

Measurement of Allergens Associated with Dust Mite Allergy

I. Development of Sensitive Radioimmunoassays for the Two Groups of *Dermatophagoides* Mite Allergens, *Der* I and *Der* II

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Abstract. Sensitive radioimmunoassays were developed to measure the two groups of major allergens (*Der* I and *Der* II) of *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, in which radiolabeled protein A served as a general tracer. Glass rods covalently coupled with F(ab')₂ fragments of rabbit antiserum IgG were incubated first with mite or house dust extracts, and then with affinity-purified rabbit antibodies. The bound allergen-antibody complex was detected with a ¹²⁵I-labeled protein A.

The two *Der* I allergens, *Der p* I and *Der f* I, were measured separately with the use of immunoabsorbed rabbit antibodies directed against species-specific determinants, while the two *Der* II allergens, *Der p* II and *Der f* II, were measured as '*Der* II' with the use of antibodies directed against common determinants on both allergens.

Each assay demonstrated consistently parallel dilution curves with mite and house dust extracts. The mean intraassay and interassay coefficient of variation for each assay ranged from 4.3 to 7.7% and from 3.6 to 9.0%, respectively.

The *Der p* I and *Der f* I assays were shown to be highly species-specific so that the ratio of *Der p* I : *Der f* I would provide a good index of the distribution of the two mite species in a dust sample. The concentrations of the *Der* I and *Der* II allergens in different types of mite extract and dust samples from houses were compared using these assays. The results demonstrated that the ratio of *Der* I : *Der* II in the mite body extracts varied markedly from those in the whole culture extracts, while the ratio in individual dust samples was nearly constant and was close to that in the mite body extracts.

These assays will be useful in standardizing mite and house dust extracts and for assessments of mite allergen exposure. Furthermore, the high assay sensitivity will make it possible to use these assays to measure mite allergens associated with airborne particles.

Introduction

The most important sources of house dust allergens in many parts of the world are mites of the genus *Dermatophagoides* [1-4]. It has been generally recognized that the *Dermatophagoides* mites have two groups of major allergens, the *Der* I group (*Der p* I, *Der f* I and *Der m* I) and the *Der* II group (*Der p* II and *Der f* II) [5-14]. We have isolated these two

groups of allergens from both *D. pteronyssinus* and *D. farinae* [12, 13].

The measurements of individual mite allergens by the use of immunochemical assays [15-18] can be useful for the standardization of mite and house dust extracts [19-21]. In addition, mite allergen measurements can help to assess the level of exposure to mite allergens [22-28].

In the present study, we have developed sensitive

radioimmunoassays for these mite allergens. *Der p* I and *Der f* I were measured separately using species-specific antibodies, and *Der p* II and *Der f* II were measured as '*Der* II' using antibodies directed against common determinants on both allergens. These assays were used to compare the concentrations of the two groups of allergens in mite extracts and dust samples from houses. In addition, the distribution of the two mite species in dust samples was examined by using the species-specific assays for the *Der* I allergens.

Materials and Methods

Purified Mite Allergens

Four mite allergens, *Der p* I, *Der f* I, *Der p* II and *Der f* II, were purified from whole cultures of *D. pteronyssinus* and *D. farinae* as described previously [12, 13]. The concentrations of the purified allergens used as the standards for radioimmunoassays were determined by using their $E_{1\text{cm}}^{1\%}$ values [13]. Cyanogen-bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) was coupled with each preparation of purified mite allergens (0.5 mg/ml gel) according to the manufacturer's instructions.

Mite and House Dust Extracts

Mite extracts were prepared by extracting 1 g of either defatted whole culture or defatted and grounded mite body of either *D. pteronyssinus* or *D. farinae* in 100 ml of phosphate-buffered saline (PBS), pH 7.4, for 24 h at 4°C. The extracts were centrifuged (2,000 g for 20 min) and the supernatants were stored at -20°C.

The WHO international standard for house dust mite (*D. pteronyssinus*) extract (82/518) [20] was obtained from the National Institute for Biological Standards and Control (London, UK).

Forty-seven dust samples were obtained from household vacuum cleaner bags of the houses of patients with asthma in and around Sagami-hara. Dust samples were sieved through a 0.3-mm mesh screen, and the fine dust (20 mg) was extracted for 24 h at 4°C with 2 ml of PBS containing 0.2% Tween 20, 0.05% NaN_3 and 0.2% bovine serum albumin (PBS-T-BSA). The extracts were centrifuged and the supernatants were stored at -20°C.

