

Allergens, IgE, mediators, inflammatory mechanisms

Cloning and expression of complementary DNA coding for an allergen with common antibody-binding specificities with three allergens of the house dust mite *Blomia tropicalis*

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The mite Blomia tropicalis is a potent source of allergens in tropical and subtropical regions. So far, most of these allergens have only been studied by immunoblotting. To characterize them at the molecular level, a λ gt11 complementary DNA library was constructed from messenger RNA isolated from whole B. tropicalis mites. This library was screened by using pooled sera from patients allergic to B. tropicalis in a plaque IgE radioimmunoassay. A B. tropicalis IgE-positive clone (Bt-M) was selected for immunologic studies. After subcloning into pBluescript (Stratagene, La Jolla, Calif.), it produced a sequence of 310 bp, with a probable amino acid sequence of 72 residues for the expressed peptide. The recombinant protein was transferred to nitrocellulose filters and probed with 100 sera from patients allergic to B. tropicalis. Forty-seven percent of sera reacted with the recombinant allergen. Immunoblottings performed with allergic serum and B. tropicalis-affinity-purified IgE demonstrated that the recombinant protein shares allergenic epitopes with the 11-13, 14, and 16 kd native allergens of B. tropicalis, which are known to be important allergens of this mite. (J Allergy Clin Immunol 1996;98:573-9.)

Key words: *Blomia tropicalis*, cDNA cloning, recombinant allergen, epitopes, major allergen, IgE, immunoblotting

Blomia tropicalis is a domestic mite of the Glycyphagidae family commonly found as a component of house dust in tropical and subtropical areas of Venezuela, Brazil, Costa Rica, Barbados, Colombia, Uruguay, the United States, Suriname, Spain, Nigeria, and the Ivory Coast.¹⁻¹³ The allergenic role of this mite as an important inducer of

Abbreviations used

IPTG:	Isopropyl- β -D-thiogalactoside
PBS:	Phosphate-buffered saline
PBST:	Phosphate-buffered saline/0.1% Tween-20
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis

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allergic asthma and allergic rhinitis has been recently described.¹⁴⁻¹⁶ These reports indicate that although *B. tropicalis* is an important allergenic element of house dust in tropical regions, there is only weak cross-reactivity between this and other mites also found in the tropics, such as the pyroglyphids *Dermatophagoides pteronyssinus* and *D. farinae*.¹⁵⁻¹⁷ Therefore allergens of *B. tropicalis*

should be included among the reagents used for diagnosis and treatment of allergic respiratory diseases in these areas.

Several allergens have been identified in *B. tropicalis* extracts by using patients' sera for immunoblotting and crossed radioimmuno-electrophoresis experiments.^{17,18} Recently, we have detected 25 allergens from a whole body extract of *B. tropicalis*, among which the 11-13, 14, and 16 kd components represent clinically important allergens, as defined by their IgE-binding frequencies.^{19,20} As the first step in a program directed toward characterizing the allergenic components of *B. tropicalis* and their individual roles in the IgE immune response to this mite, we report the cloning, nucleotide sequencing, and expression of a *B. tropicalis* allergen, which shares common antibody-binding specificities with three important allergens in the mite extract.

METHODS

B. tropicalis culture

B. tropicalis mites isolated from house dust samples from homes of allergic patients living in Cartagena⁵ were deposited in a 500 ml Erlenmeyer flask with culture medium (tetramin fish food/brewer's yeast, 1:1) and kept at 27° C and 85% relative humidity. Adult mites were separated from the medium by using a modified Tullgren apparatus,²¹ immediately frozen in liquid nitrogen and kept at -80° C before RNA isolation. For immunoblotting studies, the extract of *B. tropicalis* was prepared in our laboratory as described previously.^{15,19} Lyophilized extract was reconstituted in water to obtain a concentration of 3.5 mg/ml. *D. pteronyssinus* extract was obtained from Greer Laboratories (Lenoir, N.C.) and used at a concentration of 3.4 mg/ml.

