent Substrate (Pierce, Rockford, IL) for 5 min, the membrane was incubated with peroxidase-conjugated ExtrAvidin (Sigma-Aldrich). The membrane was washed 6× with PBS-T between steps. Results were detected by autoradiography using the Kodak Biomax Light ML film (Eastman Kodak Co., Rochester, NY).

Epitope mapping

The location of the epitopes recognized by the six Blo t 11 mAbs were mapped using seven Blo t 11 overlapping peptides (f1–f7) as described previously (16). In brief, the 7 Blo t 11 fragments were amplified by PCR; cloned into pGEX-4T-1 expression vector (Pharmacia Biotech, Buckinghamshire, UK); transformed into *E. coli* BL21 (Promega Corp., Madison, WI); expressed as GST-fusion peptides; and were purified using the Glutathione-agarose column (Sigma-Aldrich). Epitope mapping was performed by ELISA as described above.

Isolation of native Blo t 11

The Blo t 11 mAb P2E7-G1 was purified from the pooled ascitic fluid by affinity chromatography using Protein A-agarose column (Kirkegaard and Perry Laboratories, Gaithersburg, MD), analyzed under denaturing conditions by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), dialyzed against 0.1 M NaHCO₃, 0.5 M NaCl (pH 8.3), and quantitated using the DC Protein Assay (BioRad). Purified P2E7-G1 mAbs were coupled to cyanogen bromide-activated Sepharose beads (Pharmacia Biotech) and packed into a 10 ml polypropylene column (Pierce) using 1× tris buffered saline (TBS) (pH 7.5). After thorough washing with 1× TBS until the OD at 280 nm was 0, 500 mg of crude Bt protein extract was gently passed through the column followed by another wash as described. Bound nBlo t 11 was eluted in 1 ml fractions using 10 ml of 5 mM glycine, 50% ethylene glycol (Merck, Darmstadt, Germany), and pH 11. Six hundred microliters of each fraction were precipitated using trichloroacetic acid (TCA; Merck) and analyzed by denaturing SDS-PAGE followed by silver staining and Western blot analysis.

MALDI-TOF mass spectrometry

Ten microliters of dialyzed nBlo t 11 (0.2 µg/ml) was dried in a Savant Automatic Environmental Speed-Vac AES2010 Centrifugal Concentrator (Holbrook, New York, NY). The dried sample was digested overnight at 37°C with 20 µl of Tryptic Digestion Buffer (0.02 µg/µl trypsin in 25 mM ammonium bicarbonate, pH 8.5) and was passed through a C18 ZipTip (Millipore, Bedford, MA). One microliter of the sample was loaded onto matrix assisted laser desorption/ionization – time of flight (MALDI-TOF) sample plate along with 1 µl of matrix solution [10 mg/ml α -cyano-4-hydroxycinnamic acid, 0.1% trifluoroacetic acid (TFA), 50% acetonitrile].

Mass analyses were carried out using the PerSeptive Biosystems Voyager-DE STR MALDI-TOF mass spectrometer (Framingham, Danvers, MA). Peptide mapping was performed by comparison with the deduced amino acid sequence of the Blo t 11 cDNA.

Sandwich ELISA

Six protein A- or G- (Kirkegaard and Perry Laboratories) purified Blo t 11 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) in PBS-T for 1 h at 22°C, test antigens (recombinant or native Blo t 11) 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 six the Blo t 11 mAbs conjugated with Biotinamidocaproate N-hydroxysuccinimide ester, C₂₀H₃₀N₄O₆S (Sigma-Aldrich) followed incubation with ExtrAvidin-Alkaline Phosphatase conjugate (Sigma-Aldrich) for 1 h. The ELISA plates were washed with PBS-T between incubation steps. Colorimetric reaction was performed using p-nitrophenyl phosphate (Sigma-Aldrich) and absorbance at 405 nm was determined using the Spectra & Rainbow ELISA reader (Tecan). The detection range of antibody pairs was determined by sandwich ELISA as described above using a serial dilution of the recombinant Blo t 11 and fD as test antigens. The amount of nBlo t 11 present in the crude Bt extract was determined using the antibody pair P2E7-G1 (capture antibody) and P2E7-G10 (detection antibody).

