Differential IgE reactivity to Der p 1 and Der p 2 allergens of *Dermatophagoides pteronyssinus* in mite-sensitized patients

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Summary. Several studies have shown that the presence of IgE antibodies to house dust mites (HDM), particularly *Dermatophagoides pteronyssinus* (Dpt), is an important risk factor for asthma. Allergen immunotherapy is indicated for patients with IgE antibodies to clinically relevant allergens. The aims of this study were to analyze the levels of specific serum IgE to Der p 1 and Der p 2 allergens in mite-sensitized atopic patients and to compare them with both *in vivo* (skin prick test) and *in vitro* (IgE-ELISA) sensitizations to Dpt crude extract. Forty-seven atopic patients with allergic rhinitis with or without intermittent or persistent mild asthma and positive skin prick test (SPT) to Dpt total extract were studied. Thirty age-matched healthy subjects with negative SPT to HDM were included as controls. Levels of total IgE and Dpt-, Der p 1- and Der p 2-specific IgE were measured by ELISAs in SPT-positive atopic patients and SPT-negative control subjects. Among 47 symptomatic atopic patients, 27 (57.4%) were double positive IgE to Der p 1 and Der p 2 allergens, 3 (6.4%) were single positive IgE to Der p 1, 4 (8.5%) were single positive IgE to Der p 2, and 13 (27.6%) were double negative IgE to both allergens. There was a significant correlation between Der p 1- and Der p 2-specific IgE levels, but not between Der p 1- or Der p 2-IgE levels and SPT results. The double negative IgE patients had the smallest skin test reactions although they showed high mean levels of total serum IgE. Therefore, the knowledge of specific IgE levels to Der p 1 and Der p 2 major allergens might support physicians for indication or follow-up in mite-sensitized patients under allergen-specific immunotherapy. These approaches might be important for obtaining improved safety and efficacy of the current clinical practice of allergen immunotherapy.

Key words*:* Allergen immunotherapy - Allergic rhinitis – Asthma **-** *Dermatophagoides pteronyssinus* - Der p 1 allergen - Der p 2 allergen - IgE antibody.

Resumen. Varios estudios han demostrado que la presencia de anticuerpos IgE frente a ácaros del polvo doméstico (APD), en concreto *Dermatophagoides pteronyssinus* (Dpt), es un factor de riesgo importante para el asma.
La inmunoterapia con alérgenos está indicada en pacientes con anticuerpos IgE frente a alérgenos clínicamente relevantes. El objetivo de este estudio fue analizar los niveles de IgE sérica específica frente a los alérgenos Der p 1 y Der p 2 en pacientes atópicos sensibilizados a los ácaros y compararlos con sensibilizaciones tanto *in vivo* (prick test) como *in vitro* (IgE-ELISA) frente a extracto crudo de Dpt. Se estudiaron a 47 pacientes atópicos con rinitis alérgica, con o sin asma leve intermitente o persistente, y prick test positivo a extracto total de Dpt. Se utilizó como control a 30 sujetos sanos apareados por edad con prick test negativo a APD. Los niveles de IgE total y de IgE específica frente a Dpt, Der p 1 y Der p 2 se determinaron mediante ELISA en pacientes atópicos con prick positivo y pacientes control con prick negativo. Entre los 47 pacientes atópicos sintomáticos, 27 (57,4%) presentaron IgE positiva frente a los alérgenos Der p 1 y Der p 2; 3 (6,4%) presentaron IgE positiva sólo frente a Der p 1; 4 (8,5%) sólo frente a Der p 2, y 13 (27,6%) presentaron IgE negativa frente a ambos alérgenos. Se observó una correlación significativa entre los niveles de IgE específica frente a Der p 1 y Der p 2, pero no entre

los niveles de IgE frente a Der p 1 o Der p 2 y los resultados de las PPC. Los pacientes con resultados IgE doble negativos experimentaron un menor grado de reacción en la prueba cutánea, aunque mostraron niveles medios altos de IgE sérica total. Por lo tanto, la identificación de niveles de IgE específica frente a los alérgenos principales Der p 1 y Der p 2 podría servir de ayuda a los médicos para indicación o seguimiento en pacientes sensibilizados a los ácaros sometidos a inmunoterapia específica con alérgenos. Estas estrategias pueden ser importantes para obtener una mayor seguridad y eficacia en la práctica clínica actual de inmunoterapia con alérgenos.

Palabras clave: inmunoterapia con alérgenos, rinitis alérgica, asma, *Dermatophagoides pteronyssinus*, alérgeno Der p 1, alérgeno Der p 2, anticuerpo IgE.

