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Short communication

IgE, IgG1, and IgG4 antibody responses to *Blomia tropicalis* in atopic patients

Background: Allergens from house dust mites (HDMs), *Dermatophagoides pteronyssinus* and *Blomia tropicalis* are clinically relevant in atopic respiratory diseases in tropical countries.
Aims of the study: To evaluate immunoglobulin (Ig)E, IgG1, and IgG4 antibody responses to *B. tropicalis* in Brazilian atopic patients.
Methods: About 110 patients with allergic rhinitis with/without asthma and 33 control subjects underwent skin prick testing (SPT) with HDM extracts, and their sera were tested for IgE and IgG subclass antibodies to *D. pteronyssinus* and *B. tropicalis* by enzyme-linked immunosorbent assay (ELISA) and immunoblotting.
Results: Most patients (56%) had positive SPT to *B. tropicalis* extract (*B. tropicalis*+ group), although 51% were reactive to both *B. tropicalis* and *D. pteronyssinus* and 6% were sensitized to *B. tropicalis* only. IgE-ELISA detected 43% *B. tropicalis* positivity with high-specific IgE levels in *B. tropicalis*+ patients. Specific IgG4 levels were higher in *B. tropicalis*+ than *B. tropicalis*− groups and correlated with specific IgE levels. The IgG1 levels to *B. tropicalis* were higher in patients than controls. The major allergenic *B. tropicalis* components recognized by *B. tropicalis*+ patient sera were the 54, 66, and 68 kDa proteins. The IgG4-binding protein profiles closely resembled that of IgE. The IgG1 antibodies recognizing multiple *B. tropicalis* protein species were detected in sera of all three patient groups.
Conclusions: A large percentage of our allergic patients are *B. tropicalis*+. They are more frequently sensitized to high-molecular weight (HMW) *B. tropicalis* components than the major low-molecular weight (11–15 kDa) allergens detected in other studies. The results suggest that HMW *B. tropicalis* antigenic components are potential candidates for evaluating allergen exposure and sensitization, and for immunotherapy treatment.

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The role of house dust mites (HDMs) as an allergen source and their contribution in allergic diseases, particularly asthma and rhinitis, has been recognized for many years (1). *Dermatophagoides pteronyssinus*, *D. farinae*, and *Blomia tropicalis* are predominant mites in house dust samples in tropical countries (2). Concurrent sensitization can occur in houses where both *D. pteronyssinus* and *B. tropicalis* are found, thus complicating etiologic diagnosis and therapeutic treatment in atopic patients. Although inhibition studies have suggested that *B. tropicalis* allergens are species-specific, antibody cross-reactivity with *D. pteronyssinus* antigens was estimated at approximately 30% (3). An important allergen from *B. tropicalis* (Blo t 5) has 43% sequence homology to Der p 5 allergen from *D. pteronyssinus*, while no cross-reactivity was found between groups 1 and 2 allergens from *D. pteronyssinus* and other *B. tropicalis* allergens (1, 3).

The role of immunoglobulin (Ig)G subclasses to HDM allergens in the pathogenesis of allergic diseases remains unclear. The IgG4 has been found to have a protective role attributable to prolonged natural antigenic exposure and antigen neutralization, or IgE blocking. This protective activity may be a significant underlying mechanism for the efficacy of immunotherapy (4, 5).

This study was aimed at evaluating the IgE, IgG1, and IgG4 antibody responses to *B. tropicalis* in patients with allergic rhinitis (AR) with/without asthma sensitized to *B. tropicalis* alone or concomitantly to both *B. tropicalis* and *D. pteronyssinus*. The IgE-, IgG1-, and IgG4-binding proteins derived from *Blomia* were also investigated by immunoblotting.