Serum samples

Sera from 100 patients with allergic asthma who not treated with immunotherapy but were highly allergic to *B. tropicalis* were used to establish the IgE-binding frequency of the recombinant protein and to make the serum pools. These sera were previously characterized by RAST and immunoblotting with a standardized *B. tropicalis* body extract.¹⁹ Patients (45 men and 55 women, aged 18 to 54 years) had positive skin test responses to a *B. tropicalis* extract (1:50 wt/vol). These individuals belonged to the prevalent ethnic group in Cartagena, mulattos.²² Allergic asthma was diagnosed according to the criteria proposed by the expert panel of the Allergy Foundation of America²³: recurrent episodes of wheezing or dyspnea, objective evidence of abnormality in pulmonary function during episodes, and positive skin test results. In addition, all patients had a clear history of atopic symptoms related to house dust exposure and had negative skin test responses to a *B. tropicalis* culture medium extract.

Construction of the *B. tropicalis* complementary DNA λ gt11 library

Total RNA (1.9 mg) was isolated from 1 gm of frozen *B. tropicalis* by using a commercial version (RNAzol-B; Cinna/Biotech Laboratory International, Inc., Friendwood, Texas) of the guanidinium thiocyanate method.²⁴ Messenger RNA (10 μ g) was obtained after adsorption to a cellulose poly(dT) column (Pharmacia LKB Biotechnology, Piscataway, N.J.). Complementary DNA was synthesized from 5 μ g of mRNA by the directional cloning method (Directional Cloning Tool Box, Pharmacia LKB Biotechnology). A *NotI*-dT₁₈ primer was used for first-strand DNA synthesis, and after the addition of *EcoRI* adaptors, the *EcoRI/NotI*-ended cDNA was ligated to *EcoRI/NotI*-digested λ gt11D (Pharmacia P-L Biochemicals, Piscataway, N.J.). The ligated DNA was packaged in *Escherichia coli* Y1090 and plated to produce a library having 3.5×10^4 recombinants.

Isolation of *B. tropicalis* allergen cDNA clones from the cDNA λ gt11 library

Screening of the library was performed by plaque radioimmunoassay²⁵ with an adsorbed (*E. coli*/Y1090) pool of 20 sera from the patients with asthma described previously. Phages were plated at 14,000 plaque-forming units (pfu) per 150 mm Petri dish. Plaques were transferred to nitrocellulose filters (Amersham Corp., Arlington Heights, Ill.) soaked in isopropyl- β -D-thiogalactopyranoside (IPTG). The filters were blocked for 1 hour with a 10% solution of skim milk powder and were washed three times in phosphate-buffered saline (PBS)/0.1% Tween-20 (PBST) and once with PBS. In addition, they were allowed to incubate overnight at room temperature, with 25 ml of the pool of human sera diluted 1:4 in PBST/0.1% bovine serum albumin/0.02% sodium azide. The filters were then washed three times in PBST and once with PBS and incubated overnight with iodine 125-labeled anti-human IgE (Sanofi Diagnostics Pasteur, Inc., Chaska, Minn.) at 2×10^6 cpm/ml, with rocking. After three washes with PBST and one with PBS, the filters were subjected to autoradiography for 24 hours (Kodak X-OMAT with intensifying screens, Eastman Kodak Co., Rochester, N.Y.) at -70° C.

Isolation of DNA from gt11 cDNA clones

Phages from immunopositive plaques were amplified by the liquid lysate method.²⁶ DNA was prepared from λ gt11 clones by using the Magic Lambda Preps DNA Purification System kit (Promega, Madison, Wis.).

