

An extensive study of human IgE cross-reactivity of Blo t 5 and Der p 5

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Background: Dual sensitization by *Blomia tropicalis* and *Dermatophagoides pteronyssinus* mites is common in tropical and subtropical countries. The human IgE cross-reactivity between clinical important group 5 allergens, Blo t 5 and Der p 5, remains controversial.

Objective: This study was undertaken to assess the levels of the IgE cross-reactivity between Blo t 5 and Der p 5 by using sera from a large cohort of asthmatic children in subtropical and tropical countries.

Methods: Purified recombinant Blo t 5 and Der p 5 were produced in *Pichia pastoris* and tested against sera from 195 asthmatic children. The IgE cross-reactivity was examined by direct, inhibitory and competitive human IgE enzyme-linked immunosorbent assay as well as skin prick tests.

Results: The Blo t 5 IgE responses were 91.8% (134 of 146) and 73.5% (36 of 49) for Taiwanese and Malaysian sera, respectively. The Blo t 5 specific IgE titers were significantly higher than those of Der p 5 ($P < .02$). The correlation of IgE reactivity between Blo t 5 and Der p 5 was low, and only limited cross-reactivity was observed. This was further confirmed by the dose-response inhibition studies. Skin prick tests performed on asthmatic children in Thailand also showed differential IgE response to Blo t 5 and Der p 5.

Conclusion: By using a large panel of asthmatic sera and a combination of in vitro and in vivo assays, the major allergen of *B tropicalis* in tropical and subtropical regions, Blo t 5, exhibits low levels of IgE cross-reactivity with homologous Der p 5. These findings suggest that highly specific clinical reagents are necessary for precise diagnosis and immunotherapeutic treatment of sensitization to group 5 mite allergens. (J Allergy Clin Immunol 2003;111:603-9.)

Key words: Blo t 5, cross-reactivity, Der p 5, IgE epitope

Abbreviations used

GST: Glutathione-S-transferase

PBS-T: Phosphate-buffered saline containing 0.05% Tween-20

House dust mite allergy is a major risk factor for allergic diseases.¹⁻³ Allergens from a number of mite species of clinical importance have been identified and categorized into groups according to their sequence homology.^{4,5} Cross-reactivity of human IgE to homologous allergens from different mite species is an important question to be addressed.⁶ It is not only for clinical importance but also for the understanding of basic immunology. Cross-reactivity of *Blomia tropicalis* with *Euroglyphus maynei*,⁷ *Lepidoglyphus destructor*,⁸ and other mite species⁹ has been studied. However, most of these studies were performed with crude mite extracts. The degree of cross-reactivity between the various specific allergenic components in the extracts was poorly defined. Hence it will be more informative to examine the cross-reactivity with individual purified native or recombinant allergens.

In recent years, a number of epidemiologic studies showed that the atopic populations in the tropical and subtropical regions are sensitized by both *Dermatophagoides pteronyssinus* and *B tropicalis*.^{10,11} Skin prick tests and in vitro IgE reactivity showed that *B tropicalis* is one of the dominant species of clinical importance in Latin America,¹²⁻¹⁶ Florida,^{17,18} and Asia.¹⁹⁻²¹ Studies carried out with mite extracts showed that the IgE cross-reactivity between *D pteronyssinus* and *B tropicalis* allergens was generally low and indicated the unique allergenic specificities of *B tropicalis* allergens.^{17,19,22} The first major *B tropicalis* allergen, *B tropicalis* group 5 allergen (Blo t 5), was cloned and expressed as a fusion protein with glutathione-S-transferase (GST).^{23,24} Both in vitro IgE binding assays and skin prick tests showed that recombinant Blo t 5 showed up to 70% of IgE reactivity with asthmatic patients.²⁵⁻²⁸ In contrast, the homologous allergen Der p 5 from *D pteronyssinus* has been shown to react with 40% to 50% of mite-sensitive asthmatic patients.^{29,30} To date there have been 3 published studies that explored the IgE cross-reactivity of Blo t 5 and Der p 5 by using purified recombinant allergens with sera of allergic patients from the tropical countries. The results from Arruda et al²⁴ showed no correlation of