Material and methods

Subjects and skin prick test

A total of 110 adult patients with AR with/without intermittent or persistent mild-to-moderate asthma were selected on the basis of the history of respiratory symptoms related to house dust exposure, physical examination, and spirometry (6) at the Allergy and Clinical Immunology Unit, Federal University of Uberlandia, Brazil. Patients underwent skin prick testing (SPT) with *B. tropicalis* and *D. pteronyssinus* extracts, and a 3×3 mm-wheal size was considered positive SPT. Thirty-three healthy adult subjects were included as control group. In parallel, blood samples were collected from all subjects for serologic testing. The study was approved by the Ethics Committee in Human Research at the Federal University of Uberlandia and informed consent was obtained from all individuals.

Mite extracts

Blomia tropicalis and *D. pteronyssinus* crude extracts were obtained from mite bodies provided by Dr Federico Montealegre (Ponce School of Medicine, Porto Rico, USA). Briefly, mite powder was triturated in liquid nitrogen and allergens were extracted in 0.1 M borate-buffered saline (pH 8.0) containing protease inhibitors (Cϕmplete^{TM}, Roche Diagnostics, Mannheim, Germany), and protein concentration was determined by Bradford assay.

IgE-, IgG1-, and IgG4-ELISA to *B. tropicalis*

The IgE-, IgG1-, and IgG4-enzyme-linked immunosorbent assay (ELISA) to *B. tropicalis* were performed as described (7), with modifications. Briefly, microtiter plates were coated with *B. tropicalis* crude extract (0.5 μg/well) and incubated with serum samples diluted 1 : 2 (IgE) or 1 : 5 (IgG1 and IgG4). Biotinylated antihuman IgE (Kirkegaard & Perry, Gaithersburg, MD, USA), IgG1, or IgG4 (Sigma, St Louis, MO, USA) were added followed by streptavidin-peroxidase. The ABTS was used for color development. The IgE titers were arbitrarily expressed as ELISA index (EI) as described (8). The IgG1 and IgG4 titers were expressed as arbitrary units (U/ml) obtained by comparison with a positive serum (subject GAOS) assigned to contain 1000 U/ml of IgG1 and IgG4 anti-*B. tropicalis*.

Competitive inhibition ELISA

The IgE-, IgG1-, and IgG4-ELISA specificity was evaluated using a competitive inhibition assay as described (7). Results were expressed as percentage of inhibition and calculated as follows: $[1.0 - (\text{sample OD}/\text{positive control OD})] \times 100$.

SDS-PAGE and IgE-, IgG1-, and IgG4-immunoblot for *B. tropicalis*

Blomia tropicalis extract (200 μg of protein) was fractionated in 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to nitrocellulose membranes as described (9, 10). Strips were blocked with 5% skim milk, incubated with serum samples diluted 1 : 2 (IgE), 1 : 10 (IgG1) or 1 : 5 (IgG4), and blotted with biotinylated antihuman IgE, IgG1, or IgG4 secondary antibodies followed by streptavidin-peroxidase. Bands were visualized by 3,3′-diaminobenzidine (Sigma Fast, Sigma-Aldrich, St Louis, MO, USA).

Statistical analysis

Differences in geometric mean values of antibody levels in patient groups were analyzed by the Mann–Whitney test. Correlation between *B. tropicalis*-specific IgE and IgG subclass levels was determined using the Pearson's correlation test. Frequencies of antigenic bands recognized by different antibodies were compared using analysis between two proportions by Z-statistics. Differences were considered statistically significant when $P < 0.05$.

Results

SPT to mites

Sixty-two (56.4%) patients had positive SPT to *B. tropicalis*, 56 (50.9%) were positive to both mites, and six (5.5%) were positive to *B. tropicalis* only. Of 48 patients with negative SPT to *B. tropicalis* (43.6%), 14 (12.7%) were reactive to *D. pteronyssinus* only and 34 (30.9%) showed no reactivity to either mite (Table 1).