Introduction

Allergic airway diseases are characterized by the presence of specific IgE antibodies directed to the inhalant allergens, including those derived from the house dust mites (HDM), such as the *Dermatophagoides pteronyssinus* group 1 (Der p 1) and group 2 (Der p 2) allergens [1,2]. Specific allergen immunotherapy has been shown to be highly effective in selected patients with IgE-mediated disease, particularly in respiratory allergy, when properly implemented [3-6]. The purpose of allergen-specific immunotherapy is to induce immunological changes or tolerance against the administered allergens and thus is indicated for patients with IgE antibodies to clinically relevant allergens [7-9].

Mite allergen crude extracts are currently used throughout the world for both diagnostic and specific immunotherapy procedures. Thus, skin prick test (SPT) positive to a certain mite crude extract will indicate that the patient is sensitized to mite allergens, but without identifying the allergenic components [10]. Mite extracts contain at least 15 well characterized allergens, and mite-sensitized patients could have differential IgE reactivity to the major allergens Der p 1 and Der p 2. In this context, a previous study has demonstrated a different profile of contribution of *D. pteronyssinus* major allergens (P1 and Dp X) for inducing IgE antibodies, with IgE anti-P1 frequently higher than the contribution of IgE anti-Dp X [11]. Furthermore, mite-sensitized patients with no detectable IgE levels to either P1 or Dp X allergens have been found [11], indicating the contribution of other allergens contained in the extract.

Recently, the development of new IgE sensitivities has been reported during specific immunotherapy for pollen allergy [12, 13], although this issue still remains a controversial field, particularly for mite specific immunotherapy.

The aim of this study was to analyze the levels of specific serum IgE to Dpt, Der p 1 and Der p 2 allergens in mite-sensitized atopic patients and to compare to both *in vivo* (SPT) and *in vitro* (IgE-ELISA) sensitizations to Dpt crude extract.

Materials and methods

Patients

Forty-seven atopic patients (mean age 43 years, range 18-60) with allergic rhinitis with or without intermittent or persistent mild asthma and positive SPT to *D. pteronyssinus* (Dpt) crude extract (30,000 AU/mL; Bayer Co., Spokane, WA, USA) were selected at the Allergy Clinic of the Clinical Hospital of the Federal University of Uberlândia (Brazil). This extract contained 410 μg/mL of Der p 1 and 211 μg/mL of Der p 2 as measured by a two-site monoclonal antibody ELISA [14]. Skin test reactions were evaluated based on the mean wheal diameter at 15 minutes after application of the extract, considering values > 3 mm as a positive SPT. Atopic asthma and rhinitis were diagnosed according to the medical history, physical examination and cutaneous sensitization to HDM. Thirty age-matched healthy subjects with negative SPT to HDM were included as controls. All individuals were negative for intestinal parasites in three independent and alternative stool samples. A written consent was obtained from all volunteers after being fully informed about the purpose and nature of the study, which was approved by the Ethics Committee in Research of the Federal University of Uberlândia.

ELISA for measuring mite-specific IgE

All sera were assessed by ELISA for measuring levels of specific serum IgE to Dpt, Der p 1, and Der p 2 allergens as previously described [15]. For Dpt allergen, a conventional ELISA (cELISA) was used. Briefly, high-binding microtiter plates were coated with 1.0 μg/well of Dpt crude extract in 0.06 M carbonate buffer (pH 9.6). Plates were incubated with serum samples (1:2) for 2 h at 37°C. Subsequently, biotinylated secondary antibody anti-human IgE (1:1000; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA), was added and incubated for 1 h at 37°C. Next, the streptavidin-peroxidase conjugate (1:500; Sigma) was added and incubated for 30 min at room temperature (RT). The assay was developed by adding the enzyme substrate (0.01M ABTS and 0.03% H_2O_2). Optical density (OD) values were determined in a plate reader (Titertek Multiskan, Flow Laboratories, McLean, VA, USA) at 405 nm. Results were expressed as ELISA units per milliliter (EU/mL) and compared with a control curve (500 to 0.5 EU/mL) obtained by measuring HDM-specific IgE levels in parallel, using a standard mite allergic serum pool (UVA 89/01, University of Virginia, USA). ELISA sensitivity was 5 EU/mL and established

as the cut off value, since negative control sera showed values of EU/mL ranging from 0.3 to 2.5 EU/mL as previously reported [15]. ELISA-reactivity classes were arbitrarily assigned as follows: class 0 (< 5 EU/mL; negative IgE); class 1 (5 to 20 EU/mL; low positive specific IgE); class 2 (20 to 100 EU/mL; moderate positive specific IgE); class 3 (100 to 500 EU/mL; high positive specific IgE) and class 4 (> 500 EU/mL; very high positive specific IgE).

