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Biological Activity of Recombinant Der p 2, Der p 5 and Der p 7 Allergens of the House-Dust Mite *Dermatophagoides pteronyssinus*

Abstract

Der p 2, Der p 5 and Der p 7 are three allergens of the house-dust mite *Dermatophagoides pteronyssinus* that have been cloned and expressed in *Escherichia coli* as fusion proteins with glutathione-S-transferase (GST). We showed that these recombinant allergens produced immediate hypersensitivity skin-test reactions in 70, 60 and 52% respectively of a group of mite-sensitive allergic patients who were strongly positive to whole mite extract (WME). Comparable positivities were found for serum levels of specific IgE antibody against these allergens, as measured by the radioallergosorbent test (RAST). Overall, for the group of allergic patients that we evaluated, the serum IgE antibody concentrations against Der p 2, 5 and 7 were calculated to represent about one third, one quarter and one fifth respectively of the levels measured against the WME. However, for some patients the activity determined against the separate allergens was far higher than that detected against the WME, thus indicating that the concentration of these can be limiting in the WME. We found no significant correlations between the RAST levels against Der p 2 and either Der p 5 or 7, and RAST-inhibition tests indicated a lack of cross-reactivity between Der p 2 and the other two allergens. In contrast, the RAST results revealed the existence of a significant immunological relationship between Der p 5 and 7. Although a certain degree of reactivity against the GST fusion partner was found in the allergic patients studied, this was not a significant influence in determining the positivity against the recombinant allergens. These results confirm the in vivo biological activity of recombinant Der p 2, 5 and 7, and indicate that whilst Der p 2 is undoubtedly a major mite allergen, both Der p 5 and 7 make important contributions toward the overall allergenic activity of house-dust mites.

Key Words

House-dust mite
Dermatophagoides pteronyssinus
Recombinant allergens
IgE
Allergy

Introduction

House-dust mites are important sources of environmental allergens that stimulate allergic disease in sensitive patients [1, 2], and these organisms contain a large number of

components that react with the IgE of such persons [3–6]. Considerable information is available on many of the major mite allergens, particularly those of the genus *Dermatophagoides*, either through physicochemical studies of the native proteins [7–10], or the analysis of the cDNA coding for

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these, and the evaluation of the expression products of this cDNA [11, 12]. A particularly important allergen of *Dermaphagoides pteronyssinus*, designated Der p 2, has been extensively studied [13, 14], and has been cloned and expressed in *Escherichia coli* as a fusion protein with glutathione-S-transferase (GST) [15, 16]. We have shown that the recombinant material is closely comparable to the native protein in its capacity to bind specific IgE and to elicit immediate hypersensitivity reactions in the skin of house-dust mite-allergic patients [17]. Two less well characterized mite allergens, Der p 5 [18, 19] and Der p 7 [20], have also been cloned, and the recombinant fusion proteins shown to bind IgE and produce positive skin tests.

The objective of the present study was to quantitatively compare the IgE reactivities against recombinant Der p 2, 5 and 7 in a group of mite-sensitive patients, and to explore possible allergenic relationships between these.

Materials and Methods

Study Subjects

A total of 170 allergic patients were selected from the Allergy Clinic of the Institute of Biomedicine, on the basis of their evident clinical sensitivity to house dust, and strong positivity in immediate-hypersensitivity skin tests to whole mite extract (WME). The mean $\pm$ SD age of the group was 29 ± 6 years, and 60% were female. A group of 12 persons who reported no history of allergic disease, and were skin-test negative to WME, were also evaluated. This study was approved by the Ethical Committee of the Institute of Biomedicine, and was ratified by the Academic Council of the Faculty of Medicine of the Central University of Venezuela, and the National Council for Scientific and Technological Investigation (CONICIT).

Whole Mite Extract

Cultured *D. pteronyssinus* (Commonwealth Serum Laboratories, Parkville, Australia and Biopol Inc, Spokane, Wash., USA) were defatted by Soxhlet extraction with diethyl ether, extracted for 48 h at 4°C with 0.05 M NH_4HCO_3 saturated with chloroform and then concentrated/dialyzed using a hollow-fibre filter with a nominal molecular weight cut-off of 5 kD (Amicon Corp., Mass., USA). Total protein concentrations were determined by the Folin-Lowry method, then the extracts were membrane filtered and sterility tested by routine bacteriological methods.

