

Generation of monoclonal antibodies against Blo t 3 using DNA immunization with *in vivo* electroporation

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Summary

Background House dust mite allergy is closely associated with allergic diseases. *Blomia tropicalis* mite species is an important clinical species in the tropics. The cDNA clone encoding Blo t 3, a group 3 allergen from *B. tropicalis*, has been isolated in our laboratory.

Objective This study was designed to generate Blo t 3-specific monoclonal antibodies (mAbs) for the detection, characterization and purification of this allergen.

Methods Mice were immunized intramuscularly with naked plasmid DNA encoding Blo t 3 gene with *in vivo* electroporation. Hybridomas were generated by the fusion of the splenocytes to X63-Ag8.653 myeloma cells. Purified native Blo t 3 was obtained by mAb immuno-affinity purification and the allergenicity of native Blo t 3 was determined by human IgE enzyme-linked immunosorbent assay (ELISA).

Results A panel of class-switched and high-affinity mAb recognizing a wide spectrum of Blo t 3 epitopes have been generated. These mAbs are useful for western immunoblot assay, sandwich ELISA and affinity purification of native Blo t 3. Allergenicity of native Blo t 3 protein was examined with 44 mite-allergic sera and approximately 57% of the tested sera had positive serum IgE reactivity to the native Blo t 3.

Conclusions These results demonstrated that intramuscular injection of naked DNA encoding Blo t 3 gene combined with *in vivo* electroporation is an effective and simple method to raise monoclonal antibodies that can be used for characterization and purification of Blo t 3.

Keywords *Blomia tropicalis*, Blo t 3, DNA immunization, *in vivo* electroporation, monoclonal antibody

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Introduction

House dust mites are widely recognized as major factors involved in the triggering of allergic diseases such as asthma and allergic rhinitis. Mite bodies and mite faeces are the sources of many allergens which are divided into specific groups (groups 1–15). Group 1 and 2 allergens are two major allergens from *Dermatophagoides pteronyssinus* (Dp) and *D. farinae* (Df) mites, which have been extensively studied [1–5]. However, previous reports of IgE reactivity to the *Dermatophagoides* group 3 allergens have varied considerably, with frequencies reported as low as 16% [6] to as high as 100% [7].

Besides *Dermatophagoides* species, *Blomia tropicalis* (Bt) is also an important source of allergen in tropical and subtropical regions. A large number of Bt mites were found in the domestic environment and about 70–80% of asthmatic patients showed positive IgE reactivity to Bt allergens [8–12]. The cDNA clone encoding Blo t 3, the group 3 allergen from Bt, has been isolated and characterized by our laboratory [13]. The amino acid

sequence of Blo t 3 showed approximately 50% homology with the serine protease trypsin. The homology between Der p 3 and Der f 3 is 81% [14, 15], while Blo t 3 cDNA sequence show 47% identity with Der p 3 and 48% identity with Der f 3. In order to further characterize Blo t 3, generation of Blo t 3 monoclonal antibodies (mAbs) is highly desirable. Here we report the generation of Blo t 3 mAb by DNA immunization approach instead of the conventional protein immunization strategy. The main reason for adopting such gene immunization strategy is that the native Blo t 3 was unavailable then and the only available form of recombinant Blo t 3 was produced as a fusion protein with glutathione-S-transferase (GST-Blo t 3). The GST component of GST-Blo t 3 could not be cleaved off and it has been shown that immunization using the fusion protein was undesirable because the hybridoma produced were predominantly GST-specific (unpublished data). Besides, our attempts made to produce Blo t 3 as a nonfusion protein in other expression systems were unsuccessful (unpublished data).

DNA immunization technology involves the inoculation of genetic material into mammalian hosts, in the context of a suitable mammalian expression vector encoding the protein of interest, leads to *in vivo* expression and subsequently to the development of an antigen-specific antibody response [16].