IgE, IgG1, and IgG4 levels to *B. tropicalis*

Mean *B. tropicalis*-specific IgE levels (Fig. 1A) were significantly higher in *B. tropicalis*+ (EI = 11) than *B. tropicalis*− (EI = 0.9), or control (EI = 0.8) groups ($P < 0.0001$). The IgE-ELISA was positive in 42 (67.7%) *B. tropicalis*+ patients, five (10.4%) *B. tropicalis*− patients, and two (6.1%) control subjects. When *B. tropicalis*-specific IgE levels were compared against SPT reactivity to *D. pteronyssinus* (Fig. 1B), positive IgE-ELISA results were found in 40 (71.4%) *B. tropicalis*+/*D. pteronyssinus*+ patients, and two (33.3%) *B. tropicalis*+/*D. pteronyssinus*− patients. Among *B. tropicalis*− patients, one (7.1%) and four (11.7%) had positive IgE-ELISA in *B. tropicalis*−/*D. pteronyssinus*+ and *B. tropicalis*−/*D. pteronyssinus*− subgroups, respectively. Mean specific IgE levels among the subgroups showed no significant difference ($P > 0.05$).

Mean *B. tropicalis*-specific IgG1 levels (Fig. 1C) were significantly higher in both *B. tropicalis*+ (52 U/ml) and *B. tropicalis*− (43 U/ml) groups than control subjects (29 U/ml) ($P < 0.01$). The IgG4 levels (Fig. 1D) were

Table 1. Skin prick test (SPT) results to *Blomia tropicalis* and/or *Dermatophagoides pteronyssinus* in 110 patients presenting allergic rhinitis (AR) with/without asthma

SPT results	Number (%)
B. tropicalis+	62 (56.4)
B. tropicalis+/*D. pteronyssinus*+	56 (50.9)
B. tropicalis+/*D. pteronyssinus*−	6 (5.5)
B. tropicalis−	48 (43.6)
B. tropicalis−/*D. pteronyssinus*+	14 (12.7)
B. tropicalis−/*D. pteronyssinus*−	34 (30.9)

+, positive; −, negative.