Subcloning of λ gt11 insert into a plasmid vector

The insert from a restriction enzyme (*EcoRI* and *NotI*) digest of λ DNA was purified with the Magic PCR Preps kit (Promega), ligated to pBlueScript vector (Stratagene, La Jolla, Calif.) and transformed into *E. coli* XL1-Blue (Stratagene) according to the method of Sambrook et al.²⁷ Color selection of the transformed

clones was done on LB (Luria-Bertani) plates containing 100 µg/ml ampicillin, 12.5 µg/ml tetracycline, 80 µg/ml X-gal, and 20 mmol/L IPTG. Recombinant clones were confirmed by restriction enzyme analyses of miniprep from transformed XL1-Blue. Analysis of the expression of allergenic proteins by the recombinant clones was carried out by colony radioimmunoassay with the pool of sera from allergic patients to detect IgE binding.

DNA sequencing and analysis

Complementary DNA sequencing of clone Bt-M, subcloned into pBlueScript, was performed in both directions by polymerase chain reaction–dideoxy sequencing method, based on the dideoxynucleotide chain–termination method of Sanger et al.²⁸ In addition, with the use of λgt11 primers, Bt-M was isolated and subcloned into pCR II Vector by using the TA Cloning Kit (Invitrogen Corporation, San Diego, Calif.). Transformed *E. coli* (INV αF') was infected with M13, and single-stranded DNA was sequenced by using Sequenase Version 2.0 (Amersham Corp.).

IgE-binding frequency of a recombinant *B. tropicalis* allergen

The recombinant λgt11 was densely plated, and plaques with recombinant β-galactosidase fusion protein were transferred to nitrocellulose filters (Amersham Corp.) soaked in IPTG. Transferred plaques were cut into several sectors and probed with the 100 sera from patients allergic to *B. tropicalis* as described previously. Bound serum IgE was detected with ¹²⁵I-labeled horse anti-human IgE (Sanofi Diagnostics Pasteur, Inc.).

Affinity purification of IgE antibodies

To prepare affinity-purified IgE, cDNA clone Bt-M was densely plated on agarose, and fusion protein production was induced by overlaying the plates with IPTG-impregnated nitrocellulose filters. The filters containing the recombinant protein were then blocked in 10% skim milk, washed, and incubated overnight in the pooled allergic sera described earlier, which had already been adsorbed with *E. coli* Y1090 extract. The filters were washed twice with PBST and then with PBS. The bound antibodies, including IgE, were eluted with 0.1 mol/L glycine hydrochloride (pH 2.6) containing 1% bovine serum albumin and neutralized to pH 7.5 with Tris 1 mol/L, pH 8.0. The *E. coli*-adsorbed pool and the purified antibodies were used to probe Western blots of whole body extracts of *B. tropicalis* and *D. pteronyssinus*. The same experiment was performed with an individual allergic serum (AA1), also adsorbed with *E. coli*.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

SDS-PAGE and immunoblotting were accomplished according to standard methods.^{29,30} The extracts of *B. tropicalis* and *D. pteronyssinus* were loaded at 50 µg

protein/track (as determined by Bradford method (Bio-Rad Protein Assay; Bio-Rad Laboratories, Richmond, Calif.), and electrophoresis was carried out in 12% and 15% gels in a Mini-Protean II cell (Bio-Rad, Hercules, Calif.). For immunoblotting, proteins were transferred to a nitrocellulose membrane (Trans-blot NC, pore size 0.45 µm; Bio-Rad). Each nitrocellulose strip was incubated overnight with 3 ml of serum. Binding of the IgE was visualized by using ¹²⁵I-labeled anti-human IgE (Sanofi Diagnostics Pasteur, Inc.), followed by autoradiography (24 to 48 hours) with Kodak X-OMAT films.