Results

Immune responses of DNA immunized mice

Four of five mice immunized intramuscularly with the pCI-Dp5LS-Blo t 11 construct showed significant Blo t 11-specific antibody titer (data not shown). The kinetics of Blo t 11 antibody production of the four responder mice showed a steady increase of antibody production after the first boost. Interestingly, the majority of the antibodies produced by the immunized mice are specific to the Blo t 11 immunodominant peptide fD. The production of fD-specific antibodies started to plateau at day 35, 1 week after the second boost (day 28). Analyses of the isotypes present in the anti-Blo t 11 polyclonal antibodies revealed that the majority of the antibodies are of IgG1 and IgG2a subclasses (data not shown). Blo t 11-specific IgG2b, IgM and IgA antibodies were also detected. Two of the high responder mice were killed on day 40 and splenocytes were isolated for fusion.

Blo t 11 mAbs

A total of 256 hybridoma clones were picked from the methyl cellulose-based medium D after 14 days of incubation. Initial screening of hybridoma clones by ELISA resulted in the identification of 76 total Ig-positive clones specific to recombinant and native Blo t 11. Isotyping of these clones by ELISA identified 10 IgG1-, 1 IgG2a-, 1 IgG3-, 12 IgM- and 5 IgA-secreting clones. Further screening of the Blo t 11 hybridoma clones identified six stable antibody-producing clones.

Figure 1 shows the titer of the Blo t 11 mAbs, their corresponding isotypes/subclasses, and the location of their epitopes. All six Blo t 11 mAbs registered slightly

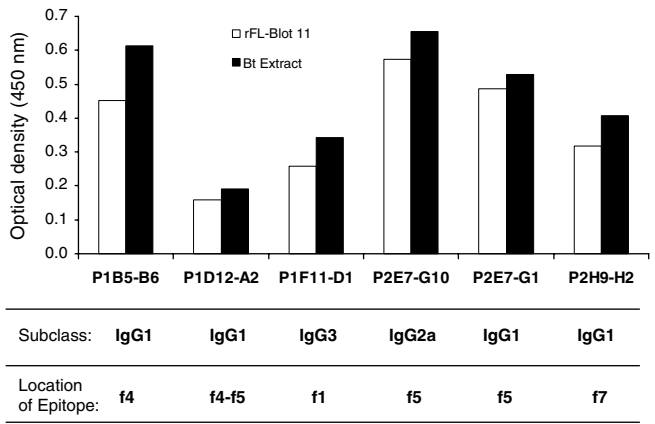


Figure 1. Summary of the titer of anti-Blo t 11 antibodies present in the ascites produced with the six stable Blo t 11 hybridoma clones. Results of the isotyping and epitope mapping by enzyme-linked immunosorbent assay were shown.

higher reactivity to the nBlo t 11 present in crude Bt extract as compared to the reactivity with the rFL-Blo t 11. The reactivity of the six Blo t 11 mAbs to the recombinant and native forms of the allergen was confirmed by Western blot analysis (Fig. 2), which showed that all except one clone (P1D12-A2) recognized the recombinant allergen. All clones reacted to the nBlo t 11 and its degraded forms of approximately 66 kDa present in the crude Bt extract.