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IgE responses between Blo t 5 and Der p 5 in *B tropicalis*-exposed and -unexposed patients. Similar studies by Chew et al³¹ using FAST inhibition assay with pooled sera from allergic asthmatic patients showed that the two group 5 allergens shared low to moderate IgE cross-reactivity. In contrast, the study conducted by Caraballo et al³² using a truncated Blo t 5 protein with pooled sera from allergic asthmatic patients in Colombia showed that truncated Blo t 5 and Der p 5 exhibited a high degree of cross-reactivity. The question of IgE cross-reactivity between the group 5 mite allergens remains controversial. In the present study, we investigated the cross-reactivity of Blo t 5 and Der p 5 by using proteins expressed from *Pichia pastoris* expression system. Sera from a large cohort of doctor-diagnosed asthmatic children living in subtropical Taiwan, where sensitization to *D pteronyssinus* and *B tropicalis* shares similar clinical significance,¹⁹ were used. We evaluated the cross-inhibition with individual serum from 146 asthmatic children to both allergens and provided data of quantitative difference of cross-inhibition with competitive ELISA. In addition, we also assessed the IgE reactivity profiles of asthmatic children from Malaysia and Thailand to group 5 mite allergens by ELISA and skin prick tests, respectively.

MATERIALS AND METHODS

Human sera

The sera of 146 Taiwanese asthmatic children (age, 7 to 15 years), 49 Malaysian asthmatic children (age, 7 to 16 years), and 7 control sera from healthy volunteers without history of allergy (age, 20 to 40 years) were used in this study. The Taiwanese asthmatic children had skin test positive results to *D pteronyssinus* extract and underwent immunotherapy treatment with *D pteronyssinus* extract in the Allergy Clinic of Department of Pediatrics, National Taiwan University Hospital, Taipei, Taiwan. The sera were provided by the laboratory of Professor K. H. Hsieh (deceased). The sensitization profile to *B tropicalis* mite was not assessed at the time when the sera were collected. The Malaysian sera were from asthmatic children without immunotherapy treatment attending the Allergy and Asthma Clinic in the University Hospital, University of Malaya, Malaysia. The sera were provided by Drs J. A. de Bruyne and C. K. Sam. The control sera were tested negative to *D pteronyssinus* and *B tropicalis* extracts, native Der p 1, recombinant Der p 2, Der p 5, and Blo t 5 by ELISA. All sera were used for in vitro IgE binding assays with recombinant Blo t 5 and Der p 5 proteins. The cross-inhibition studies were performed with the Taiwanese sera only.

Human subjects

A cohort of 45 asthmatic children (age, 6 to 16 years) were recruited for skin prick tests performed in the Department of Pediatrics, Chiang Mai University, Chiang Mai, Thailand. As indicated by skin prick test results, these children showed higher prevalence of sensitization to *D pteronyssinus* than to *B tropicalis* mites. The Research Ethics Committee of the Faculty of Medicine, Chiang Mai University, approved the protocol for skin prick tests.

Recombinant protein preparation

Recombinant Blo t 5 and Der p 5 proteins were produced from the *P pastoris* yeast expression system.^{25,33} Briefly, the yeast transformants carrying the allergen cDNA were grown in 5 mL of buffered glycerol-complex medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base with ammonium sulfate, 1% glycerol, 0.4 mg/L biotin, and 0.1 mol/L potassium phosphate, pH 6.0) and subsequent-

ly transferred to a final 2-L culture until the OD_{600nm} = 6. Cells were harvested by centrifugation at 1000g and gently suspended in 400 mL of buffered methanol-complex medium containing 0.5% methanol. The cells were cultured for another 2 days to induce the protein production, and methanol was replenished to 0.5% final concentration every 24 hours. The yeast media containing the recombinant allergens were processed by conventional fast performance liquid chromatography. Purified proteins were analyzed by SDS-PAGE.