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Naked DNA can be delivered intramuscularly (i.m.) with a needle or intracutaneously (i.c.) with a particle-mediated helium-driven gene-delivery device, a so-called 'gene gun'. Using this technology it is possible to circumvent laborious antigen purification and still obtain a specific immune response. Generation of mite allergen-specific antibodies in rodents by DNA-based immunization was first published by our laboratory [17]. It has been demonstrated that DNA immunization is an alternative immunization strategy for the generation of mAbs [18, 19]. Although i.m. injection is simple and direct, it is still relatively inefficient. Recently, *in vivo* electroporation has attracted considerable attention because such technology significantly improved the efficiency of gene delivery [20–22]. Another advantage of DNA immunization for the antibodies production is that a full spectrum of native epitopes of an antigen can be expressed *in vivo* and better conformation or protein presentation can be achieved as compared with administration of recombinant protein expressed *in vitro*.

Materials and methods

Mice

Seven-week-old female BALB/cJ mice housed under conventional conditions were used for DNA immunization and generation of mAbs. Animal experiments were performed according to Institutional Guidelines for Animal Care and Handling, National University of Singapore.

Plasmid construction

The plasmid pCI/Dp5L containing the Der p 5 leader sequence for secretion was modified from the eucaryotic expression vector pCI (Promega Corporation, Madison, WI, USA) as described [23]. The Der p 5 leader sequence was generated by annealing complementary synthetic oligonucleotides of the sequence 5'-TCGAGATCAATCATGAAATTCATCATTGCTTTCTTTGTTGCCACTTTGGCAGTTATGACTGTTTCCGGA-3'. The predicted coding sequences for mature Blo t 3 was amplified with the forward primer 5'-ATCCGGAATTGTTG GTGGATCGAATGCTGCT-3' and reverse primer 5'-GCGC GGCCGCTTAACTGGAACCTCTT-3'. The PCR product was inserted into the *BspEI* and *NotI* sites of pCI/Dp5L vector. Plasmid pCI/Dp5L-Blo t 3 was amplified in *Escherichia coli* strain DH5 α , purified using Qiagen® Plasmid Giga Kit (Qiagen, Chatsworth, CA, USA) and dissolved in phosphate-buffered saline (PBS) at 2.0 mg/mL for use in DNA immunization.

Preparation of GST-Blo t 3 fusion protein

Plasmid pGEX4T-1 (Invitrogen, Carlsbad, CA, USA) was used for expression of the Blo t 3 fused with GST. GST-Blo t 3 fusion protein was induced with 0.05 mM isopropyl 1-thio- β -D-galactopyranoside at 30 °C for 1 h in *E. coli* strain TG-1 and purified by glutathione-sepharose 4B (Amersham Biosciences, Uppsala, Sweden) affinity chromatography as described previously [13].

Preparation of whole mite and spent mite extracts of *B. tropicalis*

Whole mite extract was prepared from the pure Bt mite culture as described previously [24]. One gram of whole mites were

frozen in liquid nitrogen and ground with mortar and pestle and then extracted with 12.5 mL of cold PBS pH 7.4 with 2 mM phenylmethylsulphonyl fluoride (PMSF) and 1 mM EDTA. Spent mite extract was prepared by suspending 1 g of spent mite medium in 10 mL of Tris-buffered saline (TBS) pH 7.4 plus 2 mM PMSF at 4 °C for 2 h. Supernatants were separated by centrifugation at 17 000 *g* in 4 °C for 30 min. The extracts aliquots were stored at –80 °C. Protein concentration was quantified with the Bio-Rad Protein Assay reagent (Bio-Rad, Richmond, CA, USA) with bovine serum albumin (BSA; Sigma, St Louis, MO, USA) as a standard.

Human sera

A total of 78 human sera (36 males and 41 females, mean age of 32.96 years) were used for the study. Serum total and specific IgE antibodies were measured using Pharmacia Unicap System (Pharmacia & Upjohn Diagnostic AB, Uppsala, Sweden); levels more than 0.35 kU/L indicated as positive results. Among these sera, 44 were positive to Bt, 44 to Dp and 43 to Df mites. The mean level of total IgE in the cohort is 374.54 kU/L.