Epitopes of Blo t 11 mAbs

Four of the six Blo t 11 mAbs recognized epitopes located on f4 and/or f5 (Fig. 1). Clone P2H9-H2 recognized an epitope on the C-terminus fragment (f7) while clone P1F11-D1 contains an epitope located at the N-terminus fragment (f1). Clone P1D12-A2 reacted to both f4 and f5 indicating that the epitope recognized by this antibody is found in the region of overlap between the two fragments. All epitopes recognized by the six Blo t 11 mAbs appeared to be linear as shown by the comparable reactivity between the FL-Blo t 11 and peptides containing the epitopes for each clone (data not shown). Interestingly, the reactivity of the six mAbs to the purified

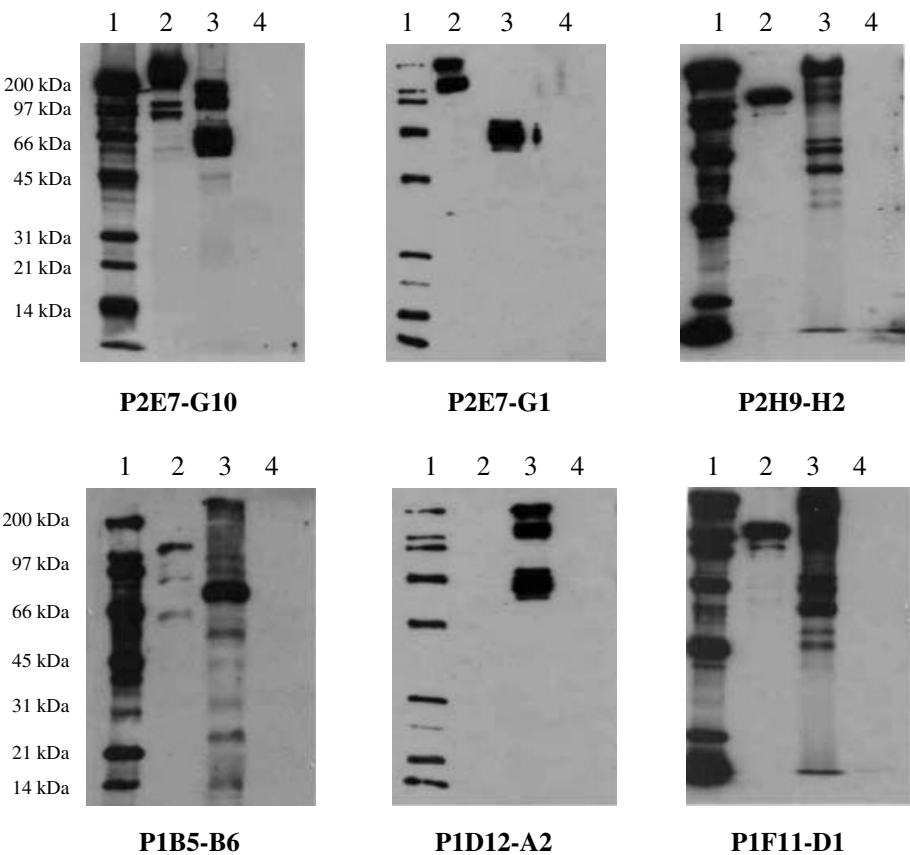


Figure 2. Western blot analysis of rFL-Blo t 11 (1), Bt extract (2) and GST (3) using the six Blo t 11 mAbs.

nBlo t 11 and to its unpurified form present in the Bt extract is relatively higher compared with the reactivity with the recombinant allergen (data not shown).

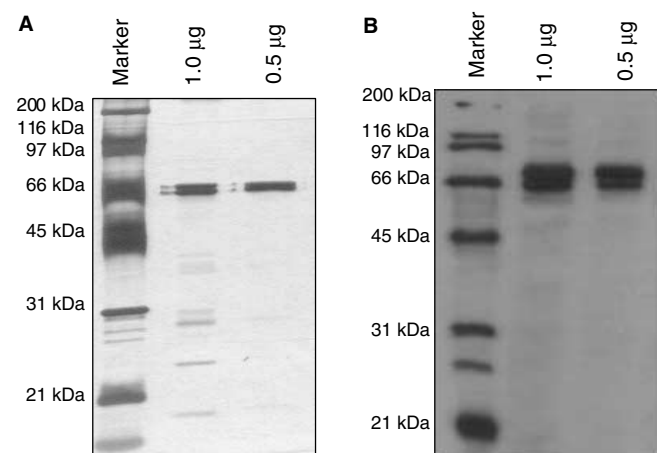


Figure 3. The native Blo t 11 isolated by affinity chromatography using Blo t 11 mAb P2E7-G1 on silver stained sodium dodecyl sulphate-polyacrylamide gel electrophoresis gel (A) and Western blot analysis (B).