Commercial allergen extracts of short ragweed, orchard grass and Japanese cedar pollens were obtained from Hollister-Stier Laboratories (Spokane, Wash., USA) and allergen extracts of cat and dog danders, silk and molds (*Alternaria*, *Aspergillus*, *Candida*, *Cladosporium* and *Penicillium*) were from Torii (Tokyo, Japan).

Rabbit Antisera, $F(ab')_2$ Fragments and Affinity-Purified Antibodies

Preparation of antisera to *Der p* I and *Der f* I has been described elsewhere [13]. Rabbit antisera used for the radioimmunoassay measuring the *Der* II allergens were prepared by immunizing rabbits with a 1:1 mixture (200 µg) of *Der p* II and *Der f* II in complete Freund's adjuvant (Difco, Detroit, Mich., USA). The rabbits were injected 4 times at 4-week intervals and bled 10 days after the last injection.

$F(ab')_2$ fragments were prepared from the IgG fraction of rabbit antisera by pepsin digestion followed by chromatography on Sephadex G-150 (Pharmacia) [29]. The trace undigested IgG remaining in the $F(ab')_2$ fragments was removed by absorption with protein A-Sepharose CL-4B (Pharmacia).

Species-specific antibody to *Der p* I was prepared from rabbit anti-*Der p* I serum by affinity chromatography on *Der p* I-Sepharose followed by absorption with *Der f* I-Sepharose. The IgG fraction from 6 ml of antiserum was applied to a *Der p* I-Sepharose column (2 ml). After extensive washing with borate-buffered saline (BBS), pH 8.0, the bound antibody was eluted with 0.17 M glycine-HCl, pH 2.5, at 4°C. The antibody solution was neutralized and passed through a *Der f* I-Sepharose column (2 ml). The fraction eluted unretarded from the column was collected and further absorbed extensively with *Der f* I-Sepharose until little or no cross-reaction with *Der f* I was detected in the *Der p* I radioimmunoassay. A similar method was used for the preparation of species-specific antibody to *Der f* I. Antibody to common determinants on the two *Der* II allergens was prepared from rabbit antiserum immunized with a mixture of *Der p* II and *Der f* II by affinity chromatography on a *Der p* II-Sepharose column and then on a *Der f* II-Sepharose column.

Activation of Glass Rods and Conjugation of $F(ab')_2$ Fragment to Glass Rods

Pyrex glass rods, 3 mm in diameter and 5 mm in length, were treated with 3-aminopropyltriethoxysilane (Tokyo Kasei, Tokyo, Japan) according to the method of Robinson et al. [30]. Amino groups on the glass rods were then converted to isothiocyanate groups by treatment with *p*-phenylene diisothiocyanate (Wako Pure Chemical, Osaka, Japan), as described by Wachter et al. [31]. The activated glass rods were stored at -20°C under nitrogen atmosphere until used.

$F(ab')_2$ -fragment-coupled glass rods were prepared by linking 2 mg of $F(ab')_2$ fragment to 500 pieces of the activated glass rods in 25 ml of BBS, pH 8.5, for 48 h at 25°C and stored in PBS-T-BSA at 4°C.

Radioimmunoassays

$F(ab')_2$ -fragment-coupled glass rods were added to 200 µl of either standard allergen solutions or mite or house dust extracts in a polystyrene tube (75 mm × 12 mm) and incubated overnight at room temperature. The glass rods were washed twice with 2 ml of PBS containing 0.2% Tween 20 and 0.05% NaN_3 (PBS-T), then incubated for 3 h with either 100 ng of the species-specific antibodies to *Der p* or *Der f* I or 400 ng of the antibody to common determinants on the two *Der* II allergens in 100 µl of PBS-T-BSA. Then the glass rods were washed 3 times with PBS-T. Two nanograms (100,000–150,000 cpm) of ^{125}I -labeled protein A (specific activity ~40 mCi/mg; IM 144, Amersham, Buckinghamshire, UK) in 100 µl of PBS-T-BSA was added to the glass rods. After a 3-hour incubation at room temperature, the glass rods were washed 3 times with PBS-T. The radioactivity bound was then quantified in a gamma counter (ARC-300; Aloka, Tokyo, Japan). Values were obtained from control curves with serial dilutions of the standard allergen solutions with concentrations in the range of 10–5,000 pg/ml. House dust extracts were routinely assayed at two dilutions, 1:10³ and 1:10⁴ (w/v). When it was necessary, extracts were further diluted and assays were repeated.