DNA immunization and *in vivo* electroporation

Mice were anaesthetized with intraperitoneal injection of 20 μ L of a mixture containing 1.25 mg/mL midazolam (Roche, Basel, Switzerland), 2.5 mg/mL fluanisone and 0.079 mg/mL fentanyl citrate (Janssen Pharmaceutica, Beerse, Belgium). The skin overlying the anterior tibialis muscle was shaved and a dose of 100 μ g DNA in 50 μ L PBS was injected using an insulin syringe with a 27-gauge needle. The two-needle array electrode (5 mm) was inserted into the muscle encompassing the DNA injection site to a depth of 2 mm. Four pulses of length 20 ms with 200-ms interval at 82 V were delivered using the BTX ECM830 square wave electroporator (BTX, San Diego, CA, USA) [23]. Mice were immunized three times with 2-week intervals.

Production of mAbs

DNA-immunized mice were boosted intravenously via the tail vein with 5 μ g GST-Blo t 3, 3 days prior to fusion. Pooled splenocytes from three immunized mice were fused with X63-Ag8.653 myeloma cells using the ClonaCell-HY™ system (StemCell Technologies Inc., Vancouver, Canada) as described by the manufacturer. Hybridoma clones were screened with both GST-Blo t 3 and whole mite extract using enzyme-linked immunoabsorbant assay (ELISA). Positive clones were selected after three rounds of screening and ascites fluids were produced [25]. Immunoglobulins were enriched by caprylic acid precipitation [26] and used for further experiments. The biotin labeling was performed using biotin hydroxysuccinimide ester (Sigma) as described by the manufacturer.

ELISA analysis

In all of the ELISA protocols, 50 μ L of reagents were used unless specified and samples were assayed in duplicate. Costar high binding plates (Costar, Corning, NY, USA) were coated with 5 μ g/mL GST, GST-Blo t 3, or 200 μ g/mL whole mite extract in coating buffer 0.1 M NaHCO₃, pH 8.2. Plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 100 μ L of 1% BSA (Sigma) in PBS-T. For Blo t 3-specific IgG₁ and IgG_{2a} determination of immunized mice, 1 : 500 diluted mouse sera were added and incubated at 4 °C for

overnight. For screening and isotyping of hybridomas, neat culture supernatants were added. After being washed three times, plates were incubated with biotin-conjugated anti-mouse isotypic antibodies (1 : 2000; Serotec) for 1 h. After washing off the secondary antibodies, ExtrAvidin Alkaline Phosphatase (1 : 2000; Sigma) was added and incubated for 1 h. Finally, plates were washed six times and developed with paranitrophenyl phosphate substrate (pNPP; Sigma). Optical density at 405 nm was determined on a Spectra (Shell) & Rainbow (Shell) Readers (Tecan, Tecan Austria GmbH, Salzburg, Austria). For the human IgE reactivity assay, native Blo t 3 was coated and incubated with human sera (diluted 1 : 5 in blocking buffer) at 4 °C for overnight. Biotin-conjugated anti-human IgE (1 : 2000; Southern Biotechnology Associates Inc., Birmingham, AL, USA) was used as secondary antibody [11].

Western immunoblot

Purified recombinant GST-Blo t 3 (100 ng), Bt whole mite extract (80 µg), spent mite medium extract (24 µg), were separated on 7.5% Tris-tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [27] and blotted onto Hybond-C nitrocellulose (Amersham Biosciences). After blocking with 5% non-fat milk in PBS-T, incubation with unconjugated or biotin-conjugated mAbs was performed at 4 °C for overnight. Biotin-conjugated anti-mouse immunoglobins (1 : 10 000; Sigma) was added if the primary mAbs were unconjugated. The signals were developed with further incubation with ExtraAvidin-Peroxidase (1 : 10 000; Sigma) followed with SuperSignal chemiluminescence substrate (Pierce, Rockford, IL, USA).

