
A sensitive reverse ELISA for the measurement of specific IgE to Der p 2, a major *Dermatophagoides pteronyssinus* allergen

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Background: Epidemiologic studies have shown that the presence of IgE antibodies to house dust mite and other indoor allergens is an important risk factor for asthma.

Objective: The aim of this study was to develop a reverse ELISA (rELISA) for measuring specific IgE to Der p 2, a major *Dermatophagoides pteronyssinus* (Dpt) allergen, as a potential tool for followup of allergen immunotherapy.

Methods: Recombinant Der p 2 allergen or a monoclonal antibody to Der p 2 was used to coat plates in conventional ELISA (cELISA) and rELISA, respectively. Sera from 48 asthmatic patients with positive skin prick test (SPT⁺) to *D. pteronyssinus* extract were analyzed for total IgE and specific IgE to Der p 2, and the results were compared with a group of 41 SPT⁻ asthmatic and 30 SPT⁻ control subjects.

Results: The sensitivity of the two assays for Der p 2-specific IgE was 3.9 EU/mL and their specificities were confirmed by inhibition tests, in a dose-dependent manner. There was a significant positive correlation between cELISA and rELISA ($r = 0.74$; $P < 0.0001$). However, rELISA was more sensitive than was cELISA, regarding both the positive sera percentage (70.8% vs 52.1%) and the Der p 2-specific IgE levels (28.4 vs 4.5 EU/mL) in SPT⁺ asthmatic patients.

Conclusions: rELISA has shown to be a sensitive and alternative method for measuring Der p 2-specific IgE without using radioactive techniques. Detection of specific IgE to major allergens and relevant peptides, and identification of B cell epitopes in allergens will provide valuable information for the design of allergen analogs and peptides for immunotherapy.

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INTRODUCTION

The importance of house dust mites (HDMs) as a source of indoor allergens and their role in the development of allergic diseases have been recognized for many years.¹ Epidemiologic studies from tropical countries including Brazil have shown that the pres-

ence of IgE antibodies to HDM and other indoor allergens is an important risk factor for asthma.^{2,3} Mites of the genus *Dermatophagoides* (*D. pteronyssinus*, *D. farinae*, and *D. microceras*) account for >90% of the mites found in house dust samples and are the principal cause of mite allergy.⁴ The majority of HDM allergic patients have high levels of IgE antibodies to group 1 ($\approx 80\%$) and group 2 ($\approx 90\%$) allergens of *D. pteronyssinus* (Dpt) and *D. farinae* (Df).

Crude mite extracts are currently used in assays for measuring HDM-specific IgE, in part because of difficulties in obtaining large amounts of purified group 1 and 2 allergens.⁵ The sensitive RAST has still been used for

measuring specific IgE to *Dermatophagoides* sp. However, radioactive materials are relatively unstable, expensive, and potentially hazardous, requiring special facilities.⁶ These disadvantages have prompted investigators to develop alternative methods for IgE measurements.

Kemeny et al⁷ have used allergen-specific monoclonal and polyclonal antibodies to measure IgE by ELISA. These authors reported that sandwich ELISA using polyspecific rabbit antibody was more sensitive than was conventional ELISA (cELISA) and also slightly more sensitive than RAST. Mastrandrea et al⁸ reported that IgE antibodies to Dpt whole extract and to the natural major allergens Der p 1 and Der p 2, could also be measured by ELISA. A new automated fluoroimmunoassay system using reagents suitable for increased speed and accuracy (UniCAP; Pharmacia & Upjohn, Uppsala, Sweden) was evaluated by Paganelli et al.⁹ They showed that the UniCAP system for IgE measurement is efficient for routine diagnostic testing of allergy. Recently, Peng et al⁵ have described a monoclonal antibody-based capture ELISA for measuring Der f 1-specific IgE. This method has shown to be more sensitive and specific than the conventional Der f 1-capture ELISA to diagnose dust mite allergy.