Human IgE ELISA

All the ELISA procedures with 50 μ L per well were carried out in room temperature for 1 hour unless specified.²⁵ ELISA plates (Costar Co, Cambridge, Mass) were coated with 5 μ g/mL recombinant Blo t 5 or Der p 5 in 0.1 mol/L NaHCO₃, pH 8.2. After washing with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T), plates were blocked with 100 μ L 1% bovine serum albumin in PBS-T. Human sera were diluted (ranging from 1:5 to 1:50) in blocking buffer and incubated with allergens in coated wells in duplicate at 4°C for 16 hours. Plates were washed and incubated with biotin-conjugated monoclonal mouse anti-human IgE (1:2000 in blocking buffer) (Southern Biotechnology, Birmingham, Calif). After washing with PBS-T, ExtrAvidin-alkaline phosphatase (Sigma, St Louis, Mo), diluted 1:2000 in blocking buffer, was added. Signals were developed with *p*-nitrophenylphosphate substrate. Absorbance was measured at 405 nm by using microtiter plate reader (Ges mBH, Tecan, Salzburg, Austria). The IgE titer was defined as OD_{405nm} value $\times$ serum dilution factor. The mean plus 3-fold of the standard deviation of the ELISA titer from 7 control sera was used as the cutoff value.

Absorption and competitive ELISA

For absorption assay, a final concentration of 10 μ g/mL of Blo t 5 or Der p 5 was included in the diluted individual serum and incubated at 4°C for 16 hours. For competitive ELISA, diluted sera were incubated with varying concentrations of Blo t 5 or Der p 5 (from 40 pg/mL to 10 μ g/mL) at 4°C for 16 hours. The preabsorbed sera were then reacted with both plate-bound Blo t 5 and Der p 5 at 4°C for 16 hours. The plate-bound IgE were developed as the ELISA methods described above. The percentage of inhibition was calculated as $(1 - OD_{405nm} \text{ with inhibitor} / OD_{405nm} \text{ without inhibitor}) \times 100$.

Skin prick tests

Skin prick tests were performed according to the procedures described previously.²⁵ Purified Blo t 5 and Der p 5 proteins were dissolved in normal saline and used for skin prick test at the concentration of 25 μ g/mL. A drop of 10 μ L of protein solution was applied on the volar of the forearm, and the skin was pricked with a disposable lancet. Histamine (1 mg/mL) and normal saline were included as positive and negative controls. A wheal reaction of greater than 3 mm in diameter after subtracting the negative control after 30 minutes was regarded as a positive skin reaction.

Statistical analysis

Statistical differences were determined by using SPSS, Version 11.0.1 (SPSS Inc, Chicago, Ill). Correlations were analyzed with the Spearman rank method. A P value of less than .05 was considered statistically significant.

RESULTS

Production of recombinant Blo t 5 and Der p 5 from *P pastoris* culture media

The recombinant mite allergens Blo t 5 and Der p 5 were produced as secretory proteins with the help of

yeast α -factor signal. The yield of production was above 0.1 g/L. The purified recombinant Blo t 5 was migrated above 14.4 kd, and the purified recombinant Der p 5 was migrated just below 14.4 kd (Fig 1). The mobility of these 2 proteins was higher than the calculated molecular weight of Blo t 5 (13,891.69 Da) or Der p 5 (13,586.68 Da).

