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Cloning and IgE Binding of a Recombinant Allergen from the Mite *Blomia tropicalis*, Homologous with Fatty Acid-Binding Proteins

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Key Words

Blomia tropicalis
 Allergens
 Allergy
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 Immunoglobulin E

Abstract

To characterize the allergens of *Blomia tropicalis*, a cDNA library was constructed and screened with allergic sera from asthmatic patients. One clone, Bt6, was subcloned and sequenced. The nucleotide sequence of 934 bp length shows a 390-bp reading frame which encodes a 130-amino acid protein with a MW 14.8 kD. No potential glycosylation site was found in the predicted protein. The inferred amino acid sequence has no homology to known allergens. It has a cytosolic fatty acid-binding protein (FABP) signature at 5–22 amino acid residues, 42.3% identity with the Sm14-FABP of *Schistosoma mansoni* and 36% identity with FABPs from rat, mouse, bovine and human. The protein was expressed as a GST fusion protein and the purified GST-Bt6 used for dot blot, RAST and RAST inhibition assays. The frequency of IgE binding of allergic sera to Bt6 was low (11%) and usually weak. One positive serum did, however, show strong reactivity by RAST and dot blot and Bt6 could inhibit 60% of the IgE binding of this serum to the *B. tropicalis* extract. These data show that Bt6 encodes a mite FABP with allergenic properties, which are pronounced in some atopic subjects.

Introduction

The mite *Blomia tropicalis* is an important source of allergens inducing allergic asthma (AA) in tropical regions. Some reports indicate that, while *B. tropicalis* is an abundant allergenic element of house dust, there is only weak cross-reactivity between this and other mites also found in the tropics like *Dermatophagoides pteronyssinus*. Therefore, allergens of *B. tropicalis* should be characterized and be included among the reagents for diagnosis and treatment of allergic respiratory diseases in these areas. Allergens of the *Blomia* mite have been partially characterized by immu-

nochemical techniques [1, 2] and some of them have been cloned and sequenced [3–6].

Mite allergens have several molecular structures and functions [7]. DNA cloning procedures have allowed a better analysis of these proteins, so that besides those characterized as proteolytic enzymes and amylases, some mite allergens share important sequence homology with molecules like heat shock proteins [8], tropomyosin [9], glutathione S-transferase (GST) [10], epididymal protein homologue [11] and paramyosin [12]. Here we describe a recombinant allergen of *B. tropicalis* with important amino acid sequence identity to several fatty acid-binding proteins.

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Materials and Methods

Culture

B. tropicalis mites isolated from house dust samples from homes of allergic patients resident in Cartagena [13] were deposited in a 500-ml Erlenmeyer flask with culture medium (fish food/brewer's yeast, 1:1) and kept at 27°C and 85% relative humidity. Adult mites were separated from the medium using a modified Tullgren apparatus [14], immediately frozen in liquid N₂ and kept at -80°C before RNA isolation. For RAST inhibition studies, the extract of *B. tropicalis* was prepared in our laboratory as described previously [15]. Lyophilized extract was reconstituted in water to obtain a concentration of 3.5 mg/ml.

Subjects and Serum Samples

A total of 95 sera from non-immunotherapy-treated AA patients highly allergic to *B. tropicalis* were used in this study. Fifty of these sera were used to establish the IgE-binding frequency of the recombinant protein cloned in lambda phage and to make the serum pool for library screening. The sera were previously characterized by RAST and immunoblotting with a standardized *B. tropicalis* body extract [2]. Another set of 45 sera was used for RAST with Bt6 expressed as a GST fusion protein (GST-Bt6). All the allergic sera were donated by asthmatic patients who were skin test-positive to a *B. tropicalis* extract (1:50 wt/vol). AA was diagnosed following the criteria proposed by the expert panel of the Allergy Foundation of America [16], namely, recurrent episodes of wheezing or dyspnea; objective evidence of abnormality in pulmonary function during episodes, as well as positive skin tests. In addition, all patients had a clear history of atopic symptoms related to house-dust exposure and were negative when skin-tested with a *B. tropicalis* culture medium extract. All donors were informed volunteers. Control sera come from nonallergic subjects with negative skin test and RAST to *B. tropicalis*.