Immuno-affinity purification

mAbs were coupled with cyanogenbromide-activated Sepharose® 4B (Amersham Biosciences) according to the manufacturer's instruction. Whole mite extract (36 mg each) was passed through different mAb affinity columns by gravity. The columns were washed with TBS and bound native Blo t 3 was eluted with acidic buffer 0.1 M glycine, 0.15 M NaCl, pH 2.6 or alkaline buffer 10 mM glycine, 50% of ethylene glycol, pH 11. The eluents were neutralized with one-tenth of 1 M Tris-HCl pH 8.8, or 1 M Tris-HCl pH 7.5. The eluents (60 µL) precipitated with trichloroacetic acid and 5 µL of the eluted resins were used for western immunoblot analysis as described above.

Development of sandwich ELISA

Eight unconjugated mAbs were coated at 5 µg/mL on ELISA plates as capture antibodies. After blocking, serially diluted GST-Blo t 3 proteins were added in duplicate and incubated at 4 °C for overnight. Forty-two different combinations were tested with six biotin-conjugated mAbs as detection antibodies (5 µg/mL). Plates were washed three times with PBS-T and incubated with ExtrAvidin Alkaline Phosphatase (1 : 2000; Sigma). After final washing steps, signals were developed with pNPP (Sigma).

Results

DNA immunization and antibody responses

Five mice received three doses of i.m. immunization with pCI/Dp5L-Blo t 3 naked DNA followed by *in vivo* electroporation

produced antigen-specific antibody response with relatively high titres (Fig. 1). It was found that the titre of IgG_{2a} was higher than that of IgG₁.

Generation of mAbs to Blo t 3

To further amplify the immune response, three mice with high titre immune response were boosted intravenously via the tail vein 3 days prior to spleen cells fusion. As a result of splenocytes fusion with parental myeloma cells, more than 800 hybridomas were obtained and screened for the secretion of anti-Blo t 3-specific antibodies by ELISA. Because the mice were boosted with GST-Blo t 3, it is possible that the mAb produced could be reactive to either Blo t 3 or GST. To identify hybridomas secreting mAb against Blo t 3, the reactivity to GST-Blo t 3, GST and whole mite extract were determined. BSA was used as an antigen negative control. Clones with high reactivity to GST were discarded. After the third screening, 30 clones with varying degree of reactivity to both whole mite extract and GST-Blo t 3 as we aimed to obtain clones that can recognize native Blo t 3. Isotyping of the mAbs for these 30 hybridoma clones were determined and the results were summarized in Table 1. Six clones of the IgG_{2a} (mAbs 29, 65, 157, 243 and 281), one clone of the IgG₁ (mAb 35) and two clones of IgM isotype (mAbs 37 and 330) were selected for ascites production.

Characterization of mAbs to Blo t 3

Western analysis revealed that mAb 157 (IgG_{2a}), 35 (IgG₁) and 243 (IgG_{2a}) reacted well with native Blo t 3 in whole mite extract (Fig. 2a) and spent mite extract (Fig. 2c). The size of the protein band is approximately 25 kDa, which is in good accordance with the predicted size deduced from its cDNA sequence. The 21.5-kDa band in whole mite extract was confirmed to be non-specific binding because a mAb Ag8-38-4 against Der p 1 (Fig. 2b) also reacted to it. Besides the reactivity to the 54-kDa full-length GST-Blo t 3, mAb 157 and 35 also recognized smaller bands that could be attributed to degraded products of the recombinant fusion protein. None of the selected Blo t 3 mAbs showed reactivity to GST (data not shown).