We have developed a sensitive reverse ELISA for measuring Der p 2-specific IgE in serum. In this article, we report the results of measurement of IgE antibodies to Der p 2 by a cELISA, in which purified antigen was bound directly to microtiter plates, and by a reverse ELISA (rELISA), in

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which antigen was captured by anti-Der p 2 monoclonal antibody bound to the plates. In addition, we compared the sensitivity and specificity of the two ELISAs for measurement of Der p 2-specific IgE in asthmatic patients with positive or negative skin prick test (SPT) to dust mite extract, and in non-allergic healthy control subjects.

METHODS

Subjects

Eighty-nine patients (19 males), ages 18 to 60 years, with mild intermittent to severe persistent asthma, seen at the Allergy and Infectious Diseases Unit of the Immunology Division of Federal University of Uberlândia, Brazil, were included. The study was approved by the human investigation committee at the Federal University of Uberlândia and informed written consent was obtained from patients. Diagnosis of asthma was based on medical history, physical examination, and spirometry.^{10,11} Patients were submitted to SPT with HDM extract (*Dermatophagoides pteronyssinus*; Bayer Corporation, Spokane, WA) and venipuncture. Skin test reactions were evaluated based on the mean wheal diameter at 15 minutes after application of the extract, considering values ≥ 4 mm as a positive SPT.

Among the 89 patients, 48 had positive SPT to Dpt (asthma SPT⁺ group), and 41 patients had negative SPT (asthma SPT⁻ group). Thirty healthy subjects without asthma or clinical history of atopic disease and with negative skin tests to Dpt were included as controls (control group).

Mite Extract

Crude Dpt extract was prepared from dried material kindly provided by Dr. Larry Arlian (Wright State University, Dayton, OH) and contained 285 $\mu\text{g/mL}$ of Der p 1 and 121 $\mu\text{g/mL}$ of Der p 2. Recombinant Der p 2 (rDer p 2) was produced by means of a bacterial expression system.¹² Dpt extract, 50% glycerin, 10,000 allergen units (AU)/mL, was purchased from Bayer Corporation.

Der p 2-Specific IgE by rELISA

High-binding microtiter plates (Corning Laboratories Inc, New York, NY) were coated (50 $\mu\text{L}/\text{well}$) with mouse monoclonal antibody to Der p 2 (clone 1D8) at 1 $\mu\text{g}/\text{well}$ in 0.06 M carbonate buffer (pH 9.6) overnight at 4° C. As control of the mouse monoclonal antibody, plates were coated in parallel with purified mouse IgG at 10 $\mu\text{g}/\text{mL}$. Plates were washed three times with 0.01 M phosphate buffered saline (PBS; pH 7.2) containing 0.05% Tween 20 (PBS-T) and active sites were blocked (200 $\mu\text{L}/\text{well}$) with PBS-T plus 1% bovine serum albumin (BSA; Sigma, St. Louis, MO) for 1 hour at room temperature. Subsequent steps were carried out using 1% BSA-PBS-T as diluent (ELISA buffer). After blocking, wells were washed and subsequently incubated (50 $\mu\text{L}/\text{well}$) with Dpt extract (40 $\mu\text{g}/\text{mL}$) for 1 hour at room temperature. Plates were washed five times and incubated (50 $\mu\text{L}/\text{well}$) with serum samples diluted at 1:2.5 and 1:5 for 2 hours at room temperature. After washing as above, biotinylated goat anti-human IgE (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted at 1:4,000 was added to the wells (50 $\mu\text{L}/\text{well}$) and plates were incubated for 1 hour at room temperature. After washing, 50 $\mu\text{L}/\text{well}$ of streptavidin-peroxidase conjugate (Sigma) diluted at 1:1,000 were added and incubated for 30 minutes at room temperature. After washing, the assay was developed by adding 50 $\mu\text{L}/\text{well}$ of the enzyme substrate 0.01 M 2,2'-azino-bis-(3-ethyl-benz-thiazoline sulfonic acid; Sigma) in 0.07 M citrate-phosphate buffer (pH 4.2) containing 0.03% H_2O_2 . The reaction was read at 405 nm with a Titertek Multiskan Plus MK II plate reader (Flow Laboratories, McLean, VA). Results were expressed as ELISA units (EU)/mL and compared with a control curve obtained by measuring HDM-specific IgE levels in parallel, using a standard mite allergic serum pool (UVA 89/01; University of Virginia, Charlottesville, VA). The UVA serum pool contained 1,000 RAST units

(RU)/mL of specific IgE to *Dermatophagoides farinae* (1 RU is equivalent to 0.1 ng of IgE).¹³ The control curve was included in each plate with duplicate samples, and values ranged from 500 to 0.5 RU/mL.