Construction of the *B. tropicalis* cDNA Lambda gt11 Library

Total RNA (1.9 mg) was isolated from 1 g of frozen *B. tropicalis* using a commercial version (RNAzol-B, Cinna/Biotech Lab. International, Inc., Friendwood, Tex., USA) of the guanidinium thiocyanate method [17]. mRNA (10 µg) was obtained following adsorption to a cellulose poly(dT) column (Pharmacia LKB Biotechnology, Piscataway, N.J., USA). cDNA was synthesized from 5 µg 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 lambda gt11D (Pharmacia P-L Biochemicals, Piscataway, N.J., USA). The ligated DNA was packaged in *Escherichia coli* Y1090 and plated to produce a library having 3.5 × 10⁴ recombinants.

Isolation of *B. tropicalis* Allergen cDNA Clones from the cDNA lambda gt11 Library

Screening of the library was performed by plaque radioimmunoassay [18] using an adsorbed (*E. coli*/Y1090) pool of 20 sera from the asthmatic patients described above. Phages were plated at 14,000 plaque-forming units (pfu) per 150-mm Petri dish. Plaques were lifted onto nitrocellulose filters (Amersham, Ill., USA) soaked in isopropyl-β-D-thiogalactopyranoside (IPTG). The filters were blocked for 1 h using a 10% solution of skimmed milk powder and were washed 3 times in sodium phosphate buffer 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% BSA/0.02% sodium azide. The filters were then washed 3 times in PBST and once with PBS, and incubated overnight with ¹²⁵I-labelled anti human IgE (Kallestad Diagnostic, Inc., Chaska, Minn., USA) at 2 × 10⁶ cpm/ml, with rocking. After three washes in PBST and one with PBS, the filters were subjected to autoradiography for 24 h (Kodak X-OMAT with intensifying screens) at -70°C.

Isolation of DNA from gt11 cDNA Clones

Phages from immunopositive plaques were amplified by the liquid lysate method [19]. DNA was prepared from lambda gt11 clones using the Magic Lambda Preps DNA Purification System kit (Promega, Madison, Wisc., USA) and digested with *EcoRI* and *NotI*. The insert of clone Bt6 (approximately 900 bp) was selected for subcloning.

Subcloning of Bt6 Inserts into Plasmid Vectors

The insert from *EcoRI* and *NotI* digest of lambda DNA was purified with the Magic PCR Preps kit, ligated to pBlueScript vector (Stratagene, La Jolla, Calif., USA) and transformed into *E. coli* XL1-Blue (Stratagene) according to Sambrook et al. [20]. Color selection of the transformed clones was done on LB (Luria-Bertani) plates containing 100 µg/ml of ampicillin, 12.5 µg/ml of tetracycline, 80 µg/ml of X-gal and 20 mM IPTG. Recombinant clones were confirmed by restriction enzyme analyses of miniprep from transformed XL1-Blue. The purified insert from *EcoRI* and *NotI* was also ligated to pGEX-4T-3 vector (Pharmacia Biotech) and transformed into *E. coli* BL21. Recombinant clones were confirmed by restriction analysis of a miniprep from transformed BL21. The expression of Bt6 as a fusion protein with GST was induced with 1 mM IPTG and purification from bacterial lysate was done over glutathione Sepharose (Pharmacia Biotech) followed by elution with 10 mM reduced glutathione as suggested by the GST Purification Module (Pharmacia Biotech). Digestion with thrombin (10 units/mg protein, for 18 h at room temperature) released the recombinant 17-kD Bt6 protein, which was collected in the flowthrough after further purification over glutathione Sepharose (fig. 1). The yield of the fusion protein was 0.7 mg/l. The IgE-binding to the fusion protein (GST-Bt6) and to Bt6 was established by immunoblotting using a patient's serum and ¹²⁵I-labelled horse antihuman IgE. IgE-binding was also detected by RAST and dot blot. GST was also produced and purified from pGEX-4T-3 transformed *E. coli* BL21.