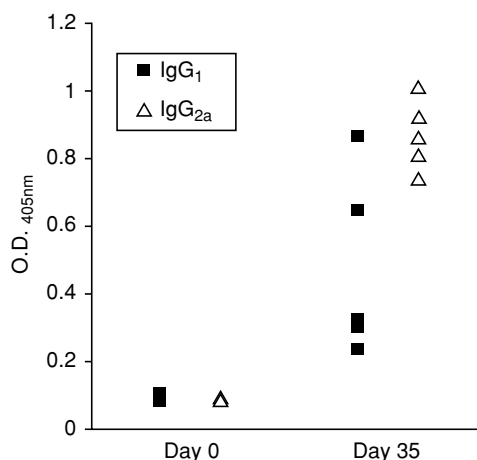
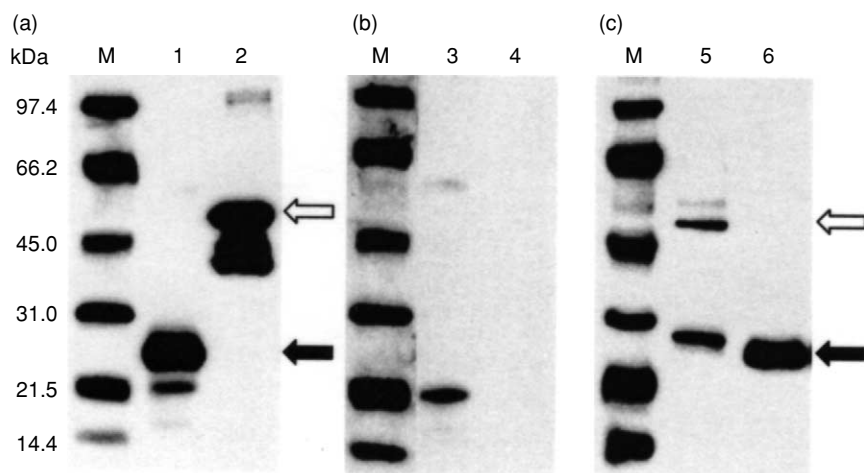


Fig. 1. Anti-Blo t 3 antibody [IgG₁ (■) and IgG_{2a} (△)] titres after three times of DNA immunization. Three doses of pCI/Dp5L/Blo t 3 plasmid DNA (100 µg) were injected intramuscularly into each of the five BALB/cJ mice at days 0, 14 and 28, and sera were collected at days 0 and 35 for antibody assays by ELISA.

Table 1. Screening and characterization of the selected hybridoma clones

	Total number of clones picked	1st Screening	2nd Screening	3rd Screening	Isotyping (<i>n</i> = 30)				
					IgM	IgG ₁	IgG _{2a}	IgG _{2b}	IgG ₃
Number of selected clones	800	406	45	30	15	2	8	3	2

**Fig. 2.** Identification of native Blo t 3 in whole mite and spent mite extracts by western blot immunoassay with Blo t 3 mAbs. Bt whole mite extract (80 µg) and spent mite extract (24 µg) were separated on a 7.5% reducing SDS-PAGE gel and then blotted for western analysis with (a) anti-Blo t 3 mAb 157; (b) anti-Der p 1 mAb Ag8-38-4 (isotype control); (c) anti-Blo t 3 mAb 35. M: molecular weight markers (kDa); lanes 1 and 3: Bt whole mite extract; lanes 2, 4 and 5: GST-Blo t 3; lane 6: Bt spent mite medium extract. Native Blo t 3 (approximately 25 kDa) is indicated by black arrows and GST-Blo t 3 (approximately 54 kDa) by open arrows.

Development of sandwich ELISA for Blo t 3

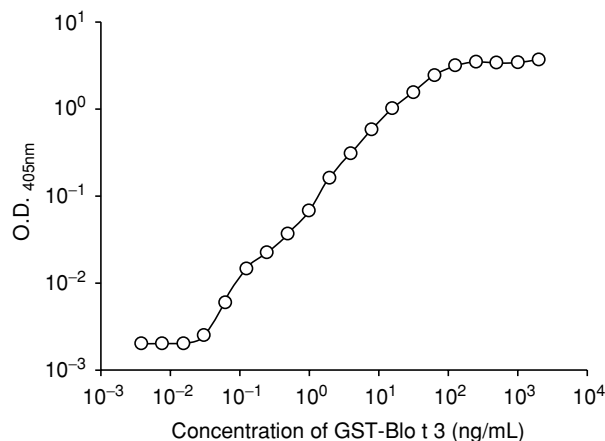
For the development of sandwich ELISA, a total 42 combinations with eight different mAbs were tested against GST-Blo t 3. All eight mAbs were treated by caprylic acid precipitation and six were biotin-conjugated to serve as detection antibody. Results indicated that mAb 35 and mAb 157 performed best as capture and detection antibody, respectively. The linear detection range of GST-Blo t 3 using this mAb pair is from 30 pg/mL to 250 ng/mL (Fig. 3).