Levels of Der p 2-specific IgE were arbitrarily classified in ELISA reactivity classes based on the sensitivity of the assay. Thus, the following classes were determined using 5-fold serial factor: class 0: <3.9 EU/mL (negative specific IgE); class 1: 3.9 to <19.5 EU/mL (low positive specific IgE); class 2: 19.5 to <97.5 EU/mL (moderate positive specific IgE); class 3: 97.5 to <487.5 EU/mL (high positive specific IgE); class 4: ≥ 487.5 EU/mL (very high positive specific IgE).

Der p 2-Specific IgE by cELISA

cELISA for Der p 2-specific IgE was carried out according to Mastrandrea et al,⁸ with modifications. High-binding microtiter plates (Corning Laboratories Inc) were coated overnight at 4° C (50 $\mu\text{L}/\text{well}$) with 10 $\mu\text{g}/\text{mL}$ of recombinant rDer p 2 allergen in 0.06 M carbonate buffer (pH 9.6). Plates were washed three times with PBS-T and blocked with 1% BSA-PBS-T for 1 hour at room temperature. Subsequent steps were carried out using ELISA buffer. After washing, 50 $\mu\text{L}/\text{well}$ of serum samples diluted at 1:2 were added in duplicate to the wells and incubated for 2 hours at 37° C. Plates were washed five times and incubated with 50 $\mu\text{L}/\text{well}$ of biotinylated goat anti-human IgE (Kirkegaard & Perry Laboratories) diluted at 1:1,000 for 1 hour at 37° C. Subsequent steps (addition of streptavidin-peroxidase conjugate and enzyme substrate) were similar to those described for rELISA. Results were expressed as EU/mL, compared with a control curve obtained as previously mentioned. Levels of Der p 2-specific IgE were arbitrarily classified in ELISA reactivity classes as described for rELISA.

Specificity of cELISA and rELISA

Specificity of cELISA and rELISA was evaluated using inhibition tests. Dpt extract (Bayer Corporation) was

10-fold serially diluted, from 15,000 to 15 AU/mL in ELISA buffer. Each dilution of the extract was mixed 1:1 with a 1/50 dilution of serum that was designated as containing 1,000 EU/mL of Der p 2-specific IgE, and further incubated at 37° C for 2 hours. The reference serum incubated with buffer only was used as positive control. Der p 2-specific IgE was determined by measuring the absorbance in two ELISAs as described above. Percentage of inhibition was calculated as follows: $[1.0 - (\text{test sample absorbance} / \text{positive control absorbance})] \times 100$.

Total Serum IgE

Total serum IgE was measured by a monoclonal antibody-based ELISA modified from a previously described radioimmunoassay.¹³ Briefly, polystyrene plates (Immulon II; Dynatech Laboratories, Chantilly, VA) were coated with mouse monoclonal anti-human IgE (Sigma) diluted 1:5,000 in 0.06 M carbonate buffer (pH 9.6) overnight at 4° C. Plates were washed and blocked as previously described. Serum samples diluted 1:5, 1:25, and 1:125 in 1% BSA-PBS-T were incubated for 1 hour at room temperature. After washing, biotinylated goat anti-human IgE (Kirkegaard & Perry Laboratories) diluted 1:4,000 was added and plates were incubated for 1 hour at room temperature. Subsequent steps (streptavidin-peroxidase and enzyme substrate) were similar to those described for rELISA. Results were expressed as international units (IU)/mL of serum (1 IU/mL = 2.4 ng/mL of IgE) and were calculated based on a control curve obtained by serial 2-fold dilutions of a serum that was designated as containing 3,000 IU/mL of total IgE. The control curve values ranged from 0.3 to 300 IU/mL.