DNA Sequencing and Analysis

cDNA sequencing of clone Bt6, subcloned into pBlueScript, was done in both directions by PCR-dideoxy sequencing methodology, based on the dideoxynucleotide chain-termination method of Sanger et al. [21]. The complete sequence was obtained using T3, T7 and internal primers. The sequence was confirmed when clone Bt6, subcloned into pGEX-4T-3, was also sequenced using the appropriate primers for this vector.

IgE-Binding Frequency of the Recombinant Allergen Bt6

IgE-binding frequency was first determined by plaque radioimmunoassay. The recombinant lambda gt11 was densely plated and plaques with recombinant beta-galactosidase fusion protein were lifted onto nitrocellulose filters (Amersham) soaked in IPTG. Lifts were cut into several sectors and probed with the sera described above. Bound serum IgE was detected with ¹²⁵I-labelled horse antihuman IgE (Kallestad Diagnostics Inc). In addition, the IgE-binding frequency of Bt6 was confirmed by RAST, using a purified GST-Bt6 fusion protein.

Dot Blot Immunoassay

To better define the pattern of reactivity of allergic sera against GST-Bt6, GST and Bt6, dot blot analysis was done with 5 sera (A21, A217, CB6, A242, A118) that were reactive to Bt6 expressed as a gt11-recombinant protein; a serum from a nonallergic control (CAR106), a serum allergic to Bt but RAST-negative to Bt6 (CB34), a serum highly allergic (ST) to grass pollen but negative to mites (DGB) and PBS. Each serum was adsorbed (overnight 1:10 dilution) with a lysate of noninduced *E. coli* BL21 transformed with pGEX-4T-3. Dot blot analysis was performed as follows: nitrocellulose was cut to size, soaked in PBS (pH 7.4) and placed on 3-mm Whatman paper before being clamped into a BioDot apparatus (Bio-Rad Laboratories, Richmond, Calif., USA). Samples containing approximately 10 µg of GST-Bt6, GST, a lysate of noninduced pGEX-4T-3 transformed *E. coli* cells, *B. tropicalis* extract and 3 µg of purified Bt6 recombinant protein were applied in a volume of 200 µl and drawn through the nitrocellulose by suction. Each well was washed with 200 µl of PBS. The nitrocellulose was then washed with 10 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl and 0.1% Tween-20 and incubated with sera diluted 1:5 in PBS-0.1% BSA, overnight at room temperature. Then, the nitrocellulose was washed 3 times for 4 min with PBS-0.1% Tween-20, and once with PBS. Strips from the nitrocellulose membrane were then incubated with ¹²⁵I-labelled horse antihuman IgE (100,000 cpm/strip), overnight at room temperature. Autoradiography was done overnight with intensifying screens at -70°C.

RAST and RAST-Inhibition Assays

RAST for specific IgE of sera to *B. tropicalis* extract was performed as described elsewhere [22]. A RAST with purified GST-Bt6 was standardized using the set of 45 sera described above. Four positive and 4 negative sera selected from the screening with plaque radioimmunoassay, as well as PBS were used as controls. Microtiter plates (Immulon-4 Dynatech Laboratories, Alexandria, Va., USA) were coated with 0.5 µg/100 µl of purified GST-Bt6 in 0.2 M sodium carbonate/bicarbonate, pH 9.2 and incubated overnight at room temperature. After washing 3 times with PBS-0.1% Tween-20, 100 µl of serum, diluted 1:5, were added to each well and incubated overnight in a humid chamber. This was followed by three washes and overnight incubation with 100 µl of ¹²⁵I-labelled horse antihuman IgE (25,000 cpm/well). The radioactivity of each well was then counted in a gamma counter (Gamma 5500, Beckman) and the results expressed as the mean of total counts bound (TCB). RAST was also done to the same sera, using GST. The RAST was considered positive when a serum bound more than 1% of TCB.