For Der p 1 and Der p 2 allergens, a reverse ELISA (rELISA) was used. Briefly, high-binding microtiter plates were coated with mouse monoclonal antibody to Der p 1 (clone 5H8) or to Der p 2 (clone 1D8) at 1.0 μg/well in 0.06M carbonate buffer (pH 9.6). Plates were then incubated with Dpt extract (40 μg/mL) for 1 h at RT. Next, plates were incubated with serum samples, biotinylated secondary antibody, streptavidin-peroxidase conjugate, and enzyme substrate as above described for cELISA. Results were expressed as ELISA units per milliliter (EU/mL) as established in cELISA for Dpt.

Total serum IgE

Total serum IgE was measured by a monoclonal antibody-based ELISA as previously described [15]. In brief, microtiter plates (Immulon II, Dynatech Laboratories Inc., USA) were coated with monoclonal anti-human IgE (1:5000; Sigma) in 0.06 M carbonate buffer, pH 9.6 overnight at 4 °C. Plates were washed

and blocked as previously described, and subsequently incubated with serum samples (1:5, 1:25 and 1:125) for 1 h at RT and biotinylated goat anti-human IgE (1:4000; Kirkegaard & Perry) for 1 h at RT. Subsequent steps were similar to those described for rELISA. Results were expressed as international units per milliliter (IU/mL) and were calculated based on a control curve (300 to 0.3 IU/mL) obtained by serial 2-fold dilutions of a serum that was designated as containing 3,000 IU/mL of total IgE.

Statistical analysis

Unpaired t tests were used to compare specific IgE values between groups and obtained by different techniques. The χ^2 test was used to compare percentages of positives within the groups. Levels of specific IgE measured by cELISA and rELISA were analyzed by Spearman's correlation test. P values <0.05 were considered statistically significant.

Results

Among 47 symptomatic atopic patients, 27 (57.4%) were double positive IgE to Der p 1 and Der p 2 allergens, 3 (6.4%) were single positive IgE to Der p 1, 4 (8.5%) were single positive IgE to Der p 2, and 13 (27.6%) were double negative IgE for both allergens (Table 1). There

Table 1. Skin reactivity to *D. pteronyssinus* (Dpt), levels of serum total IgE and IgE to Dpt, Der p 1 and Der p 2 allergens in 47 symptomatic atopic patients.

Patients	SPT Mean Wheal (mm)	Total IgE (IU/mL)	Specific IgE (EU/mL)		
			Dpt	Der p 1	Der p 2
Der p 1+/Der p 2+ IgE (n = 27; 57.4%)					
Mean (SD)	8.7 (2.3)	1115.0 (1226.0)	78.1 (86.3)	428.4 (698.0)	730.2 (1271.0)
Range	4.5-12	73-4209	3.4-277.8	6.6-3111.1	5.8-5317.7
Positivity			96.3%	100%	100%
Der p 1+/ Der p 2- IgE (n = 3; 6.4%)					
Mean (SD)	9.7 (4.7)	545.5 (437.2)	6.4 (7.9)	201.6 (337.3)	2.3 (1.7)
Range	6.0-15	58-904	1.5-15.6	6.7-591	1.1-4.3
Positivity			33.3%	100%	0%
Der p 1- / Der p 2+ IgE (n = 4; 8.5%)					
Mean (SD)	10.7 (6.9)	444.3 (314.1)	10.2 (10.1)	2.3 (1.4)	114.1 (109.0)
Range	6.0-21.0	51-818	2.2-23.7	0.7-4.1	7.3-244
Positivity			50%	0%	100%
Der p 1-/Der p 2- IgE (n = 13; 27.6%)					
Mean (SD)	5.3 (0.5)	434.3 (647.7)	1.8 (0.8)	1.4 (0.8)	1.0 (0.8)
Range	4.5-6.0	23-2081	0.7-3.2	0.5-4.2	0.2-3.5
Positivity			0%	0%	0%

SPT = skin prick test; SD = standard deviation.

was no significant correlation between serum IgE levels to Dpt, Der p 1 or Der p 2 and SPT results in all subgroups studied ($P > 0.05$). However, a significant positive correlation was found between Der p 1 ($r = 0.5781$; $P = 0.0016$), but not Der p 2 ($r = 0.3315$; $P = 0.0912$) specific IgE and total IgE levels in the subgroup of double positive IgE patients only. In addition, this latter subgroup showed a significantly higher mean wheal size (8.7 ± 2.3 mm) on SPT than double negative IgE patients (5.3 ± 0.5 mm; $P < 0.0001$). There was no significant difference between the subgroups with regards to the total serum IgE levels, although the double positive IgE patients had higher mean levels of total IgE (1115 IU/mL) when compared to those of double negative IgE patients (434 IU/mL; $P = 0.0689$).