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 as statistically significant.

RESULTS

Sensitivity and Specificity of ELISAs

Control curves of cELISA and rELISA obtained with the standard serum (UVA 89/01) were analyzed for intra- and interassay variations. The sensitivity of the two ELISAs for Der p 2-specific IgE was 3.9 EU/mL (Fig 1). The average coefficients of variation for each standard serum dilution assayed in duplicate were 12.7% and 5.8% in cELISA and rELISA, respectively. For repeated assays, the average coefficients of variation were 16.5% and 9.6% in cELISA and rELISA, respectively.

Specificities of cELISA and rELISA for the Der p 2 allergen were confirmed by inhibition tests (Fig 2). Both ELISAs were inhibited in a dose-dependent manner, when reference serum was mixed with increasing concentrations of crude Dpt extract. Inhibition was up to 80% with the highest concentration of mite extract (15,000 AU/mL).

Levels of Total IgE and Der p 2-Specific IgE

Total serum IgE was significantly higher in the SPT⁺ asthmatic patients (geometric mean; GM: 375.2 IU/mL) compared with either the SPT⁻ asthmatic patients (GM: 141.3 IU/mL; *P* < 0.01) or the control subjects (GM: 17.8 IU/mL; *P* < 0.0001).

The relationship between levels of Der p 2-specific IgE (EU/mL) obtained by the two assays in SPT⁺ asthmatic patients is shown in Figure 3. There was a significant positive correlation between cELISA and rELISA (*r* = 0.74; *P* < 0.0001).

As presented in Figure 4, the GM of Der p 2-specific IgE obtained by rELISA in SPT⁺ asthmatic patients (28.4 EU/mL) was significantly higher than in SPT⁻ asthmatic patients (0.6 EU/mL) or control subjects (0.6 EU/mL; *P* < 0.0001). Using cELISA, the mean levels of Der p 2-specific IgE were also significantly higher in the SPT⁺ asthmatic patients (4.5 EU/mL), compared with SPT⁻ asthmatic patients and control subjects (0.2 EU/mL and 0.3 EU/mL, respectively; *P* < 0.0001). In addition, levels of Der p 2-specific IgE measured by rELISA in SPT⁺ asthmatic patients were significantly higher than those obtained by cELISA (*P* < 0.01).

Serodiagnostic Performance of ELISAs

The serodiagnostic performance of the cELISA and rELISA was evaluated for sensitivity and specificity in relation to skin prick testing. Positivity rate of Der p 2-specific IgE measured by cELISA and rELISA was calculated based on the sensitivity of both assays (3.9 EU/mL), in asthmatic patients (SPT⁺ and SPT⁻ groups) and control subjects. Thus, rELISA for Der p 2 showed significantly higher sensitivity (70.8%) than did cELISA (52.1%; *P* <

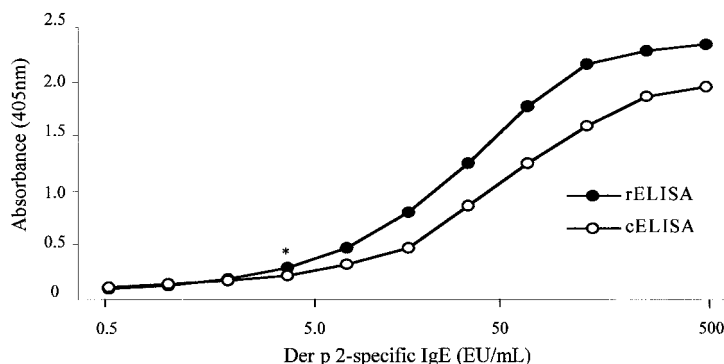


Figure 1. Standard curves of cELISA and rELISA for the measurement of Der p 2-specific IgE. *The sensitivity of the two assays was 3.9 EU/mL.