Recombinant Allergens

The recombinant allergens were synthesized as fusion proteins with GST, using pGEX vectors in *E. coli* [21]. Der p 2 cDNA was cloned in the BamHI site of pGEX-1, to begin synthesis from the Asp N-terminal of the mature protein [15, 16]. Der p 5 was synthesized from a pGEX-2T construct in which a PCR product [19] obtained with Pfu polymerase (Stratagene, La Jolla, Calif. USA) was cloned into the EcoRI site to begin at the predicted N-terminal Glu of the mature protein [19], although it is now known that the Asp following the Glu is the mature N-terminus [22]. Der p 7 cDNA was a Pfu polymerase PCR

product cloned into the EcoRI site of pGEX-1, starting with the predicted Asp N-terminal [20]. Bacteria carrying the p-GEX vector, but without any cDNA insert corresponding to mite allergens, were used to provide the GST control material.

The fusion proteins were prepared as previously described [16]. Thus, log-phase *E. coli* were induced by the addition of 0.1 mM isopropyl β -D-thiogalactopyranoside (Promega, Madison, Wisc., USA) for 1 h at 37°C. The bacteria were sedimented at 9,000 g for 15 min, washed with Tris-HCl (10 mM)-NaCl (150 mM)-EDTA (0.5 M), pH 7.5, and resuspended in this buffer, plus 5 mM MgCl_2 , 20 $\mu\text{g}/\text{ml}$ DNase (Boehringer Mannheim, Germany), 0.12 TIU/ml aprotinin (Sigma Chemical Co., St. Louis, Mo., USA) and 1% Triton X-100. The suspension was homogenized 5 times for 1 min in a Braun MSK homogenizer (Braun Instruments, Burlingame, Calif., USA) with 0.1-mm glass beads, with phenylmethylsulphonyl fluoride being added to 0.5 mM before each homogenization. The lysate was centrifuged at 20,000 g for 30 min and the supernatant added to columns of glutathione-agarose (Sigma). The fusion proteins were eluted with 5 mM reduced glutathione in 50 mM Tris-HCl, pH 8, dialyzed against 0.05 M ammonium bicarbonate, and freeze-dried. The homogeneity of these products was checked by electrophoresis in polyacrylamide gels that were stained by Coomassie Blue 250, and also by Western blotting [16].

Skin Tests

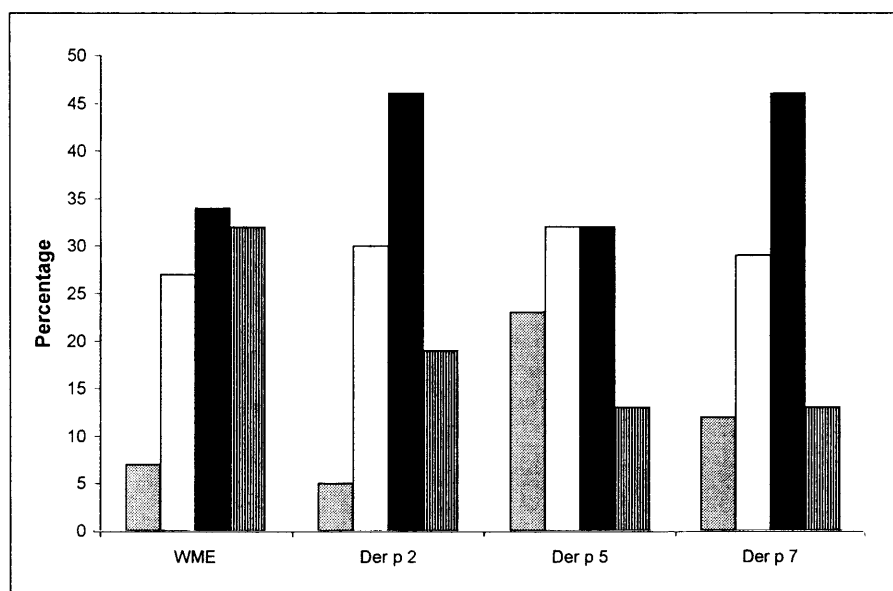
Skin-prick tests were performed with WME at 100 $\mu\text{g}/\text{ml}$ in saline containing 50% glycerol and 0.4% phenol. The recombinant allergens were tested at concentrations that started at 1 $\mu\text{g}/\text{ml}$, with negative tests being repeated at higher concentrations, up to a maximum of 100 $\mu\text{g}/\text{ml}$. Control tests were performed with the diluent, and 1% w/v histamine dihydrochloride. The wheal diameters were measured at 20 min, and diameters ≥ 3 mm were considered positive. Any antiallergic medication was suspended for 3 days before the tests. We should note here that prick tests were used in preference to the more sensitive and precise intradermal tests, because the risks of provoking systemic reactions are higher with the latter. This is of particular importance for uncharacterized allergen preparations such as the recombinant materials, and in the present study the tests in any given patient were stopped at allergen concentrations that produced wheal sizes ≥ 10 mm. Despite this, most of the patients were tested at up to 100 $\mu\text{g}/\text{ml}$ of the recombinant allergens, with no excessively large reactions, or other adverse effects, being observed.