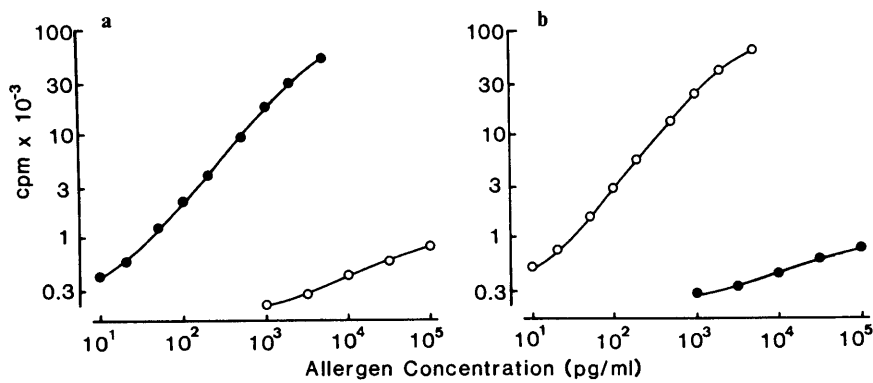


Fig. 1. Typical dose-response curves for *Der p* I (●) and *Der f* I (○) in the *Der p* I assay (a) and the *Der f* I assay (b).

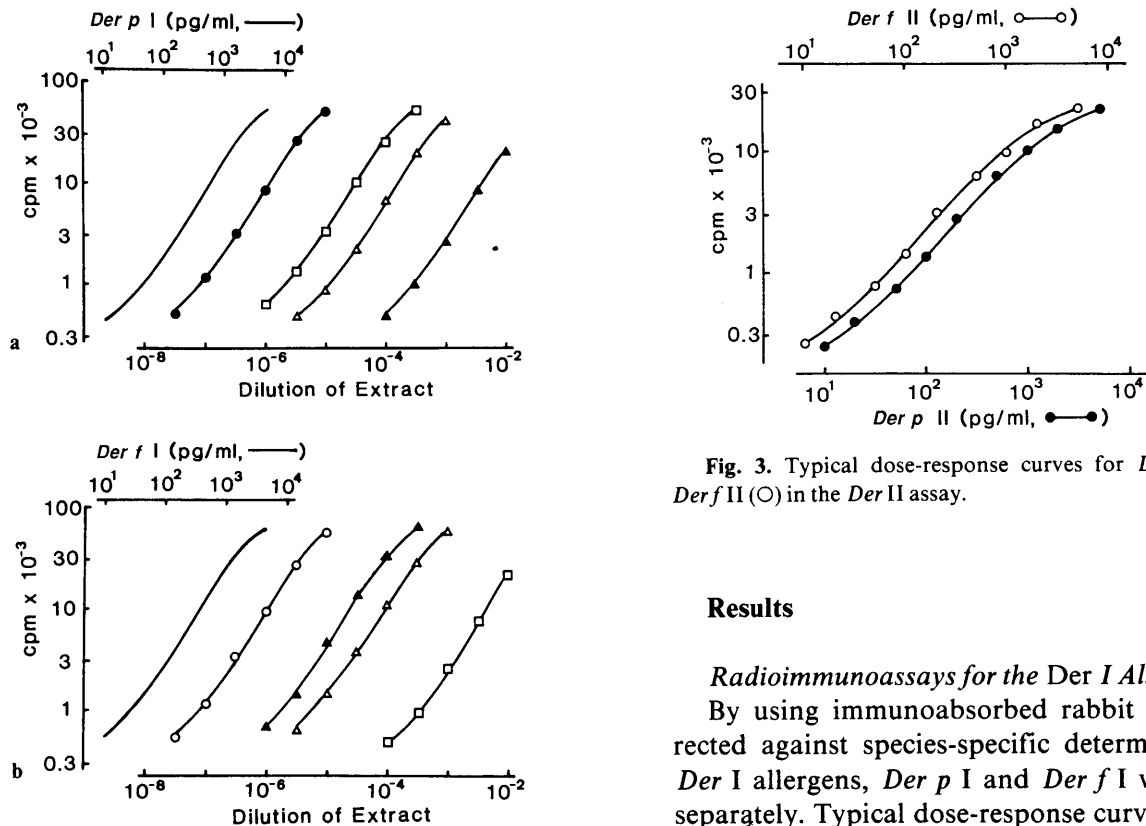


Fig. 2. Comparison of the dilution curves of mite body extracts of *D. pteronyssinus* (●) and *D. farinae* (○), and 3 extracts of house dust (▲, △, □) with the *Der p* I or *Der f* I standard curve (solid line) in the *Der p* I assay (a) and the *Der f* I assay (b).