0.05). Specificity was 100% for both ELISAs (Fig 5).

Der p 2-Specific IgE by cELISA and rELISA at Different Reactivity Classes

Der p 2-specific IgE levels were analyzed according to reactivity classes (classes 0, 1, 2, and ≥ 3) obtained by cELISA and rELISA in the SPT⁺ asthma, SPT⁻ asthma and control groups, and the results are shown in Table 1.

Among the 34 positive samples (70.8%) for Der p 2-specific IgE by rELISA in the SPT⁺ asthma group, the majority (41.7%) displayed high or very high ELISA reactivity (class ≥ 3), whereas a lower proportion of samples (20.8%) showed ELISA reactivity class ≥ 3 when using cELISA ($P < 0.05$). In contrast, in the SPT⁻ asthma and control groups, neither ELISAs demonstrated any reactivity (class 0), resulting in total concordance with SPT and no difference in their specificity.

DISCUSSION

ELISA has shown to be a sensitive and alternative method for measuring specific IgE antibodies to mite allergens without using radioactive techniques. cELISA is usually performed using crude extracts or purified antigens/allergens to coat microtiter plates. However, purified antigen or allergens are difficult to obtain in large amounts. Mite allergen-specific monoclonal antibodies are broadly used to measure environmental allergens in two-site monoclonal antibody-based ELISA.^{14,15} Few reports have described the use of monoclonal antibodies in ELISAs to detect specific IgE antibodies, and these assays are usually named mAb-capture ELISA⁵ or sandwich ELISA.⁷ In the present study, we have developed a rELISA for measuring specific IgE antibodies to Der p 2, a major *D. pteronyssinus* allergen. Because the terminology of classical capture ELISA is commonly used when monoclonal antibodies bound to microtiter plates are used to capture IgM, IgA, or IgE antibodies, we preferred to use the term rELISA because the plates were

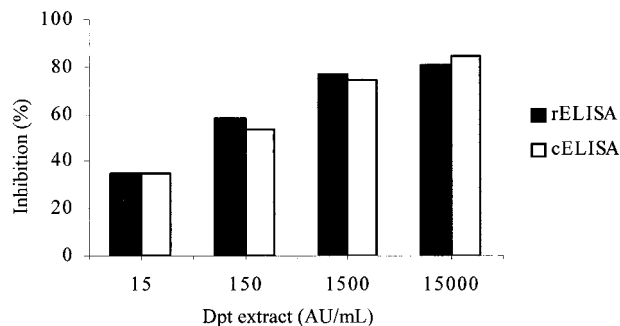


Figure 2. Inhibition tests for the measurements of Der p 2-specific IgE by cELISA and rELISA. Dpt allergenic extract was 10-fold serially diluted from 15,000 to 15 AU/mL in ELISA buffer. Each dilution of the extract was mixed 1:1 with a reference serum (20 EU/mL) and incubated at 37° C for 2 hours. As positive control, the reference serum was incubated with ELISA buffer only. Der p 2-specific IgE was determined by measuring the absorbance obtained by two ELISAs. Percentage of inhibition was calculated as follows: $[1.0 - (\text{test sample absorbance/positive control absorbance})] \times 100$.

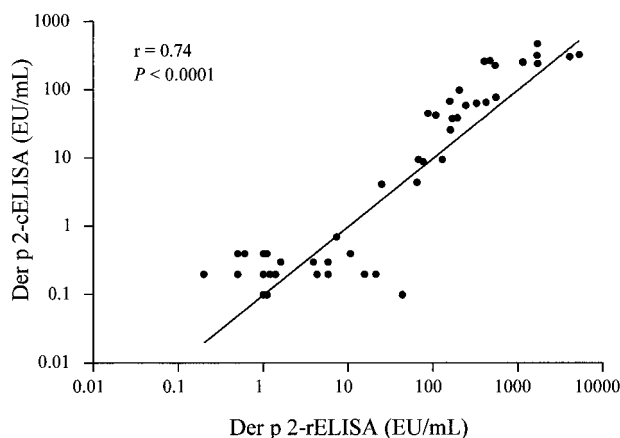


Figure 3. Correlation between rELISA and cELISA for measurement of Der p 2-specific IgE in SPT⁺ asthmatic patients (N = 48).