Human IgE reactivity to Blo t 5 and Der p 5

Direct human ELISA was used to examine the specific IgE reactivity to Blo t 5 and Der p 5. Of the 146 asthmatic sera from Taipei, 134 (91.8%) sera were positive for Blo t 5 (mean, 6.64; SD, 10.97), and 113 (77.4%) sera were positive for Der p 5 (mean, 4.01; SD, 4.56). One hundred seven sera were double positive to both Blo t 5 and Der p 5 (Fig 2, A). The percentage of IgE positive sera to the group 5 allergens, especially to Der p 5, was significantly higher than that reported previously.^{26,27,29,30} In addition, a total of 49 asthmatic sera from children without immunotherapy were tested. Thirty-six (73.5%) sera showed positive to Blo t 5 (mean, 9.05; SD, 6.96), and 19 (38.8%) sera showed positive for Der p 5 (mean, 2.40; SD, 4.41) (Fig 2, B). In Malaysian sera, the percentage of IgE reactivity to Blo t 5 or Der p 5 was comparable to that reported by others. Data derived from both Taiwanese and Malaysian sera showed that the Blo t 5 specific IgE titers were significantly higher than those of Der p 5 ($P < .02$ for Taiwanese sera; $P < .001$ for Malaysian sera). The Spearman rank correlation coefficients were 0.452 ($P < .001$) and 0.346 ($P = .015$) for Taiwanese and Malaysian sera, respectively. The results indicate that there was a positive but low correlation of IgE reactivity between Blo t 5 and Der p 5.

Heterologous absorption could not completely inhibit IgE reactivity

To determine the cross-reactivity between allergens Blo t 5 and Der p 5, inhibition assay was performed. Each individual serum was absorbed with 10 μ g/mL of Blo t 5 or Der p 5 and reacted with plate-bound Blo t 5 and Der p 5. At this concentration of inhibitor, 62 (42.7%) and 47 (31.2%) of the sera showed greater than 80% and lower than 50% homologous inhibition, respectively, for Blo t 5 specific IgE. For the heterologous inhibition of Blo t 5 specific IgE, 4 (2.7%) and 91 (62.3%) of the sera showed greater than 80% and less than 50% inhibition, respectively (Fig 3, A). For the Der p 5 specific IgE, 93 (63.7%) and 33 (22.6%) sera showed higher than 80% homologous and heterologous inhibition, respectively. The sera with less than 50% of homologous and heterologous inhibition were 2 (1.4%) and 61 (41.8%), respectively (Fig 3, B). The frequency distributions of heterologous inhibitions were significantly different from those of homologous inhibitions ($P < .0001$) in both Blo t 5 and Der p 5 specific IgE. These results suggested that the dominant IgE epitopes present in Blo t 5 and Der p 5 are unique epitopes.

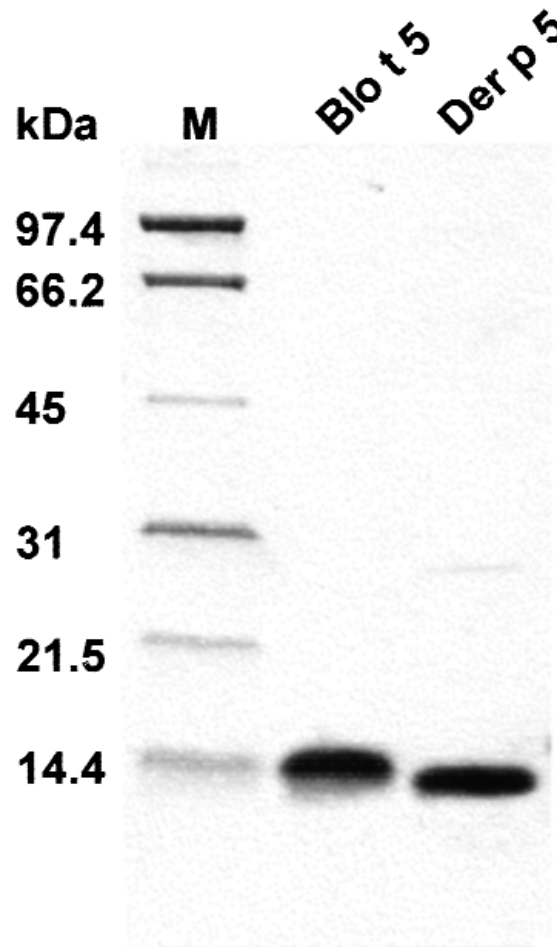


FIG 1. SDS-PAGE analysis of purified Blo t 5 and Der p 5 expressed in *P. pastoris*.