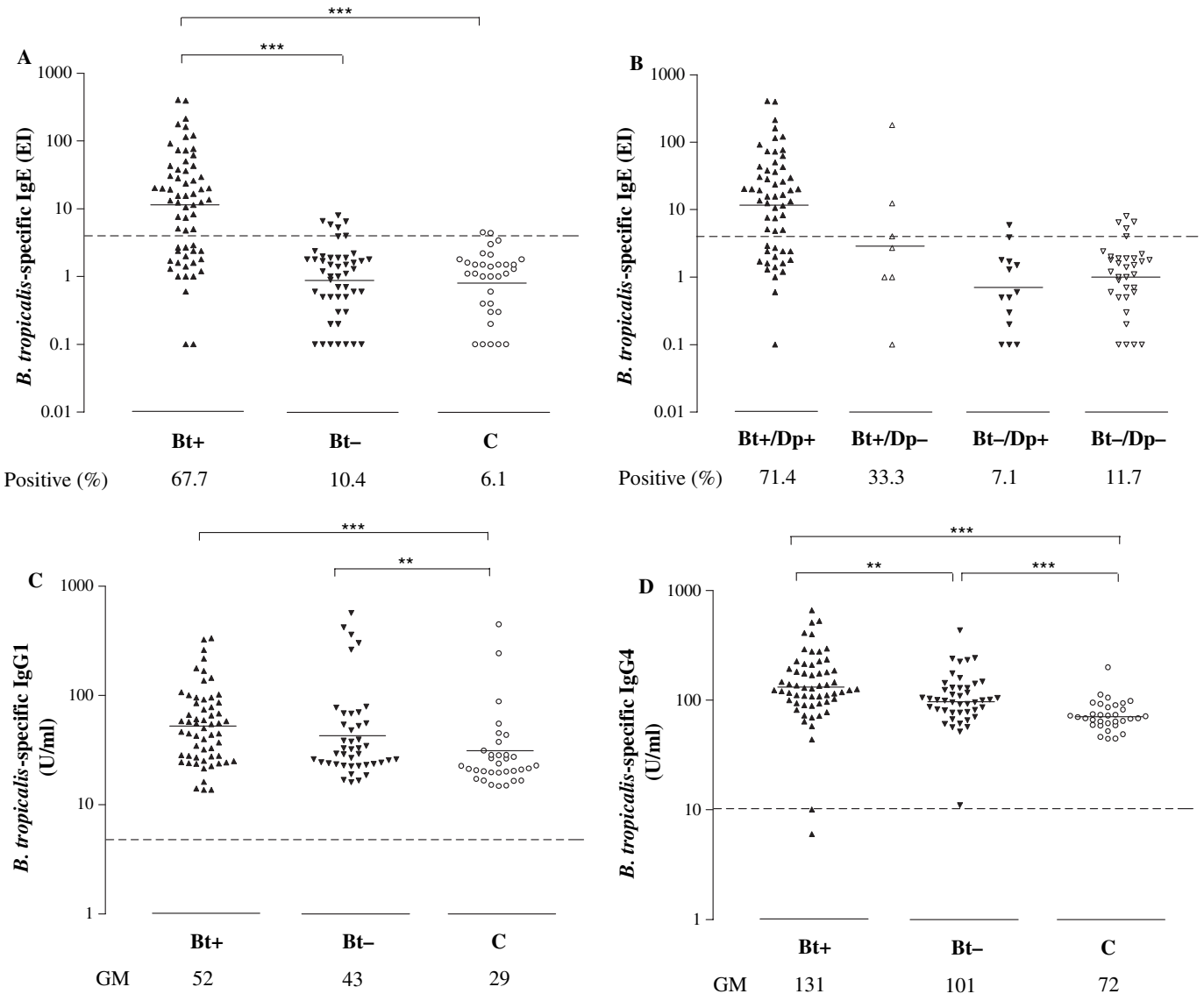

Figure 1. Levels of serum immunoglobulin (Ig)E (A, B), IgG1 (C), and IgG4 (D) to *Blomia tropicalis* (Bt) in allergic rhinitis (AR) patients and control healthy subjects grouped by skin prick test (SPT) reactivities to *B. tropicalis* or *Dermatophagoides pteronyssinus* (Dp). In panels A, C, and D, serum immunoglobulin reactivities in AR patients with positive SPT (*B. tropicalis*+, $n = 62$) and negative SPT (*B. tropicalis*−, $n = 48$) to *B. tropicalis*, and control subjects (C, $n = 33$) are shown. Panel B shows the *B. tropicalis*-specific IgE levels in AR patients with positive SPT to both mites (*B. tropicalis*+/*D. pteronyssinus*+, $n = 56$), positive SPT to a single mite (*B. tropicalis*+/*D. pteronyssinus*−, $n = 6$; *B. tropicalis*−/*D. pteronyssinus*+, $n = 14$), and negative SPT to both mites (*B. tropicalis*−/*D. pteronyssinus*−, $n = 34$). The dashed lines in A and B indicate the cut-off of positivity [enzyme-linked immunosorbent assay index (EI) = 4.0] defined as the mean of EI in controls plus two standard deviations. The dashed lines in C and D indicate the mean detection limit of each assay (5 U/ml for IgG1 and 10 U/ml for IgG4). The horizontal bars represent the geometric mean (GM) obtained for each group. Statistical significance of the differences is indicated by: $^{**}P < 0.01$; $^{***}P < 0.0001$.

significantly higher in *B. tropicalis*+ group (131 U/ml) than *B. tropicalis*− (101 U/ml) and control (72 U/ml) groups ($P < 0.01$). Additionally, *B. tropicalis*− patients had significantly higher IgG4 levels than control subjects ($P < 0.0001$).

Correlation between specific IgE and IgG1 or IgG4 levels was analyzed in patients with both positive SPT and IgE-ELISA. A moderate but significant correlation ($r = 0.55$; $P = 0.0005$) was found only between *B. tropicalis*-specific IgE and IgG4 levels (data not shown).