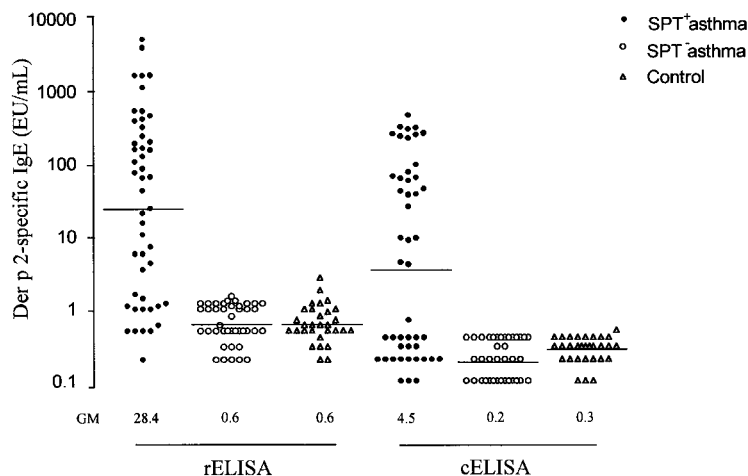


Figure 4. Levels of Der p 2-specific IgE (EU/mL) measured by rELISA and cELISA in SPT⁺ and SPT⁻ asthmatic patients and control subjects. The horizontal bars indicate the GM.

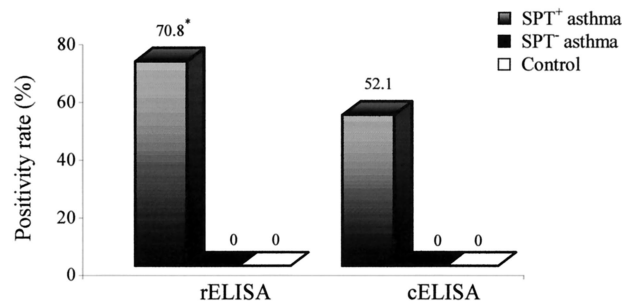


Figure 5. Positivity rate of Der p 2-specific IgE by rELISA and cELISA in asthmatic patients (SPT⁺ and SPT⁻) and control subjects. **P* < 0.05.

Table 1. Analysis of Different Reactivity Classes Obtained by rELISA and cELISA for Der p 2-Specific IgE in Asthmatic Patients (SPT⁺ and SPT⁻) and Control Subjects

Group	ELISA reactivity class*	Der p 2-specific IgE subjects (%)	
		rELISA	cELISA
SPT ⁺ asthma (N = 48)	0	14 (29.2)†	23 (47.9)
	1	7 (14.6)	5 (10.4)
	2	7 (14.6)	10 (20.8)
	≥3	20 (41.7)†	10 (20.8)
SPT ⁻ asthma (N = 41)	0	41 (100)	41 (100)
Control (N = 30)	0	30 (100)	30 (100)

* Class 0 : <3.9 EU/mL (negative specific IgE); class 1 : 3.9 to <19.5 EU/mL (low positive specific IgE); class 2 : 19.5 to <97.5 EU/mL (mild positive specific IgE); class ≥ 3 : ≥ 97.5 EU/mL (high or very high positive specific IgE).

† *P* < 0.05.

coated with monoclonal antibody to capture the antigen/allergen, and then to detect allergen-specific IgE antibodies.

Although the sensitivity of the two assays (rELISA and cELISA) was 3.9 EU/mL according to the reference serum used (UVA 89/01), the rELISA standard curve showed slightly higher absorbance values than did cELISA. In addition, the inter- and intraassay variations found for rELISA were lower than were those observed for cELISA. The specificity of both assays for Der p 2-specific IgE was confirmed by inhibition with crude Dpt extract in a dose-dependent manner. We believed that IgE-Der p 2 complexes in this inhibition assay for rELISA did not compete for binding to the plate-coated monoclonal antibody because it was obtained a dose-response curve of inhibition.