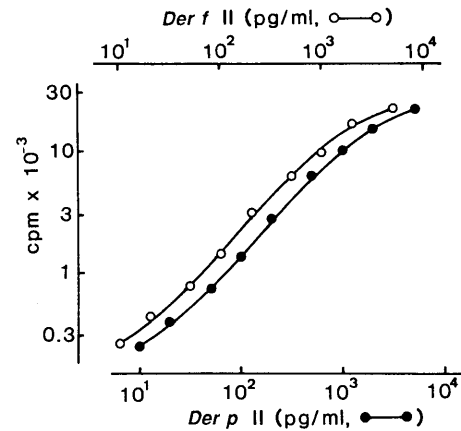


Fig. 3. Typical dose-response curves for *Der p* II (●) and *Der f* II (○) in the *Der* II assay.

Results

Radioimmunoassays for the *Der* I Allergens

By using immunoabsorbed rabbit antibodies directed against species-specific determinants on the *Der* I allergens, *Der p* I and *Der f* I were measured separately. Typical dose-response curves in the *Der p* I and *Der f* I assays are illustrated in figure 1. Both assays were highly species-specific and very sensitive. Values obtained for *Der f* I with the *Der p* I assay were less than 0.1% of the true values for *Der f* I (fig. 1a). A

similar but reversed result was observed for *Der p* I with the *Der f* I assay (fig. 1b). Standard curves for both assays were routinely made from purified *Der p* I or *Der f* I with concentrations in the range of 10–5,000 pg/ml. Nonspecific binding was usually <0.2% of the total radioactivities added and remained at <50% of the lowest standard curve point (10 pg/ml). Dilution curves for mite and house dust extracts were parallel to the standard curves in both assays (fig. 2).

Radioimmunoassays for the *Der II* Allergens

Der p II and *Der f* II were measured as '*Der II*' with the use of rabbit antibodies directed against cross-reactive determinants on both allergens, since the antigenic and allergenic determinants on the two *Der II* allergens were almost identical [13, 32]. As shown in figure 3, both *Der p* II and *Der f* II showed identical dose-response curves in the *Der II* assay. This *Der II* assay was also very sensitive as well as the assays for the *Der I* allergens. Dilution curves for the whole culture extracts and the mite body extracts from each of *D. pteronyssinus* and *D. farinae* were parallel to the *Der p* II standard curve (fig. 4a). Parallel dilution curves were also obtained with house dust extracts, which had been found by the *Der p* I and *Der f* I assays to contain either one species of mite predominantly or an equal mixture of both species (fig. 4b).

Reproducibility and Specificity of the Radioimmunoassays

The intraassay and interassay variations of the radioimmunoassays were examined using house dust extracts over the working ranges of each assay in triplicate 3 times. The mean interassay coefficient of variations of triplicate measurements for the *Der p* I, *Der f* I and *Der II* assays were 5.4, 4.3 and 7.7%, respectively. The mean interassay coefficients of variation for these assays were 4.0, 3.6 and 9.0%, respectively. In general, there is a tendency for higher intra-assay and interassay variation with lower allergen concentrations, especially <25 pg/ml.

Twelve commercial nonmite extracts including pollens, animal danders, molds and an insect were diluted 1:10 (v/v) with PBS-T-BSA and tested for their ability to bind radioactivity in the radioimmunoassays. None of these extracts demonstrated significant binding above background in any of the radioimmunoassays.