Figure 1 illustrates the distribution of Der p 1- and Der p 2-specific IgE levels according to the cut off level (Fig. 1a) and different ELISA reactivity classes (Fig. 1b). A significant positive correlation was found between the levels of Der p 1- and Der p 2-specific IgE (Spearman correlation coefficient: $r = 0.7563$; $P < 0.0001$). The results clearly show that there was a substantial number of cases with double negative IgE reactivities to Der p 1/Der p 2 and other cases with high levels (class 3 or 4) of Der p 1-specific IgE and no or low levels (class 0 or 1) of Der p 2-specific IgE or vice-versa. Control subjects showed mean levels of total serum IgE of 29 IU/mL, and specific serum IgE levels to Der p 1 (0.9 EU/mL) and Der p 2 (0.7 EU/mL) were below the cut off value.

Discussion

Our results indicate that only about 60% of the atopic patients were double positive IgE to Der p 1 and Der p 2 allergens, 6% were single positive IgE to Der p 1, 8% were single positive IgE to Der p 2, while 27% were double negative IgE to both allergens. These findings seem to suggest that our patients react differently from those in other regions and may be more biased to recognize other allergens in the mite extract or a possible sensitization to cockroaches or parasites may have occurred. In contrast, Pittner et al. [16] have demonstrated that more than 95% of the Middle European mite-allergic patients were mainly sensitized to the major Der p allergens (Der p 1 and Der p 2), while the remaining patients showed a broad sensitization profile, including highly cross-reactive allergens (Der p 10: tropomyosin) and reactivity to storage mites.

According to our findings, we could speculate that (i) single positive or double negative IgE patients could pose a risk for a potential new IgE reactivity to Der p 1 and/or Der p 2; (ii) immunotherapy with specific allergens may not be suitable for the double negative IgE patients; (iii) patients may have IgE cross-reactivities, and (iv) diagnostic tests based on the determination of the major
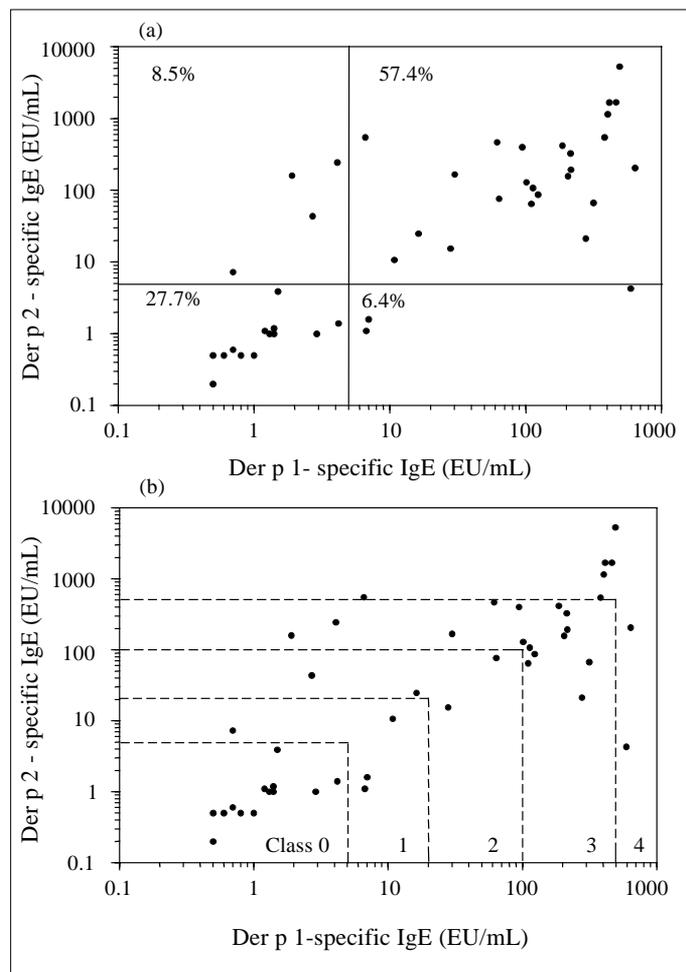

Figure 1. Distribution of Der p 1- and Der p 2-specific serum IgE levels determined by reverse ELISA (EU/mL) in 47 atopic patients. Continuous lines (a) indicate the cut off value (5 EU/mL) and dashed lines (b) indicate the thresholds of ELISA reactivity classes: 0 (< 5 EU/mL); 1 (5 to 20 EU/mL); 2 (20 to 100 EU/mL); 3 (100 to 500 EU/mL); 4 (> 500 EU/mL).

mite allergens (Der p 1 and Der p 2) might be used to improve the selection of patients for mite-specific immunotherapy.