Specificity of rELISA was further confirmed in all sera from SPT⁺ asthmatic patients, which showed no reactivity in wells coated with mouse IgG

(used as control) in contrast to the reactivity observed when using the mouse monoclonal antibody to Der p 2 (data not shown). Thus, the possibility that these sera could contain IgE isotype rheumatoid factor (IgE antibodies against Ig γ -chain) and then reacting with the plate-coated mouse IgG monoclonal antibody was eliminated.

The probability that an IgE epitope is blocked by the plate-coated monoclonal antibody in rELISA was ruled out because of the fact that the results obtained in rELISA were higher than those obtained in cELISA using Dpt crude extract instead of rDer p 2 for coating the microtiter plates (data not shown).

In contrast, a direct comparison between cELISA and rELISA using the same allergen preparation (rDer p 2) was not possible because the monoclonal antibody (clone 1D8) used in rELISA does not react to a relevant isoform of Der p 2, which has an as-

partic acid at position 114 and this is also the isoform most frequently used as recombinant Der p 2.¹⁶

Serodiagnostic performance of the two ELISAs for measuring the levels of Der p 2-specific IgE was evaluated in asthmatic patients (SPT⁺ and SPT⁻) and control subjects. Thus, rELISA was found to be more sensitive than cELISA with respect to both the positivity rate (70.8% vs 52.1%) and GM levels (28.4 vs 4.5 EU/mL) in the SPT⁺ asthmatic patients. In contrast, the specificities of both ELISAs were 100%, and total concordance with SPT-negative results was found both in asthmatic patients and control subjects.

A likely explanation for the higher sensitivity of rELISA found in the present study is provided by Kemeny et al.⁷ These authors reported that the increased sensitivity of sandwich ELISA compared with RAST and conventional ELISA is not attributable to larger amounts of IgE antibody being bound in ELISA than in RAST, but because a very high concentration of nonradioisotopic anti-IgE can saturate the small amounts of bound IgE. A further advantage of sandwich or rELISA for detection of specific IgE antibodies is that the use of a considerable excess of anti-IgE results in a much steeper binding curve and this in turn contributes to a lower interassay variation coefficient,⁷ compared with RAST, which has been associated with high interassay variation.¹⁷ This feature was also observed in our study when comparing the interassay variation coefficients between rELISA and cELISA.

Levels of total serum IgE in our SPT⁺ asthmatic patients (375.2 IU/mL) were slightly higher than those observed by Gelber et al.¹⁸ among asthmatic patients (160 IU/mL) and control subjects (44 IU/mL), living in Wilmington, Delaware. In contrast, very similar results were seen in the SPT⁻ (141.3 IU/mL) and control (17.8 IU/mL) groups.

When analyzing the different reactivity levels of the two ELISAs among SPT⁺ asthmatic patients, rELISA showed a significantly higher percentage

of reactivity classes 3 or 4 (41.7%), compared with cELISA (20.8%). Thus, the higher sensitivity (70.8%) of the rELISA, compared with cELISA (52.1%), was attributable to the presence of a larger number of positive samples with high reactivity (classes 3 or 4) as well as a smaller number of negative samples (class 0).

We demonstrated that rELISA is a sensitive alternative method for measuring specific IgE antibodies to Der p 2 mite allergen, which can be easily carried out in a clinical or research laboratory, as a valuable tool to confirm the diagnosis of atopic asthmatic patients, through the identification of the major allergens and for followup of specific allergen or peptide immunotherapy. rELISA can still be used for the detection of specific IgE to other mite allergens. Using this technique, we have found that mite allergic patients may have sensitivity to Der p 1 and not to Der p 2 or vice versa (data not shown). It is likely that rELISA could also detect IgE specific to relevant peptides. This information could be useful for the design of novel forms of allergen immunotherapy, including the use of relevant peptides or the corresponding DNA sequences.

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