To evaluate the IgE-binding capacity of Bt6, relative to that of the native *B. tropicalis* extract, RAST inhibition assays were performed with *B. tropicalis* extract in the solid phase using a serum (A21) with high levels of specific IgE to both *B. tropicalis* extract (48% TCB) and Bt6 45% TCB). The serum was adsorbed with a lysate of the strain BL21 of *E. coli* transformed with the plasmid pGEX-4T-3, with rotation overnight at 4°C and then clarified by centrifugation. Microtiter wells were coated with 5 µg of *B. tropicalis* extract in 100 µl of coupling buffer (0.2 mol/l) sodium carbonate/bicarbonate buffer, pH 9.2) and the optimal working dilution was established by serial dilutions of the serum. A dilution of 1:50 was used for the RAST-inhibition to avoid antibody excess in the fluid phase and enhance the sensitivity of the assay. One hundred microliters of diluted serum was adsorbed (overnight, with rotation at 4°C) with 10 µl of water containing increasing amounts of the GST-Bt6 fusion protein (0.5, 2.5, 5.0, 7.0, 12.5 and 25 µg) or of negative control GST at the same amounts, and incubated

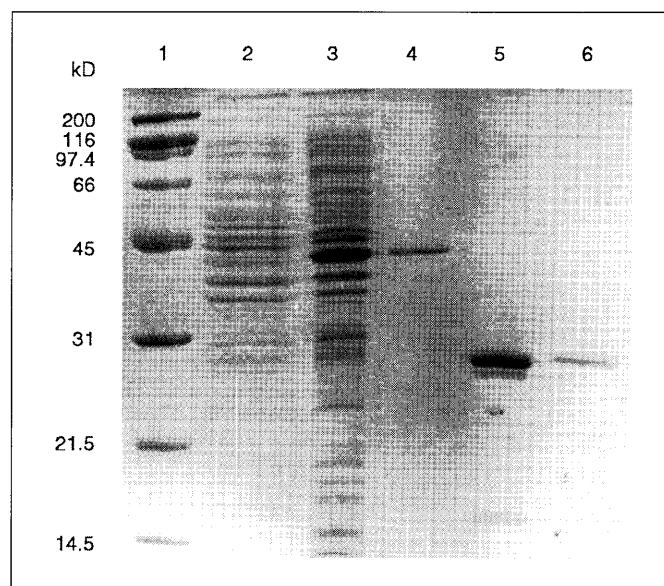


Fig. 1. SDS-PAGE (15%, reducing conditions) analysis of recombinant Bt6 protein. Bt6 was expressed as a fusion with GST in pGEX-4T-3. The gel was stained with Coomassie brilliant blue R-250. Lane 1 = Molecular mass markers (Bio-Rad Laboratories, Life Science Group, Hercules, Calif., USA); lanes 2, 3 = lysate of *E. coli* transformant before and after IPTG induction; lane 4 = fusion protein purified by glutathione affinity chromatography; lane 5 = fusion protein cleaved with thrombin to separate into a 28-kD GST and a 17-kD protein; lane 6 = purified GST.

overnight, at room temperature, with the *B. tropicalis* extract. After washing, the presence of specific IgE was detected by incubation overnight with 100 µl containing 25,000 cpm of ¹²⁵I-labelled horse antihuman IgE (Allercoat, Kallestad). The radioactivity of each well was then quantified in a gamma counter. Each experimental determination was carried out in duplicate and the mean of counts per minute used. The percent of inhibition was calculated as follows: % inhibition = cpm without inhibitor - cpm with inhibitor / cpm without inhibitor.

Results

Bt6 Is Homologous to Fatty Acid-Binding Proteins (FABP)

The complete nucleotide sequence of Bt6 (GenBank Accession number U58106) is 934 bp in length (fig. 2). This includes a 5' noncoding region of 37 bp, a 505-bp 3' untranslated region and an open reading frame of 390 bp with a stop codon (TAG) at nucleotide residues 428-430. A putative polyadenylation signal was found between nucleotide residues 892 and 897. The open reading frame includes a 130-amino acid protein with a calculated *M_r* of 14.8 kD and pI of 5.4. The Met residue (ATG) at amino acid position 38 is the most likely translation initiation site. No potential glycosylation