RESULTS

Isolation of *B. tropicalis* allergen cDNA clones

To determine whether the λgt11 library contained clones expressing mite allergens, it was screened with a pool of human sera containing IgE reacting to 25 *B. tropicalis* allergen components, as evaluated by Western blotting. After screening six filters (14,000 pfu/filter) by plaque immunoassay, 14 plaques were found to produce positive reactions by autoradiography. No signal was obtained in transferred plaques of nonrecombinant λgt11. After several screenings, one clone was purified and designated as Bt-M. This clone was amplified by the liquid lysate method. The insert size in the clone Bt-M, determined by digestion of miniprep λDNA with *Eco*RI and *Not*I, followed by electrophoretic separation of fragments in a 1.5% agarose gel, was approximately 310 bp.

Sequence analysis of λgt11/Bt-M

The DNA from clone λgt11/Bt-M was prepared, and after *Eco*RI and *Not*I digestion, the insert was isolated and subcloned into pBlueScript for DNA sequence analysis. The pBlueScript-transformed *E. coli*/XL1-Blue strain was able to express the recombinant Bt-M allergen, as demonstrated by positive colony immunoassay with a pool of sera from allergic patients.

The cDNA sequence* and the deduced amino acid sequence derived from the cDNA insert are shown in Fig. 1, A. The insert is a partial clone of 310 bp long and included a 217-base putative coding region for the recombinant protein and a 93-base long 3' noncoding region with a poly-A tail. Thus the cDNA codes for the C-terminal portion of the Bt-M polypeptide, terminating at the TAA stop codon at nucleotides 218–220. We believe that the cited amino acid sequence represents a translation of the cDNA sequence in the

*These sequence data have been submitted to Gene Bank (Accession number U27702).

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G GCA CGA GGC AAG GAA TTG CAA GAG AAA ATC   31
  A  R  G  K  E  L  Q  E  K  I

ATT CGA GAA CTT GAT GTT GTT TGC GCC ATG ATC   64
  I  R  E  L  D  V  V  C  A  M  I

GAA GGA GCC CAA GGA GCT TTG GAA CGT GAA   94
  E  G  A  Q  G  A  L  E  R  E

TTG AAG CGA ACT GAT CTT AAC ATT TTG GAA CGA   127
  L  K  R  T  D  L  N  I  L  E  R

TTC AAC TAC GAA GAG GCT CAA ACT CTC AGC AAG   160
  F  N  Y  E  E  A  Q  T  L  S  K

ATC TTG CTT AAG GAT TTG AAG GAA ACC GAA CAA   193
  I  L  L  K  D  L  K  E  T  E  Q

AAA GTG AAG GAT ATT CAA ACC CAA TAA AAA TTT   226
  K  V  K  D  I  Q  T  Q  *

AGA ATT GTA CAA TTT TAC ATT TTT GAT ATG ATT   259

AAA TGT CAA TAA ATG TTC AAT AAA TAA AAA AAA   292

AAA AAA AAA AAA ATT CCT                               310

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A

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Bt-M      4  K E L Q E K I I R E L D V V C A N I E G A Q G A L E R E L K R T D L N I
          * * s s s * * s * s * * s * s * * s * s * s * s *
Der p 5   81  K E M K D K I V A E M D T I I A M I D G V R G V L D R L M Q R K D L D I

Bt-M      L E R F N Y E E A Q T L S K I L L K D L K E T E Q K V K D I Q      70
          * s s * * * s * * s * * s * s * s * s
Der p 5   F E Q Y N L E M A K K S G D I L E R D L K K E E A R V K K I E      147

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B

FIG. 1. A, Nucleic acid and deduced amino acid (one letter coded) sequence of the Bt-M allergen. Nucleotide residues of the cDNA are numbered at the end of each row. The stop codon is represented by an *asterisk*. **B,** Protein sequence homology of the deduced amino acid sequence of Bt-M peptide and Der p 5. The alignment includes segments comprising residues 4 to 70 of the Bt-M peptide and residues 81 to 147 from Der p 5. Identical amino acid residues are indicated by an *asterisk*. Similarity between amino acid residues is indicated with an *s*.

correct reading frame, because the other two reading frames contain multiple stop codons.