IgE- IgG1-, and IgG4-ELISA specificity for *B. tropicalis*

The IgE-, IgG1-, and IgG4-ELISA specificity for *B. tropicalis* was demonstrated by competitive inhibition tests using free *B. tropicalis* or *D. pteronyssinus* extracts or tetanus toxoid (TT) as unrelated antigen (Fig. 2). All

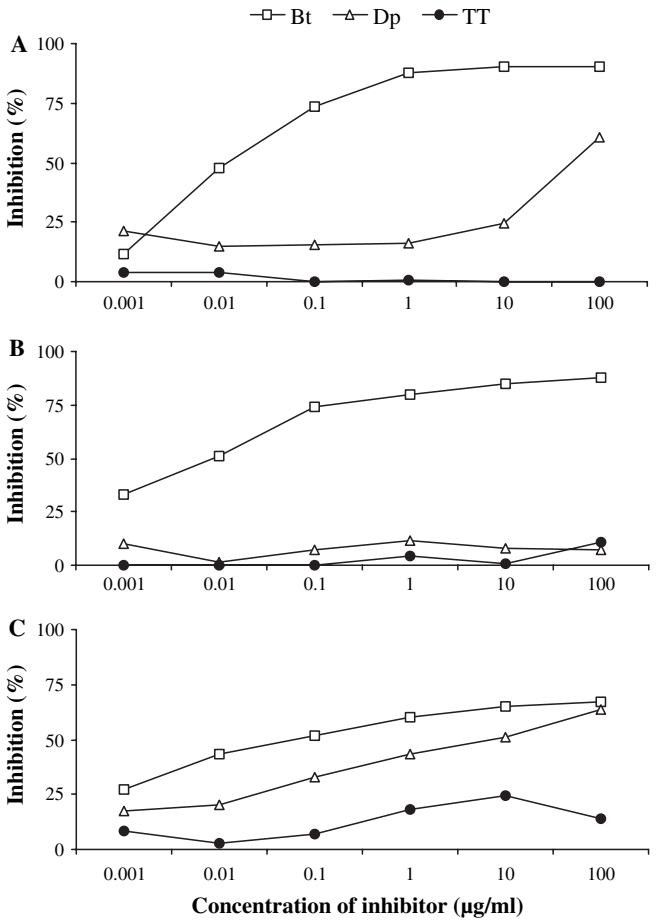

Figure 2. Enzyme-linked immunosorbent assay (ELISA) specificity for immunoglobulin (Ig)E (A), IgG1 (B), and IgG4 (C) to *Blomia tropicalis* (Bt) using competitive inhibition assays. *Blomia tropicalis* and *Dermatophagoides pteronyssinus* (Dp) extracts or tetanus toxoid (TT; negative control) were serially diluted and incubated with a panel of reference sera. The antigen–serum mixtures were assayed in IgE-, IgG1-, and IgG4-ELISA to *B. tropicalis*. Percentage of inhibition was calculated as stated in Methods.