[illegible]

site was found in the predicted protein. The deduced Bt6-peptide has a cytosolic FABP signature at amino acid residues 5–22 (fig. 2), as detected by the Prosite program of PC/Gene (IntelliGenetics, Inc., Mountain View, Calif., USA). A search, in the databases of the National Center for Biotechnology Information (NCBI), using the BLAST network server showed that the inferred amino acid sequence has no homology to known allergens. It has 35.3% identity (45% similarity) with FABPs from rat, mouse, bovine and human (fig. 3A). Furthermore, it has 42.3 (fig. 3B), 30, and 33% identity with FABPs from *Schistosoma mansoni* (Sm14), *Fasciola hepatica* (Fh15) and with the psoriasis-associated FABP, respectively.

Results of plaque radioimmunoassay for IgE-binding show that beta-galactosidase-Bt6 fusion protein reacted with 10% of allergic sera and with 0% of controls. Results of IgE-binding frequency using RAST show that GST-Bt6 fusion protein reacted with 11% of allergic sera and 0% of controls. The strength of the signals of sera in the autoradiog-

Bt6 from GST-Bt6 Digestion with Thrombin Retains IgE-Binding Capacity

A.

BT6PRO	M-PIEGKYKLEKSDNFDKFLDELGVGFMVKTAAKTLKPTLEVDVQGDITYV	49
FABHRAT	ADAFVGTWKLVDKSNFDDYMKSLGVGFATRQVASMTPPTIIIEKNGDTIT	50
FABHMOUSE	ADAFVGTWKLVDKSNFDDYMKSLGVGFATRQVGSMTKPTTIIIEKNGDTIT	50
FABHBOVIN	VDAFVGTWKLVDKSNFDDYMKSLGVGFATRQVGNMTKPTTIIIEKNGDTVI	50
FABHHUMAN	VDAFLGTWKLVDKSNFDDYMKSLGVGFATRQVASMTPPTIIIEKNGDILT	50
	..*..**.*.***.....*****..***..**..	
BT6PRO	FRSLSTFKNTEIKFKLGEEFEEDRADGKRVKTVVNKEGDNKFIQTQ-YGD	98
FABHRAT	IKTHSTFKNTEISFQLGVFEFDEVTADDRKVKSVVTLDDGG-KLVHVQKWDG	99
FABHMOUSE	IKTQSTFKNTEINFQLGIEFDEVTADDRKVKSLVTLDDGG-KLIHVQKWDG	99
FABHBOVIN	IKTQSTFKNTEISFKLGVEFDETTADDRKVKSIIVTLDDGG-KLVHVQKWNK	99
FABHHUMAN	LKTHSTFKNTEISFKLGVEFDETTADDRKVKSIIVTLDDGG-KLVHLQKWDG	99
	...*****.*..**.*..**.*..**.*..**.*..**.*..**.*..	
BT6PRO	KEVKIVRDFQGGDDVVVTASVGDVTSVRTY-KRI	130
FABHRAT	QETTLTRELSDGKLILTLTHGNVSVTRTYEKEA	132
FABHMOUSE	QETTLTRELVDGKLILTLTHGSVVSVTRTYEKEA	132
FABHBOVIN	QETSLVREMVDGKLILTLTHGTAVCTRTYEKQA	132
FABHHUMAN	QETTLVRELIDGKLILTLTHGTAVCTRTYEKEA	132
	..*..*..*..*..*..*..*..*..*..*..*..*..*..*..*..*..	

Fig. 3. A Protein sequence homology of the deduced amino acid sequence of Bt6 and the FABPs from rat, mouse, bovin and human. The alignment includes all the residues of the proteins with a consensus length of 133. Identical amino acid residues are demonstrated by an asterisk. Similarity between amino acid residues is indicated as a period. The overall identity is 35% and similarity 45.1%. **B** Protein sequence homology of the deduced amino acid sequence of Bt6 and the *S. mansoni* FABP (antigen Sm14). The alignment includes all the residues from both proteins. Identical amino acid residues are demonstrated by an asterisk. Similarity between amino acid residues is indicated as 's'. The two proteins show 42.31% of identity and 14.62% of similarity.

B.