The inferred amino acid sequence from nucleotides 2-217 suggested that the partial clone codes for a protein of 72 residues with a calculated

molecular weight of 8373 d. There are four potential N-glycosylation sites and there are no cysteine residues in the molecule. Nucleotide and amino acid sequence searches (March 1995) with the BLAST network service showed that there is 43%

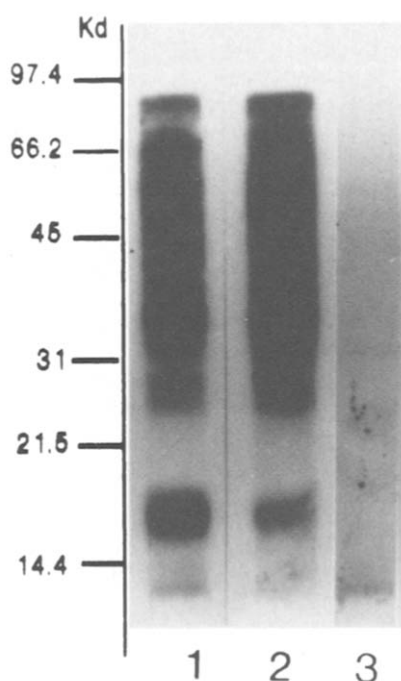


FIG. 2. Immunoblotting of *B. tropicalis* extract (15% SDS-PAGE). The gel was run under reducing conditions. Lane 1, AA1 serum detecting several allergens of the extract, including the 11-13 kD band. The signal of the 14 kD band is very weak. Lane 2, The same serum after adsorption with the Bt-M recombinant allergen. Lane 3, Affinity-purified IgE from membranes used for immunoadsorption. This photograph is overexposed to show the binding of IgE antibody to the lower molecular weight bands, especially the 11-13 kD band.

identity between residues 4-70 of this peptide and residues 81-147 of *D. pteronyssinus* allergen, Der p 5.^{31, 32} The alignment of these two sequences is shown in Fig. 1, B.

Immunologic analysis of recombinant Bt-M allergen

Among 100 characterized sera from patients with asthma who are allergic to *B. tropicalis*, 47% reacted with the Bt-M allergen transferred to nitrocellulose filters. In an additional assay with 12 noncharacterized sera from patients allergic to *B. tropicalis* (positive by skin test), seven (58%) reacted with the recombinant allergen.

To determine the relationship between Bt-M and the native allergens of *B. tropicalis*, we performed an affinity purification of IgE antibodies from both a pool of allergic serum and the individual serum, AA1. Then we used the affinity-purified anti recombinant-allergen antibodies to identify the native allergens by immunoblotting. The Bt-M allergen-adsorbed serum showed a loss