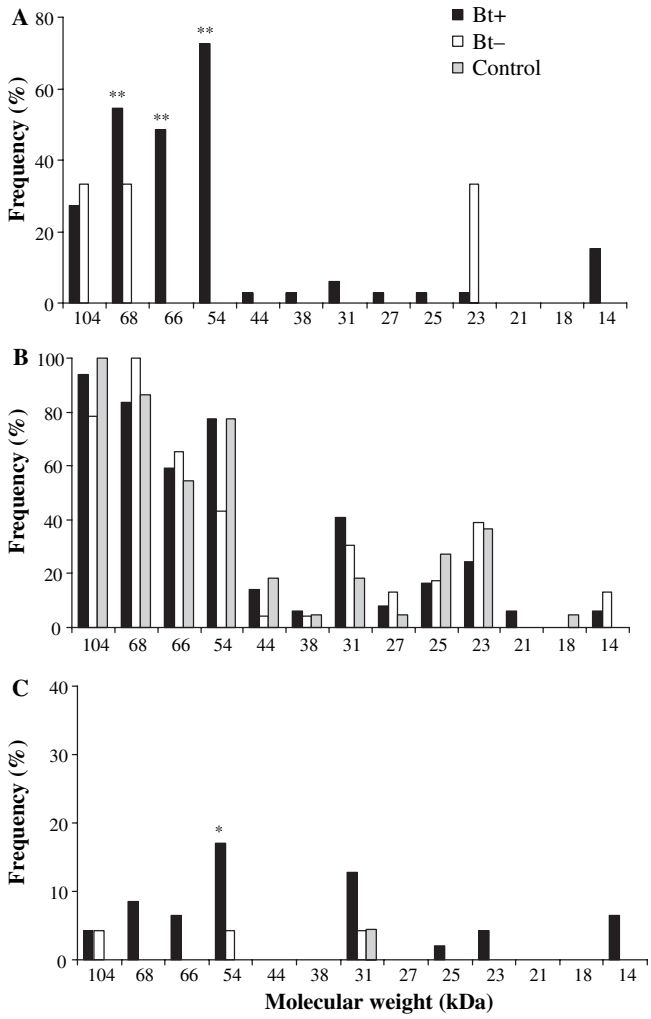

Figure 3. Frequency (%) of antigenic components derived from *Blomia tropicalis* (Bt) recognized by immunoglobulin (Ig)E (A), IgG1 (B), and IgG4 (C) antibodies in immunoblotting in sera of *B. tropicalis*+ patients, *B. tropicalis*− patients, and control individuals. Statistical significance of the differences is shown by: $*P < 0.05$; $**P < 0.001$.

three ELISAs were inhibited in a dose-dependent manner by *B. tropicalis* extract. Cross-reactivity between *B. tropicalis* and *D. pteronyssinus* antigens was seen in IgE-ELISA (Fig. 2A) but only at a high inhibitor concentration (100 µg/ml of *D. pteronyssinus* extract). The IgG1-ELISA specificity to *B. tropicalis* was confirmed in this experiment since *D. pteronyssinus* extract or TT showed only nonspecific inhibitory effects (Fig. 2B). The IgG4-ELISA showed parallel inhibition curves for both *B. tropicalis* and *D. pteronyssinus* extracts even at low inhibitor concentrations (Fig. 2C).

IgE-, IgG1-, and IgG4-immunoblot for *B. tropicalis*

At least 11 IgE-binding *B. tropicalis* proteins ranging from 14 to 104 kDa were recognized by sera from AR patients with positive results for both SPT and IgE-ELISA (Fig. 3). The major IgE-binding components were the 54, 66, 68, and 104 kDa bands, with significant staining frequency differences only for the three first bands when compared with control group ($P < 0.001$) (Fig. 3A). The 14 kDa allergen was detected less frequently but exclusively in this group. A similar profile of IgG4-binding *B. tropicalis* components was seen (Fig. 3C), albeit at lower frequencies. The 54 kDa band staining was also more frequent in *B. tropicalis*+ group when compared with control group ($P < 0.05$). A large spectrum of IgG1-binding *B. tropicalis* components was observed (Fig. 3B) with various antigenic bands in high frequencies among the different groups. Again, the four bands (104, 68, 66 and 54 kDa) referred to as IgE- and

IgG4-binding components, were also recognized with the highest frequencies as IgG1-binding proteins. However, the staining frequencies of all bands were not statistically significant among the different groups.

Discussion

Most AR patients in this study were sensitized to *B. tropicalis*, with 51% presenting with concurrent sensitization to both *B. tropicalis* and *D. pteronyssinus*, and 6% sensitized to *B. tropicalis* only. This concurrent sensitization has complicated the evaluation of the role of *B. tropicalis* in these patients, particularly in this geographic region, where both mites have commonly been found (11). However, a substantial group of patients were sensitized to *B. tropicalis* only, highlighting the need for *B. tropicalis*-hypersensitivity testing and *B. tropicalis*-specific immunotherapy in AR patients with negative SPT to routine inhalant allergens.