Affinity purification and allergenicity test of native Blo t 3

Six selected mAbs of IgG isotypes were accessed for their suitability for purification of native Blo t 3 in whole mite extract by immuno-affinity chromatography. It was found that mAb 29 (Fig. 4a) and 65 (Fig. 4b) could bind the antigen and elution was accomplished using 100 mM glycine containing 0.15 M NaCl, pH 2.6. Purified native Blo t 3 was further confirmed by western analysis using biotin-conjugated anti-Blo t 3 mAb 157. On the other hand, the mAb 243 (Fig. 4c) could bind the native Blo t 3 but the bound protein could not be eluted by either using acid (pH 2.6) or alkali (pH 11) conditions.

Human IgE reactivity of native Blo t 3

To analyse the allergenicity of purified native Blo t 3, human sera from Bt mite allergic and Bt mite non-allergic subjects were tested by human IgE ELISA. A total of 25 out of 44 Bt mite-allergic sera (57%) showed positive reactivity to native Blo t 3 (Fig. 5). All except three of the 25 subjects were also positive for Dp and Df mite extracts.

**Fig. 3.** Development of sandwich ELISA using mAb 35 and mAb 157 as capture and detection antibody, respectively. Serially diluted GST-Blo t 3 proteins were added into mAb 35-coated plates and detected with biotin-conjugated mAb 157. The O.D._{405nm} readings were plotted against different concentration of GST-Blo t 3 protein (after subtracting GST background value) showing a linear detection range from approximately 30 pg/mL to 250 ng/mL of GST-Blo t 3.

Discussion

In most reported cases of i.m., immunization with naked DNA, antibody response has been found against the expressed protein [16, 28–30]. However, the antibody titre was generally low. In fact, Krasemann et al. [31] reported that DNA-mediated immunization alone was not efficient enough to generate blastocytes for hybridomas secreting mAbs. The main reason is probably due to low level of antigen expression *in vivo*. It has

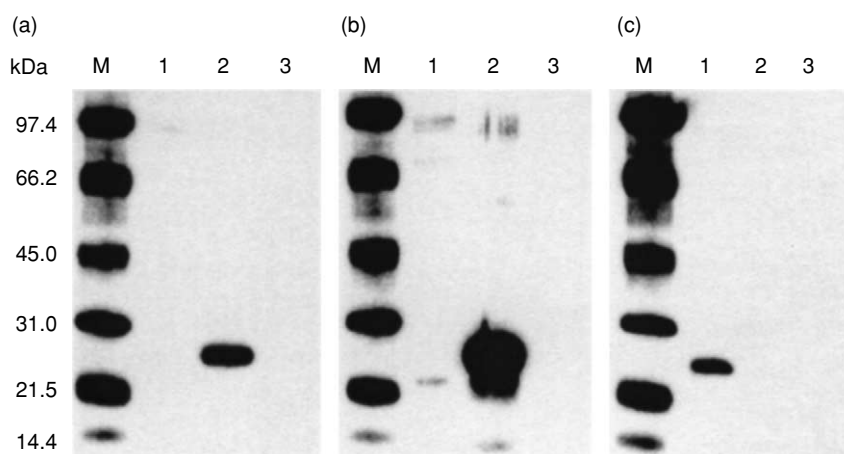


Fig. 4. Western immunoblot analysis of native Blo t 3 protein with biotin-conjugated mAb 157 as detection antibody. Results of the affinity purification of Blo t 3 from mite extract using mAb 29 (a), mAb 65 (b) and mAb 243 (c). M: molecular weight markers; lane 1: sepharose 4B resin after elution; lane 2: acidic buffer eluent; lane 3: alkaline buffer eluent.