The 170 allergic patients studied were selected for their skin-test positivity to WME, and 60 of these were tested with Der p 2, 5 and 7, and GST, whilst the remaining 110 were only tested with Der p 2 and GST.

In vitro Assays

The paper disc radioallergosorbent test (RAST) of Ceska and Lundkvist [23] was used to measure the serum levels of specific IgE antibody. In this test, filter paper discs were activated by CNBr treatment and sensitized at 50 discs/ml with 10 mg/ml of WME and 1 mg/ml of the recombinant allergens. The test sera were incubated with these and the bound IgE detected using ^{125}I -anti-IgE ('Phadebas'; Pharmacia, Uppsala, Sweden) and γ counting. The specific IgE concentrations were estimated by reference to the Phadebas reference standards, and expressed as Phadebas RAST units (PRU)/ml. These results were also classed into the widely accepted RAST scale, where values ≤ 0.35 PRU/ml are designated level 0 (negative), 0.35–0.7 PRU/ml; level 1 (probably positive), 0.7–3.5 PRU/ml; level 2

Fig. 1. The distribution of RAST classes 1–4 for the different allergenic preparations, expressed as percentages of the number of allergic sera positive ($\geq$ RAST level 1; $\geq$ 0.35 PRU/ml) for each allergen. □ = RAST level 1 (0.35–0.7 PRU/ml); ◻ = level 2 (0.7–3.5 PRU/ml); ■ = level 3 (3.5–17.5 PRU/ml); ▨ = level 4 ($\geq$ 17.5 PRU/ml).



(clearly positive), 3.5–17.5 PRU/ml; level 3 (highly positive) and $\geq$ 17.5 PRU/ml; level 4 (very strongly positive).

RAST-inhibition assays were performed by including in the disc-serum incubation step, soluble allergen at concentrations of 0.1–1,000 μ g/ml, and calculating the % inhibition caused by the competition between the soluble and solid-phase allergen.

In some of the *in vitro* studies we used a pool of sera from 8 house-dust-sensitive patients who were strongly positive in RAST to WME and the recombinant allergens. We also used a pool that has been prepared by the National Institute for Biological Standards and Control, UK, and identified as NIBSC Research Reagent 82/528, human anti-*D. pteronyssinus* serum.

Statistical Analysis

The proportions of the patient group who were positive in skin tests or RAST to the different allergen preparations were compared by the χ^2 test. The medians of the RAST values against the recombinant allergens, expressed as percentages of the WME value, were compared by the common median χ^2 test. The nature of the distribution of these percentage values was characterized by the calculation of skewness and kurtosis. Pearson correlation coefficients were determined between RAST values to the different allergen preparations. The different statistical tests were performed with the Microsoft 'Excel' and GraphPad 'InStat' programs.