Dose-response inhibition studies by competitive ELISA

Competitive ELISA was used to provide the quantitative assessment of the cross-inhibition between these 2 allergens with 5 sera individually. For the Blo t 5 specific IgE, 1 ng/mL of inhibitor achieved 16.3% to 60.4% reduction of IgE reactivity in homologous inhibition but 4.8% to 10.8% reduction in heterologous inhibition. At 1 μ g/mL inhibitor concentration, the homologous and heterologous inhibitions were 44.9% to 87% and 5.5% to 18.5%, respectively (Fig 4, A). These discrete inhibitory curves were also observed in the competitive inhibition studies of Der p 5 specific IgE (Fig 4, B). At 1 ng/mL inhibitor concentration, 23.2% to 54.6% of homologous and 4.7% to 16.2% of heterologous inhibition were obtained. At 1 μ g/mL inhibitor concentration, 49.1% to 81.9% of homologous and 8.7% to 30.9% of heterologous inhibition were obtained. The maximal heterologous inhibition in these 5 representative sera was still lower than 40%. The sera that were positive to either Blo t 5 or Der p 5 alone showed no inhibition with heterologous inhibitors (data not shown), indicating that they reacted with the unique IgE epitopes only.

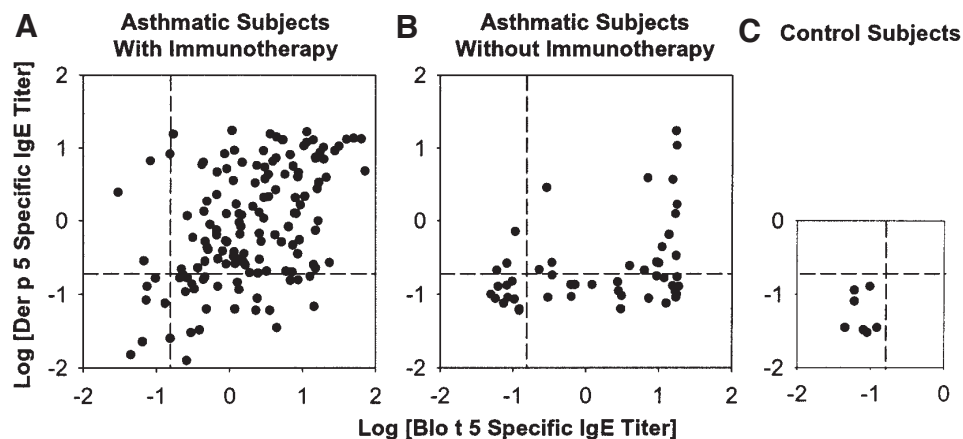


FIG 2. Human IgE reactivity to purified Blo t 5 and Der p 5. The relative titers of Blo t 5 and Der p 5 specific IgE of (A) sera from 146 asthmatic children with immunotherapy, (B) sera from 49 asthmatic children without immunotherapy, and (C) 7 control sera were evaluated by direct ELISA. Each dot on the figure indicates an individual serum. The cutoff values (dashed lines) were determined by mean plus 3-fold of SD of the control sera.