Native Blo t 11 isolated using P2E7-G1 mAb

The nBlo t 11 was isolated from the Bt crude extract by immunoaffinity chromatography using Protein A-purified P2E7-G1 (Fig. 3). A total of 15–20 µg of nBlo t 11 can be isolated from 1 g of Bt mites (wet weight). A silver-stained SDS-PAGE gel showed that the purified nBlo t 11 was partially degraded; the major derived peptide was approximately 66 kDa (Fig. 3A). The isolated nBlo t 11 reacts with P2E7-G1 on Western blot analysis (Fig. 3B).

The isolated nBlo t 11 was further verified by MALDI-TOF MS analysis. Sixteen tryptic peptides from the purified nBlo t 11 were found to match with the amino acid sequences deduced from the FL-Blo t 11 cDNA (Fig. 4). A total of 187 amino acids were identified from the 16 peptides. The amino acid sequence of these peptides exhibit 79, 79, 71, 53, 50, 49, 49, and 48% identity to the paramyosin amino acid sequences of *Sarcoptes scabiei*, *Dermatophagoides farinae*, *Boophilus microplus*, *Drosophila melanogaster*, *Anisakis simplex*, *Dirofilaria immitis*, *Onchocerca volvulus*, *Caenorhabditis elegans*, and *Taenia soleum*, respectively. Interestingly, seven of the peptides identified are located in the region coding for the immunodominant

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MAARS AKYMYQSSRAGHG GDISIEYGTDLGALTRLEDKIRLLSEDLSESERELRQRVEREKSDITVQLMNLTERLEETEGSSSESVTEMNKK 090
-----LLSEDLSESERELR-----
I
RDS ELAKLRKLLLEDVHMESEETAHHLRQKHQA AIQEMQDQLDQVQKAKNKS DKEKQKFQAEVFELLAQVETANKDKLVAQKTVEKLEYTV 180
-----
HELNIKIEEINRTVVEVTAHRQLRSQENSELIKEVHEYKISLDNANH LKGQIAQQLEDTRHRLDEDEERKRSSLENHAHTLEVELESLKVQ 270
-----GQIAQQLEDTR-----
II
LEESEARLELERQLTKANGDAASWKS KYEAE LQAHVDEVEELRRKMAQKISEYGEQLEALLNKCSALEKQKARLQSEVEVLIMDLEKAT 360
-----YEAE LQAHVDEVEELR-----LQSEVEVLIMDLE-----
III IV
AHAQALEKRVSQLEKINLDLKS KLEEVSM LLEQTQKDLRVKIADLQKLQHEYEKLRDQKEALARENKKLADDLAEAKSQLNDAHRRRIHQ 450
-----SKLEEVSM LLEQTQK-----VKIADLQK-----IHEQ
V VI VII VIII
EIEIKRLENEREELAAAYKEAETLRKQEEAKNQR LTAELAQT RHDYEKRLAQKEEEIEALRKQYQIEIEQLNMRLAEAEAKLKTEVARLK 540
EIEIK-----EEEIEALRKQYQIEIEQLNMR-----
IX X
KKYQAQITELELSLDAANKANIDLQKTIKKQALQITGLQAHYDEVHRQLQQA V DQLGVTQRRCCQALTAELEEMRVNLEQALRAKRAAEQM 630
-----QALQITGLQAHYDEVHRQLQQA V DQLGVTQR-----CQALTAELEEMRVNLEQALR-----AAEQM
XI XII XIII XIV XV
HEEAVVRVNELTTINVNLASAKSKLETEFSALQNDYDEVHKE LRISDERVQKLTIEVKSTKDLLESETERVTKLETIKKSLETEVRNLQI 720
HEEAVVRVNELTTINVNLASAKSK-----
XVI
RIEEVEANALAGGKRVI AKLESRI RDVEIEVEEERRHAET EKMLRKKDHRVKELLQNEEDHKQIQLLQEMSDKLNKVKVYKRQMMEQ 810
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EGMSQQNLTRVRRFQRELEAAEDRADQAESNLSFIRAKHRSWVTTSQVPGGTRQVFVTEQESSNF 875
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Figure 4. Alignment of the 16 nBlo t 11 peptides (I–XVI) identified by matrix assisted laser desorption/ionization – time of flight mass spectrometry with the deduced amino acid sequence of the FL-Blo t 11 (GenBank Accession No. AF525465). Peptides V and VI, IX and X, XIII and XIV, and XV and XVI overlap with one another. The amino acid sequence coding for fD (amino acid positions 336–557) is highlighted in underlined bold letters.