Results

Skin-Test Reactivity to Recombinant Allergens

Skin-prick tests were performed with recombinant Der p 2, 5 and 7 on a group of 60 house-dust-allergic patients who were positive to WME. Although the patients reacted significantly ($p < 0.001$) less frequently to the different recombinant allergens than to WME (table 1), each of these was

Table 1. Skin test and RAST positivity for WME and the recombinant allergens in 60 house-dust mite-allergic patients

	Skin test % positive ^a	RAST	
		% positive ^b	% WME ^c
WME	100	93	—
Der p 2	70	62	39
Der p 5	60	57	23
Der p 7	52	40	18

^a Percent of 60 patients studied with immediate wheals $\geq$ 3 mm.

^b Percent of sera with RAST values $\geq$ level 1 (0.35 PRU/ml).

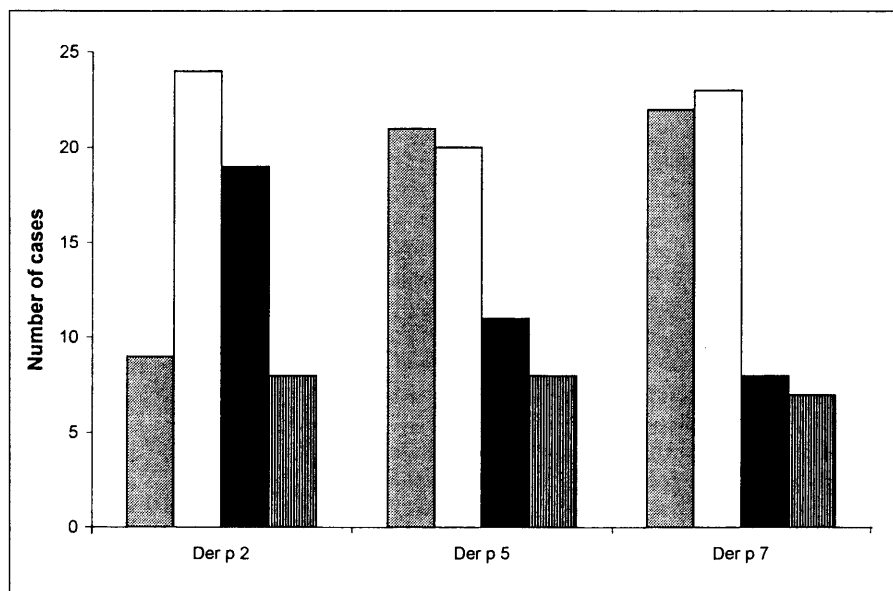
^c Median values of the distribution of RAST levels expressed as percentages of WME. The interquartile ranges were: 21–74% for Der p 2, 5–54% for Der p 5, and 3–38% for Der p 7.

positive in at least half of the group. The order of reactivity was Der p 2 > Der p 5 > Der p 7, the difference between Der p 2 and 7 being statistically significant ($p < 0.05$). No reactivity to the recombinant allergens was found in a group of 12 non-house dust-allergic patients who were skin-test negative to WME.

Specific IgE to Recombinant Allergens

The RAST positivity ($\geq$ class 1; 0.35 PRU/ml) of the sera of the 60 house-dust-allergic patients to the various allergen preparations is presented in table 1. These results reveal that, as for the skin tests, the frequency of RAST positivity to the different recombinant allergens was significantly ($p < 0.001$) lower than to WME, and the order of reactivity

Fig. 2. Distribution of RAST values to the different recombinant allergens, expressed as percentages of the level against WME for each subject. □ = 0–10%, ◻ = 10–50%, ■ = 50–100%, ▣ = >100%.



was Der p 2 $\approx$ Der p 5 > Der p 7, the difference between Der p 2 and 7 being statistically significant ($p < 0.05$). The distribution of the RAST classes within the positive sera is presented in figure 1. Compared to the recombinant allergens, a higher proportion of the patients were RAST class 4 (very strongly positive) to WME ($p < 0.01$). However, for both Der p 2 and 7 the patients were most frequently found at class 3 (strongly positive). For Der p 5 the proportion of the patients at level 1 (weakly positive) was significantly higher ($p < 0.01$) than for the other allergenic preparations.