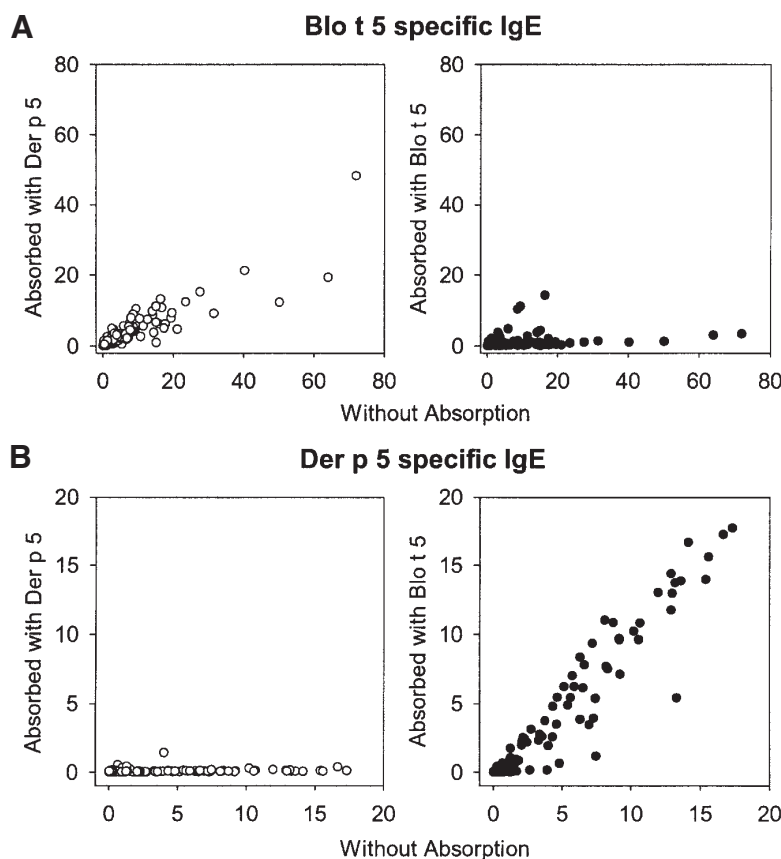


FIG 3. Absorption assay showed limited cross-reactivity between Blo t 5 and Der p 5 specific IgE. Diluted sera were preabsorbed with Blo t 5 (●) or Der p 5 (○) and reacted with plate-bound (A) Blo t 5 and (B) Der p 5.

Differential skin reactivity of recombinant Blo t 5 and Der p 5

Besides in vitro studies, the IgE cross-reactivity of Blo t 5 and Der p 5 was also evaluated in vivo by skin prick tests. Forty-five of mite-sensitive asthmatic children were tested with the 2 recombinant group 5 allergens. The Blo t 5 and

Der p 5 positive subjects were 14 (31.1%) and 11 (24.4%), respectively. When the skin reactivity of the 19 subjects who showed at least 1 positive reaction to either Blo t 5 or Der p 5 was analyzed, significant levels of differential reactivity were observed. The Spearman rank correlation coefficients for erythema and wheal were -0.561 ($P < .02$) and -0.526 ($P < .02$) at .05 level of significance (Fig 5).