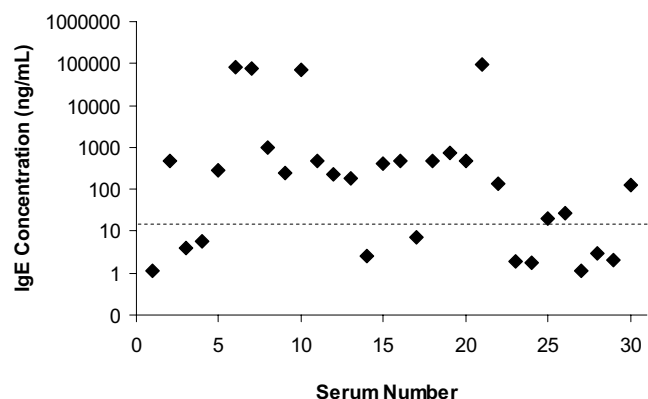


Figure 5. Summary of the profile of sensitization of 30 Singaporean asthmatic and/or atopic dermatitis patients' sera against the nBlo t 11 by hIgE enzyme-linked immunosorbent assay. All sera used are immunoglobulin E-positive to Bt extract. Cut-off value for positive reaction (horizontal bar) was computed as mean + 2 SD of the negative controls.

peptide fD (amino acid positions 336–557) as described previously (16). Our results strongly suggest that the purified *c.* 66 kDa peptide is indeed the derived product of Bt paramyosin.

The isolated native Blo t 11 was analyzed for its ability to bind IgE by ELISA. Figure 5 shows that the isolated nBlo t 11 reacts with 67% (20 of 30) of the asthmatic and/or atopic dermatitis sera tested. Eighteen of the 20 nBlo t 11-positive sera registered an IgE concentration of more than 100 ng/ml. The data presented in Fig. 5 conforms to the IgE reactivity of the isolated nBlo t 11 described previously (15).

Blo t 11 sandwich ELISA

Figure 6 shows that the best sandwich ELISA combination was obtained with mAb P2E7-G10 as the capture antibody and the biotinylated mAb P2E7-G1 as the detection antibody. The detection capacity of the P2E7-G10 and P2E7-G1 antibody pair was consistently high using both the recombinant (Fig. 6A) and the nBlo t 11 present in the crude Bt extract (Fig. 6B). A slightly lower but significant detection capacity was observed with the reverse reaction (P2E7-G1 as capture antibody and the biotinylated P2E7-G10 as detection antibody) as well as with antibody pairs P2H9-H2 and P2E7-G10, and P1D12-A2 and P2H9-H2.

To determine the detection range of the Blo t 11 sandwich ELISA pairs, a serial dilution of the rFL-Blo t 11 and fD were used for ELISA. Figure 7 shows that the detection limit of the P2E7-G10 and P2E7-G1 antibody pair and the reverse reaction are within the 100 pg/ml Blo t 11. The P2E7-G10 and P2E7-G1 antibody pair could detect the presence of approximately 100–200 ng of nBlo t 11/mg of crude Bt protein.