The levels of IgE antibody against the recombinant allergens were also expressed as percentages of the values against WME for each patient, and a very wide dispersion was found. Thus, the range for Der p 2 was from 0.5 to 192% (coefficient of skewness = 1.0), for Der p 5 was 0.7 to 1,260% (skew = 5.0) and for Der p 7 was 0.7 to 3,630% (skew = 7.7). The distributions of these percentage values are illustrated in figure 2, and the group medians are shown in table 1. The median value for Der p 2 was significantly ($p < 0.05$) higher than for Der p 7. It is of interest to note that for 17% of the patients, the RAST activity against any one of the recombinant allergens exceeded that measured against the WME. An extreme example of this is a patient who was RAST negative to WME (0.19 PRU/ml) and Der p 2 (0.20 PRU/ml), but was strongly positive to Der p 5 (2.4 PRU/ml) and Der p 7 (6.9 PRU/ml). This suggests that the amount of a given allergen on the WME RAST discs can be limiting.

Further to this, in table 2 are presented the correlation coefficients determined over the 60 test sera between the

Table 2. Correlation coefficients (r) between RAST values to WME and recombinant allergens in 60 allergic patients

	Der p 2	Der p 5	Der p 7
WME	0.65	0.39	0.42
Der p 2		0.4	0.31
Der p 5			0.87

RAST values against the different allergen preparations. There was a reasonably strong correlation between WME and Der p 2 ($p < 0.001$), but for these the correlations with Der p 5 and 7, although statistically significant ($p < 0.01$), were lower. In contrast, the correlation between Der p 5 and 7 was highly significant ($p < 0.001$).

Reactivity to GST Control

As some of the allergic patients described above reacted to the GST control material, the study group was expanded to include 110 additional patients who were positive to WME, and who were tested with recombinant Der p 2 and the GST control. The overall skin test positivity to Der p 2 was 73%, and that to GST was 23%, all of the latter being at the threshold of positivity (3 mm wheal diameters). In RAST, 60% of the sera were positive to Der p 2, and 16% to the GST. Of the sera that were positive to GST, 74% were at level 1, and the remainder at level 2. The correlation between the RAST values to Der p 2 and GST, although statistically

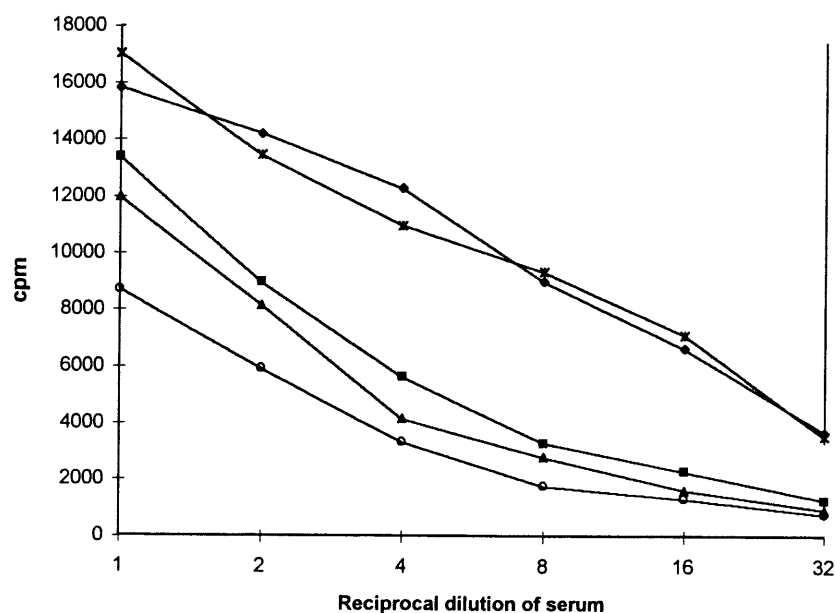


Fig. 3. RAST titration of the NIBSC human anti-*D. pteronyssinus* reference serum against WME (X) and our pool of 8 mite-sensitive sera against WME (◆), Der p 2 (■), Der p 5 (▲) and Der p 7 (○).

significant ($p < 0.01$), was relatively low ($r = 0.27$). When the RAST reactivity towards GST was subtracted from the values for the different recombinant allergens, determined in the 60 allergic patients described above, the percent positivity was 57% for Der p 2, 51% for Der p 5 and 35% for Der p 7.