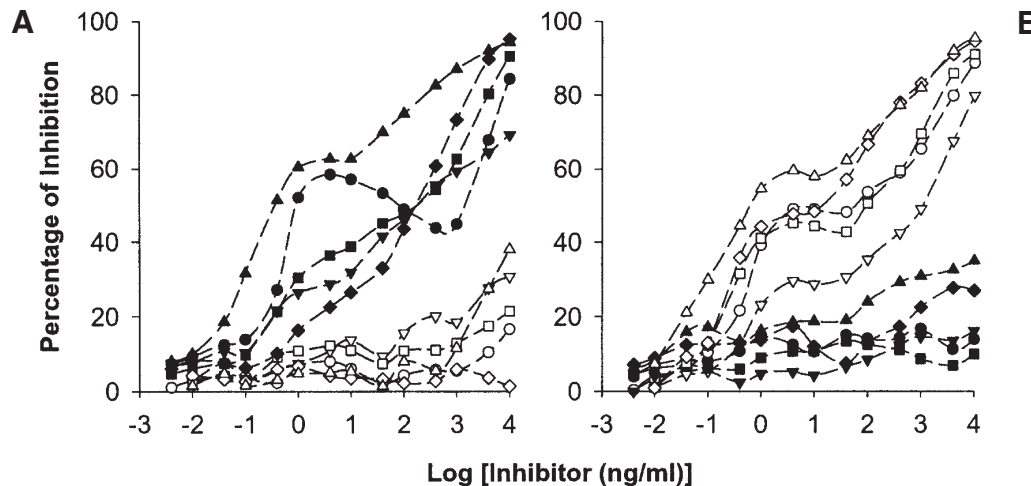


FIG 4. Competitive ELISA assay. Five representative sera (● subject 36, ▼ subject 47, ■ subject 61, ◆ subject 71; ▲ subject 84) were preabsorbed with various concentrations of inhibitors before incubation with (A) Blo t 5 or (B) Der p 5 in coated plates. Filled symbols and open symbols indicated Blo t 5 and Der p 5, respectively, were used as the inhibitor.

DISCUSSION

Our data clearly showed that the correlation of IgE reactivity between Blo t 5 and Der p 5 was low and only limited cross-reactivity was observed. The Der p 5 specific IgE was completely self-inhibited but only partially inhibited or not affected by the Blo t 5 inhibition. The Der p 5 inhibition also had limited effect on Blo t 5 specific IgE. The results were further confirmed by the dose-response inhibition curves and the skin prick tests. Taken together, our data showed that the asthmatic subjects predominantly reacted with unique IgE epitopes of the group 5 allergens. However, there was a small subset of asthmatic recognized cross-reactive IgE epitopes. The 43% identity and 72% similarity of protein sequences between these two group 5 mite allergens were considerably high.^{24,29} The lack of cross-reactivity was probably due to the fact that the identical amino acids were scattered throughout the whole protein molecules and there was no conserved peptide motif. Recently, the presence of unique IgE epitopes for group 10 mite allergens (Blo t 10 and Der p 10) has been reported,³⁴ although the highly conserved tropomyosin proteins shared up to 96% amino acid sequence identity.

The discrepancy in results relating to the cross-reactivity between Blo t 5 and Der p 5 reported by Caraballo et al³² and 2 other reports^{24,31} could be due to 2 possibilities. First, in these studies only a limited number of sera or sera pools were used for inhibition studies; thus the results obtained could be biased. The second possibility was that the difference in the conformation of recombinant proteins might attribute to different degree of cross-reactivity between the 2 allergens. It is conceivable that the truncated Blo t 5 protein (GST-BtM) used by Caraballo et al is more allergenic than the full-length allergen. It has been shown in our laboratory that the Der f 11-derived peptides containing the dominant IgE epitopes were more reactive than the full-length molecule.³⁵ This indicates

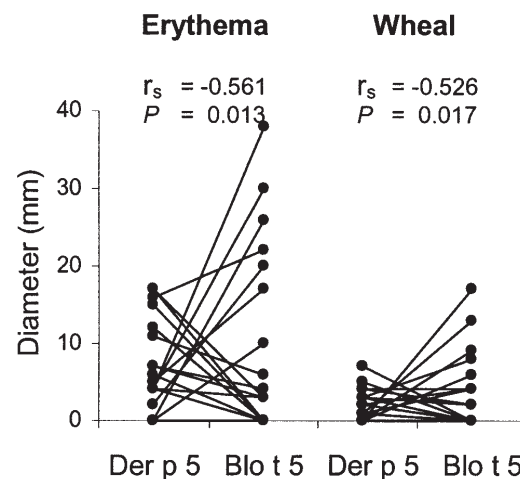


FIG 5. The comparative assessment of the allergenicity of Blo t 5 and Der p 5 based on the erythema and wheal sizes by skin prick tests in asthmatic children.