BT6PRO	- MP-IEGKYKLEKSDNFDKFLDELGVGFMVKTAAKTLKPTLEVDVQGDITYV -49
	* **s** * **s * **s s **s **s s **
SM14	- MSSFLGKWLSESHNFDVMSKLGVSATRQIGNTVPTVTFMTMDGDKMT -50
BT6PRO	- FRSLSTFKNTEIKFKLGEEFEEDRADGKRVKTVVNKEGDNKFIQTQYGDK -99
	s ***** ** **s**s * s**s **s** * s * **s *
SM14	- MLTESTFKNLSCTFKFGEEFDEKTS DGRNVKSVVEKNSESKLTQTQVDPK -100
BT6PRO	- EVK-IVRDFQGGDDVVVTASVGDVTSVRTYKRI -130
	s ** s * s* ss ***s
SM14	- NTTVIVREVGDGTMKTTVTVDVIAIRNYKRLS -133

The IgE-Binding Capacity of Bt6 is High When Evaluated with Serum A21

GST-Bt6 fusion protein was assayed for its ability to inhibit the IgE binding of serum A21 to *B. tropicalis* extract using RAST inhibition. The adsorption of serum with the recombinant allergen resulted in a dose-dependent inhibition of IgE antibody binding to *B. tropicalis* extract. Fifty percent inhibition (1,968 cpm) was obtained with 7 µg of GST-Bt6. An important inhibition of IgE-binding (62%, 1,470 cpm) was achieved with 12.5 µg of recombinant; the cpm value without inhibitor was 3,997. PreadSORption with the same amounts of GST yields no significant inhibition of IgE binding to the *B. tropicalis* extract (fig. 5). PreadSORption of a pool of four *B. tropicalis*/Bt6-reactive sera (3,255 cpm) with GST-Bt6 yields 16% of inhibition (2,730 cpm) of IgE binding to the *B. tropicalis* extract. The result of adsorption with GST was the same as for A21 serum. The inhibitory capacities of different concentrations of *B. tropicalis* extract using *B. tropicalis* allergens on the solid phase reached 90% inhibition at the highest concentration used for both A21 and the pool of sera (not shown).

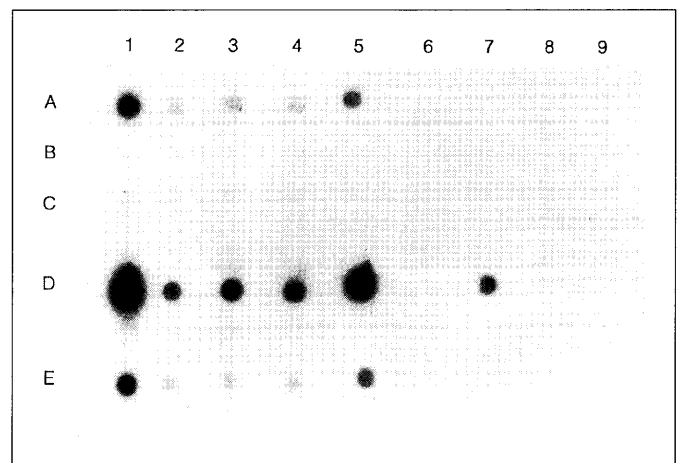


Fig. 4. Autoradiography of dot blot assay. Each lane was loaded with the following reagents: A = GST-Bt6 fusion protein; B = GST; C = lysate of noninduced pGEX-4T-3 transformed BL21; D = *B. tropicalis* extract and E = Bt6 recombinant allergen, obtained after thrombin digestion of the GST-Bt6 fusion protein. The lanes were incubated with the following sera: 1 = A21; 2 = A217; 3 = CB6; 4 = A242; 5 = A118; 6 = CAR106; 7 = CB34; 8 = GDB; 9 = PBS-BSA/01%. The exposure time of autoradiography was 24 h for all sera but A217, that was exposed 3 days.