RAST Titrations

The sera from 8 allergic patients of the study group were selected for their high RAST reactivities to the WME and the recombinant allergens, and a pool was made of these. This pool was serially diluted, and tested in RAST using discs sensitized with either WME or the recombinant allergens (fig. 3). In addition, as a comparison, the NIBSC human anti-*D. pteronyssinus* reference reagent was titrated against the WME discs. These studies showed that, firstly, our laboratory serum pool had a similar degree of reactivity toward WME as did the reference reagent. Secondly, at all dilutions of our serum pool, the order of RAST reactivity was WME > Der p 2 > Der p 5 > Der p 7. In figure 4 the RAST values at each serum dilution for the different recombinant allergens are expressed as a percentage of those against the WME. We found that when this selected serum pool was tested undiluted, the activity, relative to WME, was 84% for Der p 2, 76% for Der p 5 and 55% for Der p 7. However, as the serum pool was diluted, the relative activities against the recombinant allergens decreased, plateauing at about 35, 25 and 20%, respectively. This probably oc-

curred because at low serum dilutions the WME discs, but not those sensitized with the separate allergens, were saturated by excess antibody, thus underestimating the RAST level against the WME. As the test serum was progressively diluted, a point was reached (at about $\frac{1}{8}$ for this serum pool) where the RAST levels measured against WME and the separate allergens were in their true proportions. It is of interest to note that the percentages determined in this manner with the serum pool were closely comparable to the medians obtained for the group of 60 allergic patients when the individual RAST values to the recombinant allergens were expressed as percentages of the WME, as presented in table 1.

RAST Inhibition

The serum pool was used in RAST inhibition tests, in which the binding of the IgE antibody to the allergen in the solid phase is competitively inhibited by soluble allergen. The homologous inhibition curves are presented in figure 5. The concentrations of allergen required to produce 50% inhibition of RAST with this serum pool were 20 $\mu\text{g/ml}$ for Der p 2, 0.5 $\mu\text{g/ml}$ for Der p 5 and 84 $\mu\text{g/ml}$ for Der p 7.

The allergens were also tested in heterologous inhibitions, in which each allergen in the soluble form competes with the others in the solid phase. As the GST control material can have some inhibitory effect in these tests (for example, $16 \pm 2\%$ against WME, and $13 \pm 2\%$ against Der p 2), only heterologous inhibitions of $\geq 20\%$ were considered significant. The maximal inhibitions caused by saturating

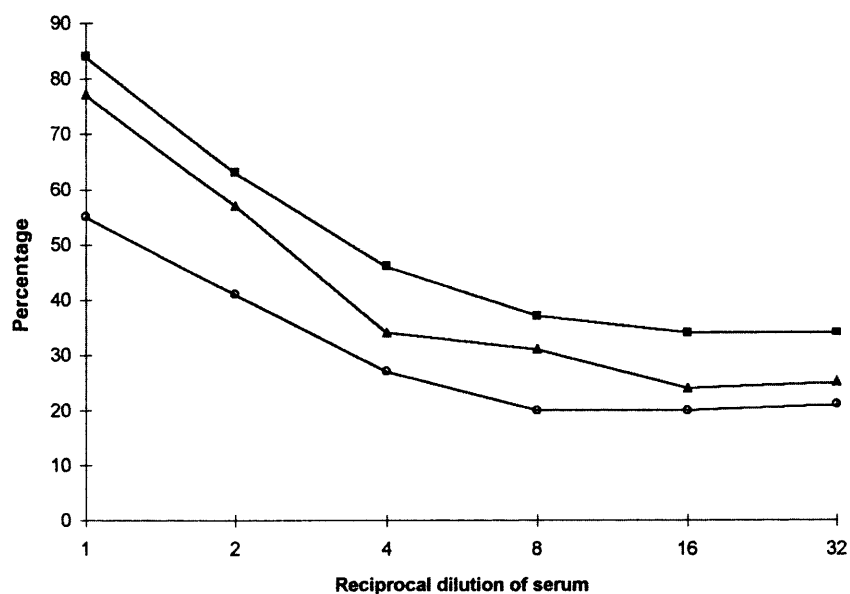


Fig. 4. The RAST values against the different recombinant allergens determined as in figure 3, but expressed as percentages of the value against WME for each serum dilution. Der p 2; ■ = ; ▲ = Der p 5; ○ = Der p 7.