that the exposure of internal or hidden epitopes from the degraded natural allergens may play a vital role in the allergen sensitization. The other possible factor is that the proteins used in these studies contained artificially introduced additional N-terminal sequences (Gly-Ser dipeptide or GST protein). It has been reported that the additional 1 alanine in the N-terminal of Der p 2 (Ala-Der p 2) affected the IgE reactivity in RAST and RAST inhibition analysis as compared to unmodified Der p 2.³⁶ The N-terminal modification may have resulted in distortion of conformational IgE epitopes. In our current study, recombinant Blo t 5 and Der p 5 were secreted and processed at correct sites without any additional N-terminal amino acid residues as confirmed by N-terminal sequencing (data not shown) to eliminate or minimize the interference on the conformational IgE epitopes.³⁷

This is the first extensive study of IgE reactivity to group 5 allergens with sera from asthmatic children living in the Taipei area. The previous mite fauna and skin test results indicated that allergic subjects in Taipei were dual-sensitized by both *D pteronyssinus* and *B tropicalis* mites.¹⁹ The *D pteronyssinus* and *B tropicalis* accounted for 52.1% and 44.3%, respectively, of the total number of mites in mite-allergic patients' houses. The skin prick test reactivities to *D pteronyssinus* and *B tropicalis* mite extracts were 88.3% and 73.3%, respectively, among asthmatic patients. Our data indicated that 91.8% of the 146 asthmatic sera had IgE reactivity to Blo t 5, and cross-inhibition studies showed that most of the sera reacted to unique IgE epitopes of the Blo t 5. We also found that 77.4% of these sera also reacted positively to Der p 5; this frequency was about 2-fold higher than that reported previously.^{26,27,29,30} Our data on Malaysian asthmatic sera from children without immunotherapy treatment also showed approximately 38.8% IgE reactivity with Der p 5. The environment natural Der p 5 content was low in the Taipei area, with geometric mean less than 100 ng/g of fine dust.³⁸ There was no seasonal variation of Der p 5 contents in allergic patients' houses, and the Der p 5 concentration was at least 10-fold less as compared to the major allergen Der p 1.³⁹ Therefore, it is unlikely that the high frequency of Der p 5 reactivity is due to the high environmental exposure to natural Der p 5. It is highly probable that the high frequency of Der p 5 specific IgE reactivity of the Taiwanese sera was boosted by immunotherapy treatment with *D pteronyssinus* mite extract. Such immunotherapy is unlikely to have an impact on the Blo t 5 specific IgE because our inhibition data confirmed that most sera exhibited low levels of IgE cross-reactivity to Blo t 5 and Der p 5. In addition to the high frequency of IgE reactivity to Blo t 5 shown by asthmatic sera from Malaysia and Taiwan, our data also showed that the IgE titers for Blo t 5 were higher than those of Der p 5 in most sera tested, suggesting that Blo t 5 is a major allergen in both subtropical and tropical regions.

In summary, we conclude that the major allergen of *B tropicalis* in tropical and subtropical regions, Blo t 5, exhibits low levels of cross-reactivity with homologue Der p 5. Unlike the scenario of group 1 and 2 mite allergens, diagnosis and therapy for sensitization by group 5 allergens require specific reagents. The advantage of expressing these recombinant allergens in the *P pastoris* system is that the host secretes very low levels of native proteins. This facilitates the purification of recombinant allergens from the culture media. The availability of such recombinant allergens with properly folded conformation is useful for the development of diagnostic and therapeutic reagents for asthmatic patients in tropical and subtropical regions.

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Correction

With regard to the January 2003 *Allergy Archives* article entitled "Coca and Cooke on the classification of hypersensitiveness" (2003;111:205-10): An earlier version of the technical part of this article included numbered references. In the final version, the list of references was replaced by a bibliography without numbered items; however, the numbered reference citations were inadvertently retained in the text of the published article. They are unrelated to the bibliography and should be ignored.