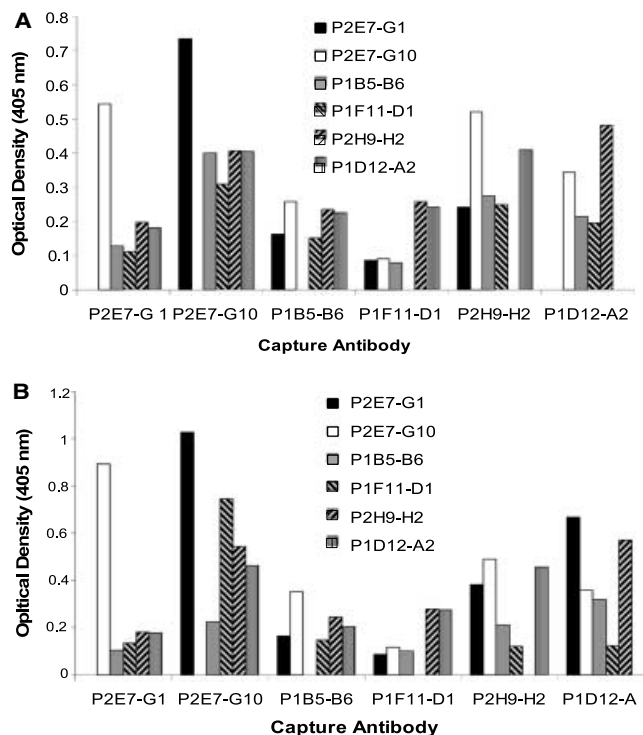


Figure 6. Summary of sandwich enzyme-linked immunosorbent assay development using rFL-Blo t 11 (A) and crude Bt extract (B) as antigen.

Discussion

This study describes the production, characterization and applications of Blo t 11 mAbs generated by DNA immunization with electroporation, an effective technique for inducing specific antibody response in mice for hybridoma production. In this study, four of five mice immunized with the FL-Blo t 11 cDNA produced high titer of specific antibodies recognizing the rBlo t 11 and fD, as well as the nBlo t 11 present in the crude Bt extract. These data showed that a strong and highly specific response to Blo t 11 could be generated by DNA immunization. It has been 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 (19, 24–26). The use of electroporation to enhance gene delivery *in vivo* resulting in increased expression and elevated immune responses has been reported (21, 22).

Administration of plasmid DNA has been proven to be an effective means to generate humoral immune responses specific to a wide array of proteins (19–26). The type of immune response generated from DNA immunization is influenced by a wide array of factors including the nature of the antigen, the expression vector used, and the route of administration (23, 27). Generally, antibody isotype induced by DNA immunization