In this context, Der p 1 or Der p 2 single positive IgE and double negative IgE patients could have a risk for developing new IgE reactivities when receiving immunotherapy with mite crude extract, since the commercial allergen preparations are based on allergen units containing effective concentrations of both Der p 1 and Der p 2 allergens [17]. Accordingly, crude allergen extracts are heterogeneous mixtures of allergenic and nonallergenic components, which can induce in some patients new sensitizations to constituents to which they were not originally sensitized. Recently, investigators have pointed out the development of new IgE specificities to allergenic components in pollen extracts during prolonged specific immunotherapy, although the clinical relevance of these new IgE reactivities is still unclear [13]. Therefore, the use of reagents with only the purified and

well-characterized allergenic proteins that are relevant to the allergic diseases would probably improve the efficacy of immunotherapy [10].

It is noteworthy that the double negative IgE patients had the smallest skin test reactions, although they showed high mean levels of total serum IgE. These findings probably can be attributed to the fact that positive SPT results and increased total IgE levels may reflect sensitization to highly conserved structures among the invertebrates, such as tropomyosin of cockroaches, shrimp, lobster, crab, molluscs, squid [18, 19] or paramyosin of *Schistosoma mansoni* and *Onchocerca volvulus* [20]. Accordingly, van Ree et al. [21] occasionally observed induction of IgE against foods of invertebrate animal origin (shrimp, snail), including tropomyosin-reactive IgE during house-dust mite immunotherapy. In addition, inhibition tests have showed that most IgE antibodies against snail were cross-reactive with house dust mite while the mite was not significantly inhibited by snail, suggesting that house dust mite was the sensitizing agent [22].

The likelihood that these patients were sensitized to other minor mite allergens is most unlikely, since no patient showed serum IgE reactivity to Dpt whole extract. In this context, discrepancies between skin test and IgE antibody assays were already reported [23], showing that greater than 80% of the discordances consisted of positive skin reactions without detectable allergen-specific IgE antibodies in serum.

Therefore, we highlight the importance of a detailed clinical and laboratory diagnosis, particularly for investigating the presence of IgE antibodies against clinically relevant major allergens to improve the diagnostic selection of patients for mite-specific immunotherapy. In this context, the use of very sensitive immunoassays, such as the reverse ELISA that we have recently developed [15], in which the allergen is captured by specific monoclonal antibodies bound to the plates, is highly desirable. This method has been shown to be much more sensitive than conventional ELISA using allergen bound directly to microtiter plates.

Although these data are important and consistent, a clinically crucial question is still unanswered, namely whether it would be better to include a too large (crude extract) or too small (recombinant allergens, possibly omitting some relevant allergens) repertoire for specific immunotherapy as previously questioned by Abramson et al. [24]. In addition, no evidence is currently available to indicate whether immunotherapy with allergen crude extract used in patients who lack IgE reactivities to major allergen components (for instance, injection of Der p 1 in patients lacking Der p 1-specific IgE) is clinically harmful. In this context, we are developing complementary studies on long-lasting allergen-specific immunotherapy trials which include atopic patients with positive SPT and negative serum IgE antibodies to Der p 1 and/or Der p 2 in order to investigate whether additional sensitization occurs and its clinical relevance.

Altogether, allergen-specific immunotherapy might be properly indicated for those patients with IgE positivity to relevant allergen components present in the crude allergen extracts if allergen avoidance is not possible or successful. Those presenting no or low levels of IgE to a specific allergen should be subjected to careful clinical and laboratory follow-up after the initiation of allergen immunotherapy when using total extracts. Treatment failures with these conventional mite extracts could be evaluated through measuring specific serum IgE to major allergens. Currently, there are several attempts to develop candidates of individual recombinant hypoallergenic allergens with reduced allergenic activity for vaccine formulation [25, 26]. Finally, the knowledge of specific IgE levels to Der p 1 and Der p 2 major allergens might support physicians for indication or follow-up in mite-sensitized patients under allergen-specific immuno-therapy. These approaches might be important for obtaining improved safety and efficacy of the current clinical practice of allergen immunotherapy.

Acknowledgments

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