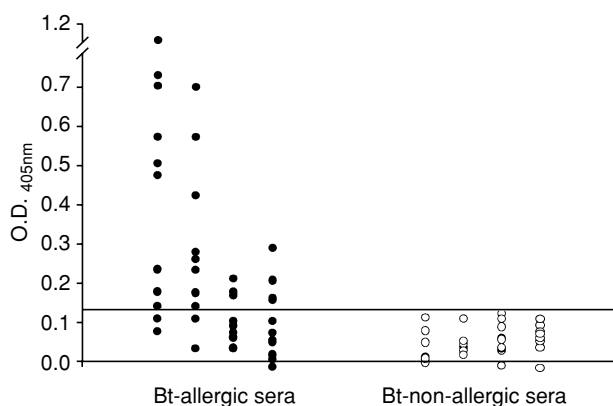


Fig. 5. *In vitro* IgE reactivity of affinity-purified native Blo t 3. Horizontal solid line represents the cut-off value that was determined as the mean + 2SD of O.D._{405nm} values obtained for Bt mite-non-allergic sera. ●, Bt mite-allergic sera; ○, Bt mite-non-allergic sera.

been shown that it is possible to achieve excellent levels of cell permeabilization compatible with cell survival by using optimal electrical parameters, and cell electroporation can be achieved locally *in vivo* by simple electrodes. Several studies demonstrated that such *in vivo* electroporation approach drastically increased the efficiency of i.m. gene transfer, resulting in a two- to four-log enhancement of gene expression [20–22]. In this study we demonstrated that i.m. DNA immunization with *in vivo* electroporation improved the efficiency of Blo t 3-specific antibody production mediated by Blo t 3 gene immunization; subsequently a panel of Blo t 3-specific mAbs were successfully generated. We found that immunized mice produced specific antibodies after one dose of i.m. DNA immunization with *in vivo* electroporation and antibody titre increased in a dose-dependent manner. Although mice were boosted with recombinant GST-Blo t 3 a total of 3 days prior to somatic cell fusion, there was no significant increase in antibody titre determined on the day when cell fusion experiment was performed (data not shown). It remains to be determined whether boosting with protein prior to fusion is necessary in order to obtain class-switched, high-affinity mAbs. The efficiency of DNA immunization for antibody production varies with nature of antigen

[32] and route of administration. It has been shown that as little as 5 µg of DNA encoding the target antigen administered with powderject gene gun could generate high-affinity mAbs successfully [33]. Recently, Moonsom et al. [34] demonstrated that intrasplenic immunization seems to be a very efficient route for production of both polyclonal and mAbs against cell surface proteins using plasmid DNA as immunizing agent. However, intrasplenic immunization required surgical procedure and may cause mortality of the mice.

In our study, out of the 30 selected mAbs, 27% and 7% of them are of IgG_{2a} and IgG₁ subtype, respectively (Table 1). The result is in accordance with the general observation that IgG_{2a} is the predominant immunoglobulin isotype detected after DNA immunization via the i.m. route. All these mAbs recognized recombinant and native Blo t 3 present in the whole mite extract when tested by ELISA. Some, but not all, of them showed good reactivity with native Blo t 3 in whole mite and spent mite extracts by western immunoblot analysis (Fig. 2). A number of the mAbs are useful for sandwich ELISA that could detect as low as 30 pg/mL of GST-Blo t 3 protein (Fig. 3). It can be a useful diagnostic tool for quick and accurate antigen detection and quantification. Two mAbs that performed less well with ELISA and western blot immunoassays worked quite well for affinity purification (Fig. 4). The data indicated that our approach of using i.m. immunization of naked DNA is a relatively simple but efficient method to generate mAbs targeting a spectrum of Blo t 3-specific epitopes. These mAbs are potentially useful for structural and functional characterization of the protein.

These mAbs are useful reagents for detection and localization of the protein in the natural source. The western blot analysis revealed that native Blo t 3 was present in both the whole mite extract (Fig. 2a, lane 1) and spent mite extract (Fig. 2c, lane 6), suggesting that Blo t 3 allergen, like other members of group 3 mite allergens, is probably a gut-associated digestive enzyme. The purified native Blo t 3 is potentially an important fecal mite allergen as indicated by the fact that greater than 50% of the tested mite allergic sera showed positive IgE reactivity with the allergen (Fig. 5). However, further study involving a larger number of mite-allergic patients is necessary to confirm the clinical importance of this allergen.

Acknowledgements

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