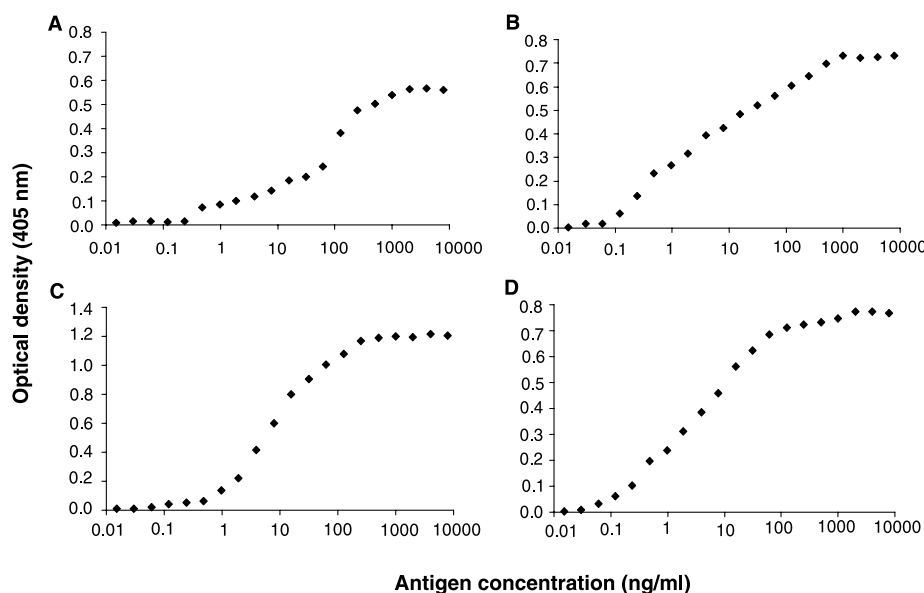


Figure 7. Standard curves for the mAb-based Blo t 11 sandwich enzyme-linked immunosorbent assay (ELISA) using the rFL-Blo t 11 (A and B) and fD (C and D). The detection range of antibody pairs P2E7-G1 (capture Ab) and P2E7-G10 (detecting Ab) (A and C) and P2E7-G10 (capture Ab) and P2E7-G1 (detecting Ab) (B and D) were determined by ELISA using a serial dilution of the antigens.

was mainly IgG, but serum IgM and IgA have also been detected (28, 29). In this study, although significant titer of IgG2a antibodies was detected, the predominant IgG subclass generated was IgG1. Similar to the observation reported by Justewicz et al. (28), IgM and IgA antibodies were also detected in this study. Of the 29 hybridoma clones produced, 41% (12 of 29), 41% (12 of 29) and 17% (five of 29) secrete IgG, IgM, and IgA isotypes, respectively.

Results of the ELISA (Fig. 1) and Western blot analysis (Fig. 2) showed the reactivity and specificity of the six Blo t 11 mAbs. However, P1D12-A2 mAb recognized the rFL-Blo t 11 in the ELISA but not in the Western blot analysis suggesting that this mAb potentially recognizes a conformational dependent epitope. Western blot results likewise showed that all six mAbs recognized the *c.* 66 kDa degraded product of the nBlo t 11 in addition to several other smaller fragments. The different profiles of reactivity to the recombinant and nBlo t 11 on the Western blots indicate that the mAbs recognized distinct epitopes.

The Blo t 11 mAbs were further characterized by mapping the location of their epitopes on the FL-Blo t 11 (Fig. 1). The finding that the six Blo t 11 mAbs recognized distinct epitopes located on the N- to the C-termini of the molecule is not surprising for a large protein like Blo t 11. Interestingly, four of the Blo t 11 mAbs possess epitopes located on fragments 4 and 5, the fragments encompassing the immunodominant fD described previously (16), indicating that fD contains epitopes capable of inducing a strong immune response. The identification of two Blo t 11 mAbs recognizing epitopes located on the

N- (P1F11-D1) and C- (P2H9-H2) termini of the Bt paramyosin agrees with previous reports which have shown that immunodominant epitopes of paramyosin are located at the ends of the molecule (30, 31).

The immunoaffinity isolation the nBlo t 11 using the mAb P2E7-G1 demonstrates the usefulness of the mAbs generated by DNA immunization. The *c.* 66 kDa nBlo t 11 is allergenic as it binds IgE in 67% of sera from asthmatic and/or atopic dermatitis patients (Fig. 5). These results are in good accordance to the data previously presented (15) and support the claim that Blo t 11 is an important allergen.

A sandwich ELISA for the detection of Blo t 11 was developed in this study. The detection limit of this assay is about 100 pg/ml of Blo t 11 (Fig. 7) which is in the same order of magnitude as most immunoassays developed to measure indoor allergens (32, 33). Using recombinant Blo t 11 as a standard, the assay was used successfully to quantitate the amount of Blo t 11 present in crude Bt extract. This assay is in line with recommendations of the WHO/IUIS Allergen Standardization Committee (33). Therefore, the sandwich ELISA for Blo t 11 is a useful tool to facilitate the quantitation of Bt extracts used for diagnosis and therapy of mite allergy. In addition, the assay could be used to monitor the concentration of environmental Blo t 11 allergen.

This study demonstrated that a DNA-based immunization protocol is effective in eliciting a strong polyclonal antibody response in mice. This approach is a useful alternative immunization strategy for the production of mAbs that recognize a spectrum of distinct epitopes throughout the antigen molecule.

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