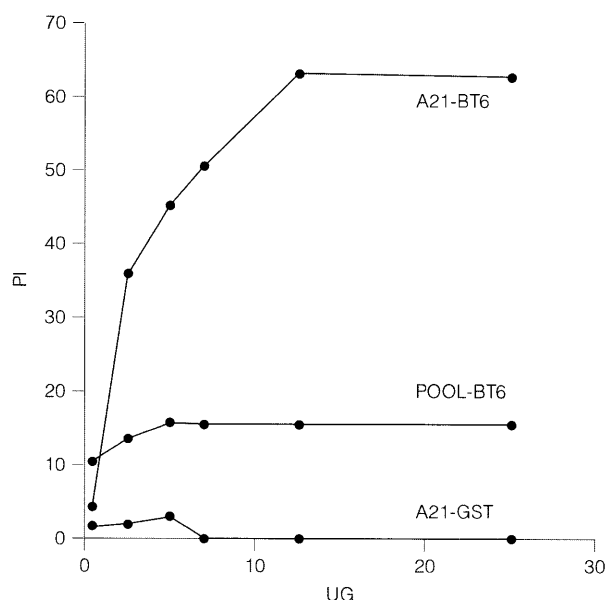


Fig. 5. Dose-response curve from RAST inhibition assays. PI = Percent of inhibition; UG = micrograms of inhibitor. *B. tropicalis* extract is in solid phase. The inhibition assays were performed with the following sera and inhibitors: serum A21 adsorbed with GST-Bt6 (A21-Bt6); pool of *B. tropicalis*/Bt6-reactive sera adsorbed with GST-Bt6 (POOL-Bt6) and serum A21 adsorbed with GST (A21-GST).

Discussion

Our data show that the clone Bt6 isolated from a library of *B. tropicalis* encodes a mite FABP with allergenic properties. The IgE-binding frequency of Bt6 protein is low, which suggests that it is a minor allergen. However, its IgE-binding capacity to the native *B. tropicalis* extract is important because it produces 62% of inhibition in RAST-inhibition experiments using 1 patient's serum, which suggests that this allergen is clinically significant to some patients, and that the equivalent native allergen is present in the extract. Although we performed the RAST with the GST-Bt6 fusion protein, the results are supported by the fact that no serum was RAST-positive to GST. In addition, the isolated recombinant Bt6 also binds IgE and could be used as a diagnostic reagent in RAST and skin test, in combination with other cloned allergens from *B. tropicalis* [3–6].

The high level of identity between Bt6 and FABPs is the first reported for a mite allergen. FABPs are involved in the intracellular transport of long chain fatty acids and their acyl-CoA esters. Several FABPs of potential interest in ex-

perimental allergy have been described. One of them is the ABA-1 allergen of *Ascaris suum* [23], however, there is no homology at the protein level between this allergen and Bt6. Other parasite antigens such as a putative vaccine candidate for schistosomiasis from *Schistosoma japonicum* [24] and *S. mansoni* (Sm14) [25]), as well as the Fh15 antigen of *F. hepatica* [26] are FABPs. In addition, a psoriasis-associated fatty acid-binding protein (PA-FABP) has been described as a new member of low-molecular-weight proteins that are highly up-regulated in psoriatic skin and that share similarity to fatty acid-binding proteins [27].

A possible synergist effect of GST of *S. mansoni* upon IgE response to the homologous *D. pteronyssinus* allergen in parasited subjects has been proposed [10]. Our finds suggest a similar immunological relationship could exist in tropical areas where parasites and *B. tropicalis* are endemic. However, there is neither schistosomiasis nor fasciolopsiasis in the Caribbean coast of Colombia. In addition, we did not find structural homology between Bt6 and ABA-1 from *Ascaris*. Therefore, it seems that the low frequency of sensitization to Bt6 observed among our asthmatic patients is mainly derived from exposure to *B. tropicalis* allergen. In this context the potential role of Bt6, as both individual and cross-reacting allergen, in the pathogenesis of some immunological diseases deserves further studies. Since the homology of Bt6 with FABPs is high, it can be assumed that its native equivalent has fatty acid-binding properties in the mite. Although this activity should be confirmed experimentally, so far this is the only allergen described from *B. tropicalis* whose biological function other than as an allergen is suggested.

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