Blomia tropicalis-specific IgE levels were significantly higher in *B. tropicalis*+ than *B. tropicalis*− patients and control individuals, suggesting that IgE-ELISA should provide an additional tool for evaluating IgE responses to *B. tropicalis* in AR patients. The IgE-ELISA was found to be relatively specific with respect to cross-reactivities with *D. pteronyssinus*. From 14 *B. tropicalis*−/*D. pteronyssinus*+ patients, only one serum was positive to *B. tropicalis* by IgE-ELISA. In addition, this single cross-reactive serum had low EI values which further validates the specificity of this assay. Arruda et al. (3) demonstrated 33% cross-reactivity of *B. tropicalis* with *D. pteronyssinus* and 43% sequence homology between Blo t 5 and Der p 5 allergens, suggesting that several IgE-binding *B. tropicalis* proteins could have overlapping antigenic reactivities with *D. pteronyssinus* antigens. Tsai et al. (12) showed that there were IgE cross-reactivities between *B. tropicalis* and *D. pteronyssinus*, but IgE binding to two major *B. tropicalis* components (14.3 and 27.3 kDa) could not be inhibited by *D. pteronyssinus* proteins, indicating that several important species-specific *B. tropicalis* allergens need to be further identified.

In this study, *B. tropicalis*-specific IgG subclasses were detected in all groups studied, indicating that these subjects were exposed to repeated *B. tropicalis* antigenic stimuli. Mean-specific IgG1 levels were higher in both *B. tropicalis*+ and *B. tropicalis*− patients than control individuals, suggesting that IgG1 antibodies might be more closely related to allergen exposure. In contrast, IgG4 levels were higher in *B. tropicalis*+ than *B. tropicalis*− groups and showed a moderate but significant positive correlation with specific IgE, indicating that IgG4 could act as potential anaphylactic and amplifying antibodies rather than blocking antibodies in allergies. However, although these observed differences are statistically significant, the results are mostly due to the large group of patients tested and do not seem to be clinically relevant. There are on-going discussions on whether IgG4 is a blocking or an anaphylactic antibody, and whether IgG1 is associated with exposure (5, 13).

In our competitive inhibition studies using a panel of sera with different spectra of antibody responses to *B. tropicalis*, little *D. pteronyssinus* cross-reactivity for IgE, none for IgG1, and parallel inhibition curves for IgG4 were observed. Thus, IgG1-ELISA was highly specific to *B. tropicalis* while IgG4-ELISA showed major cross-reactivity with *D. pteronyssinus*, suggesting that these two isotypes might be recognizing different peptide epitopes.

In IgE immunoblots, the 54, 66, and 68 kDa allergens were the major *B. tropicalis* components recognized in >50% of *B. tropicalis*+ patients, which differs from other reports (12, 14) that have identified the 14 and 11–13 kDa proteins as major *B. tropicalis* allergens. Therefore, our AR patients seem to be preferentially sensitized to high-molecular weight (HMW) components and not to the low-molecular weight (LMW) allergens, in particular to the Blo t 5 (14 kDa), BtM (11–13 kDa), or Blo t 3 (25 kDa) allergens. Additionally, IgE-binding HMW allergens have been reported by Ramos et al. (15) who found that 59% of IgE were reactive with the native Blo t 11 allergen (66 kDa) in total *B. tropicalis* extract. Therefore, the three HMW allergenic components (54, 66, and 68 kDa) should be considered immunodominant peptides in our patients sensitized to *B. tropicalis*. The IgG4 immunoblots showed an antigenic profile comparable with that of IgE immunoblots, indicating a similar Th2 cytokine-mediated switching mechanism. However, immunotherapy studies support a role for IgG4 as blocking antibodies for allergic reactions since specific IgG4 levels rise during allergen therapy (4). In contrast, IgG1-binding *B. tropicalis* proteins were widely recognized by sera of both AR patients and control individuals. Thus, IgE reactivities to HMW *B. tropicalis* antigens are potentially useful for evaluating candidate antigens for allergen exposure, sensitization, and immunotherapy efficacies.

We concluded that *B. tropicalis* is an important dust mite involved in the allergic sensitization of our AR patients. Additional studies are required to better characterize these HMW allergenic components with regard to cross-reactive B epitopes with other major *B. tropicalis* allergens, to determine their functional role in allergen sensitization and pathogenesis of allergic diseases, and to exploit the immunodominant antigenicities of these proteins in immunotherapy.

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