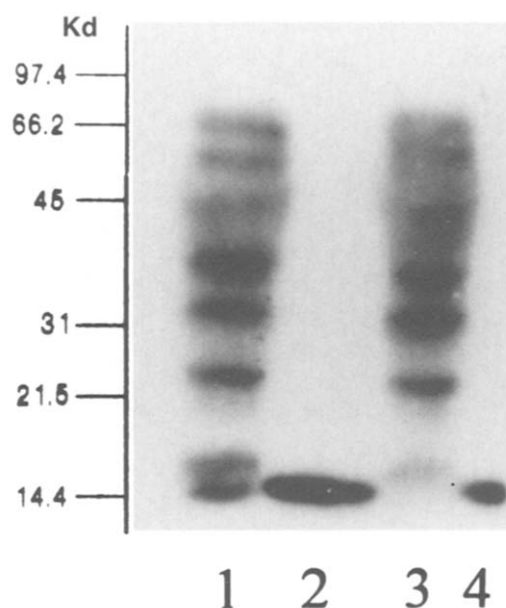


FIG. 3. Immunoblotting (12% SDS-PAGE) of *B. tropicalis* (lanes 1 and 3) and *D. pteronyssinus* (lanes 2 and 4) extracts. The gel was run under reducing conditions. Lanes 1 and 2, Pool of allergic sera. Lanes 3 and 4, The same pool after adsorption with Bt-M recombinant allergen. Both adsorbed and nonadsorbed pools were able to detect a 15 kD band in *D. pteronyssinus* extract. This band is believed to be Der p 2 on the basis of its size and strong IgE binding.¹⁹ In *B. tropicalis* extract, the pooled sera detects the 14 and 16 kD bands, which lose intensity when the pool is adsorbed (lane 3). The 11-13 kD band of *B. tropicalis* extract is not detected in this blotting.

of reactivity of the bands of 11-13 and 14 kD and a significant reduction of reactivity of the 16 kD native allergen (Figs. 2 and 3). The affinity-purified antibodies showed reactivity with two of the bands (Fig. 2, lane 3). To demonstrate the reactivity of affinity-purified IgE against the band of 16 kD, it was necessary to overexpose the strip (not shown). The results obtained when a *D. pteronyssinus* extract was used showed no serologic similarity between Bt-M and the *D. pteronyssinus* group 2 allergen (Fig. 3).

DISCUSSION

In this study we were able to clone an allergen from the mite *B. tropicalis* and characterize it by radioimmunoassay with allergic sera. According to the results of immunoadsorption studies performed with the recombinant molecule and the frequency of IgE binding with a large panel of patients' sera, the protein derived from the Bt-M clone appears to share at least one important allergenic epitope with the native major allergen of

11-13 kd and the 14 and 16 kd allergens detected by immunoblotting.^{19,20} However, the predicted molecular weight of the recombinant allergen is only 8373 d. This discrepancy (observed with both 12% and 15% SDS-PAGE immunoblotting of the *B. tropicalis* extract) could in part result from the glycosylation of the native proteins, but also the Bt-M clone is likely not full-length. Our results also show no cross-reactivity between the recombinant protein and *D. pteronyssinus* group 2 allergens, which have not yet been reported in *B. tropicalis* extracts.¹⁶

The molecular cloning of an allergen with antibody-binding specificities in common with multiple components in mite extracts has been described previously for *D. pteronyssinus*.³³ Our results suggest that Bt-M is coding for at least one important human IgE-binding epitope in *B. tropicalis* extract, which is shared by three important IgE-binding bands detectable on SDS-PAGE. Our sequence data suggest that we have identified a major allergen, which has significant homology with one important allergen of *D. pteronyssinus*,³¹ suggesting that we have obtained a partial clone of Blo t 5. This clone is different from other *B. tropicalis* allergens cloned in our laboratory. For example, the Bt11a clone codes for a protein of 14.2 kd.³⁴ However, there is complete lack of homology in a statistical comparison of these two sequences. The Bt-M peptide is able to completely absorb the IgE reactivity against both the 14 and 11-13 kd components of the *B. tropicalis* extract, which, in addition to the molecular weight of the peptide, makes it difficult to define, with the available data, to which native allergen the recombinant molecule corresponds.

Our finding is of clinical interest because of the relevance of *B. tropicalis* as a cause of allergic respiratory diseases in several tropical and subtropical areas of the world and because the recombinant peptide shares epitopes with three of the most frequent allergens of this mite, which gives this peptide an important clinical potential. As previously suggested,³⁵ allergens of *B. tropicalis* should be included among the reagents used for diagnosis and treatment of allergic respiratory diseases in regions where the mite is prevalent and clinically important. In addition, the cloning of cDNA coding for an epitope or epitopes possessed by allergens of this mite is an important step toward characterizing its allergenic components. Because Bt-M seems to be a partial clone, plans for extension of this clone include the use of a Bt-M-derived probe for recloning the cDNA library and polymerase chain reaction primers based on the Der p 5 sequence along with a 3' sequence from Bt-M.

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