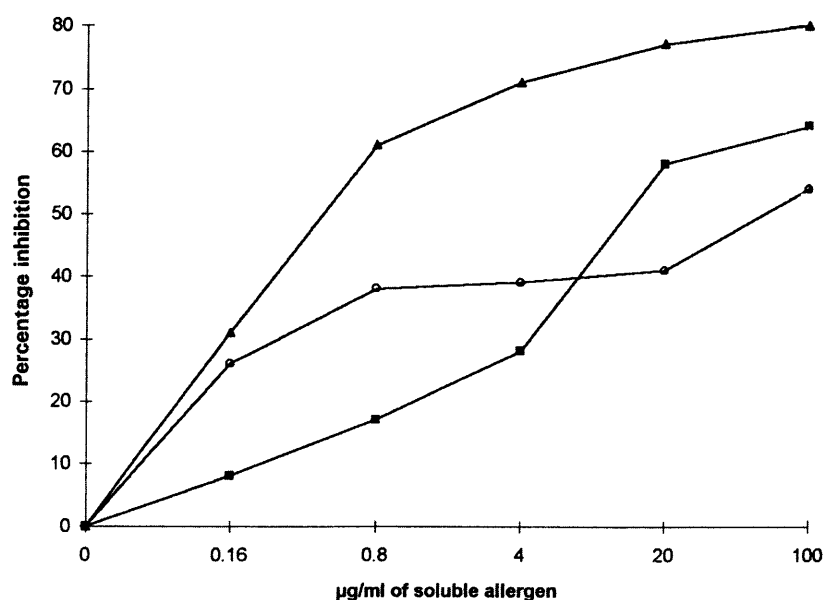


Fig. 5. Homologous RAST inhibition curves. ■ = Der p 2; ▲ = Der p 5; ○ = Der p 7.

concentrations of allergen are shown in table 3. It can be seen that of the recombinant allergens, only Der p 2 significantly inhibited the WME RAST. Inversely, WME strongly inhibited the Der p 2 RAST, but did not diminish the Der p 5 RAST, but did not diminish the Der p 7 RAST. No significant crossed inhibitions were found between Der p 2 and either Der p 5 or 7. However, Der p 5 strongly competed

for the binding of IgE antibody to solid-phase Der p 7, and the reverse inhibition (Der p 7 against Der p 5), although weaker, was also significant.

Table 3. Crossed RAST inhibition between WME and the recombinant allergens

Solide phase	Soluble allergens			
	WME	Der p 2	Der p 5	Der p 7
WME	77±6 ^a	26±6	<20	<20
Der p 2	65±4	84±7	<20	<20
Der p 5	31±12	<20	85±5	24±4
Der p 7	<20	<20	40±6	77±6

^a Maximum % RAST inhibition at saturating concentrations of soluble allergen (1,000 µg/ml).

Discussion

In two reports of the capacity of recombinant allergens to stimulate immediate reactions in the skin of Taiwanese house-dust mite-sensitive patients [19, 20], the same Der p 2 preparation used in the present study was active in intradermal tests of almost all WME-positive subjects. Similarly, we have shown [17] that a high proportion of Venezuelan mite-allergic patients are positive to this material in prick tests, which are less sensitive and precise, although somewhat safer, and less discomforting, than intradermal tests. The results of the present study confirm these findings, and add to the evidence that indicates the great importance of class 2 allergens in hypersensitivity to mites [13,14]. Like the Taiwanese studies [19, 20], our results also demonstrate that over half of the WME-sensitive patients are skin-test positive to recombinant Der p 5 and 7, and that non-mite-allergic patients do not react significantly to these.

The presence of specific IgE antibody in the serum of the Taiwanese patients has been demonstrated, with 52% reacting to Der p 5 [19] and 41% to Der p 7 [24], and, in addition, a comparable degree of IgE binding was shown for native and recombinant Der p 7 [24]. Similarly, 55% of a group of Australian mite-sensitive patients were positive in dot-blots to the polypeptide that was subsequently designated Der p 5 [18]. We used the more quantitative RAST to measure IgE antibody against these same allergen preparations, and found that 57 and 43% of the Venezuelan mite-sensitive patients were positive to Der p 5 and 7, respectively, having levels ≥ 0.35 PRU/ml, which is level 1 on the RAST scale, and indicates the presence of significant reactivity. The finding that the positivity in skin tests to the recombinant allergens was significantly higher than that determined in the in vitro tests, in both our and the Taiwanese [19, 20] stud-

ies, possibly indicates that the cut-off value for IgE against specific allergens could be taken at a lower level than that routinely used for the conventional allergenic extracts. In addition, the in vitro tests tend to measure higher affinity antibody, and this might not be such an important factor in the skin tests [25]. Considering the patients of our study group who were positive in RAST to the recombinant allergens, the majority were highly reactive, with many at RAST level 3 or 4, which indicates a degree of sensitivity that would be clinically relevant. In fact, for a significant proportion of the sera that we tested, the levels of IgE antibody against either Der p 5 or 7 exceeded those measured against the WME or Der p 2, and the RAST results indicated that the absolute amounts of Der p 5 and 7 present on the paper discs sensitized with WME were insufficient for the detection of all of the antibody against these in the serum samples of some patients. The relatively low concentration of Der p 5, and particularly Der p 7, in WME was also indicated by the inefficiency of this extract in inhibiting the RAST against these allergens, which contrasts with the case of Der p 2, where the soluble WME was strongly inhibitory. A similar suggestion has also been made on the basis of the observation that monoclonal antibodies against Der p 7 react only weakly with WME [26].

When the levels of specific IgE antibody against the recombinant allergens measured in the sera of our study group were calculated as fractions of the total RAST activity against the WME that we used, anti-Der p 2 represented about one third of the total, anti-Der p 5 one quarter and anti-Der p 7 one fifth. Considering that many other allergens of house-dust mites have been identified [3–6], these proportions could be considered somewhat high. However, calculations of this type are probably influenced by particular characteristics of the sera studied, in our case these being obtained from Venezuelan patients in a tropical situation, and also by the WME used, which can show considerable variability in allergenic composition between different sources [27]. Furthermore, although these estimations were based on tests in which the allergic sera were used at non-saturating dilutions, the great complexity of WME might also result in the under-representation of certain allergens on the solid phase of the RAST.

An important consideration in the analysis of our results with the recombinant allergens is that these materials were expressed as fusion proteins with the GST of *Schistosoma japonicum*, and that preparations of this enzyme synthesized by transformed *E. coli* showed a certain degree of allergenic activity in some of our patients. Although *Schistosoma mansoni* infection occurs in Venezuela, the study group was not drawn from an endemic area, and the patients

were negative for this parasite in faeces examinations. Occasional infections by common intestinal helminths, such as *Ascaris lumbricoides* and *Trichuris trichiura*, were, however, detected, and as the GST may be relatively conserved in evolution [28], it is possible that the positivity observed was due to cross-reactivity in the immune responses to the different helminths. Perhaps a more likely explanation is provided by the report that mite GST is also an important allergen [29], and there may be sufficient cross-reactivity between the mite and parasite enzymes to produce reactions in highly mite-sensitive patients. The degree of skin-test reactivity to the GST control material in the two previously reported groups of Taiwanese patients was 9% [19] and 0% [20]. Despite the existence of a low-grade reactivity to the GST control preparation in our study group, when this was subtracted from the RAST values to the recombinant allergens, the overall positivity to these was not appreciably altered. In addition, the lack of correlation between Der p 2 and the GST in both skin tests and RAST, indicates that the reactivity against the recombinant allergen is independent of any response that might exist to the GST fusion partner.

Low correlations were also found for the RAST results to Der p 2 and either Der p 5 or 7, indicating the independence

of the responses to these allergens, and RAST inhibition tests confirmed the lack of an immunological relationship between Der p 2 and the other two allergens. In contrast, the RAST values to Der p 5 and Der p 7 were strongly correlated, which could indicate that the magnitude of the response to these is determined by common factors. However, as significant crossed RAST inhibition was found between these, they may also share some relevant epitopes. There are no evident sequence homologies between these polypeptides that could indicate that this is the case, and monoclonal antibodies against Der 7, that do not react with Der p 5, have been prepared [26]. However, neither of these observations definitively rule out the possibility of cross-reactivity between these two allergens.

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