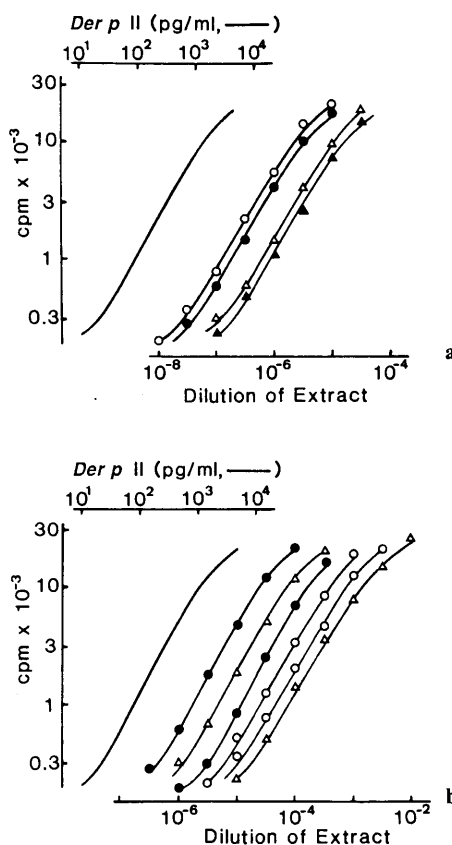


Fig. 4. Comparison of the dilution curves of mite extracts (a) and house dust extracts (b) with the *Der p* II standard curve (solid line) in the *Der II* assay. Mite extracts in a were mite body extracts of *D. pteronyssinus* (●) and *D. farinae* (○), and whole culture extracts of *D. pteronyssinus* (▲) and *D. farinae* (△). House dust extracts in b contained more than 10 times as much *Der p* I as *Der f* I (●), more than 10 times as much *Der f* I as *Der p* I (○), and a nearly equal mixture of *Der p* I as *Der f* I (△). Two dust extracts were analyzed in each of the groups containing different *Der p* I: *Der f* I ratios.

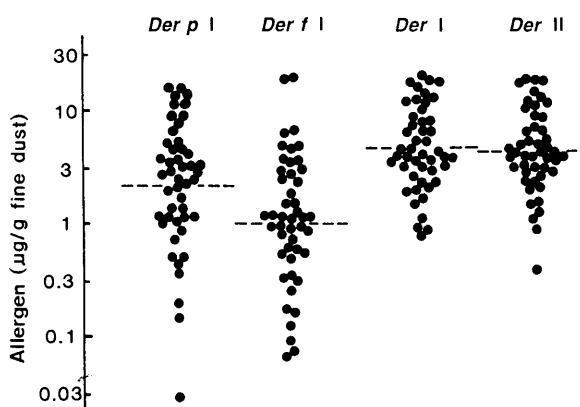


Fig. 5. Dust mite allergen levels in 47 dust samples from the houses of patients with asthma. The summed values for *Der p* I and *Der f* I assayed separately were considered as the total *Der I* levels.

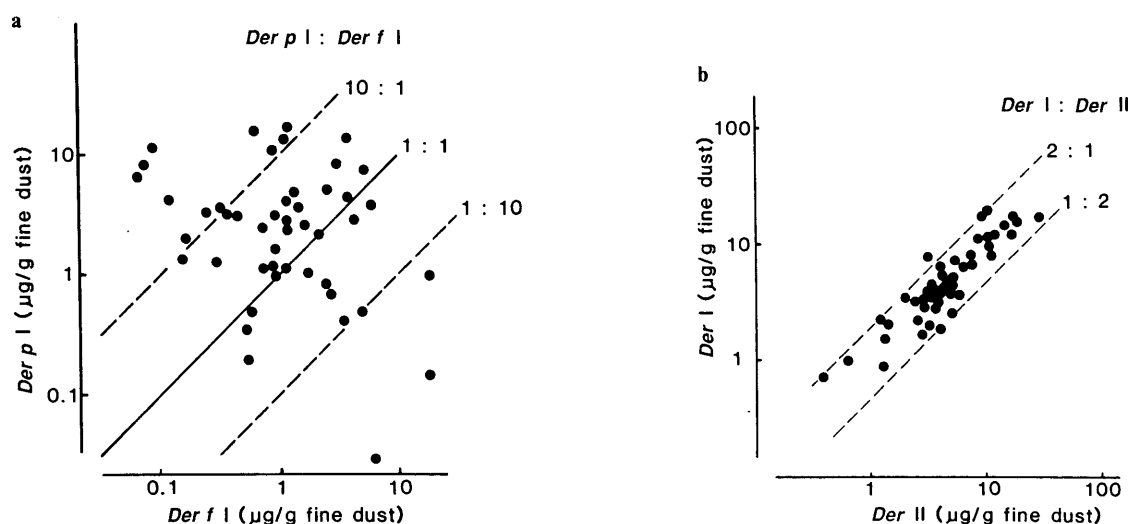


Fig. 6. Comparison of the *Der p I* and *Der f I* levels (a), and the *Der I* and *Der II* levels (b) in 47 dust samples from the houses of patients with asthma.

Table 1. *Der I* and *Der II* allergens in mite extracts

Extract	Allergen, $\mu\text{g}/\text{ml}$		<i>Der I</i> : <i>Der II</i> ratio
	<i>Der I</i>	<i>Der II</i>	
<i>D. pteronyssinus</i> mite body	3.59	4.93	0.73
<i>D. farinae</i> mite body	4.21	5.89	0.71
<i>D. pteronyssinus</i> whole culture	14.3	0.81	17.7
<i>D. farinae</i> whole culture	9.60	0.95	10.1
<i>D. pteronyssinus</i> international standard	5.16 ^a	0.31 ^a	16.6

^a Micrograms per ampoule.

Mite Allergen Levels in Dust Samples and Mite Extracts

The levels of mite allergens in 47 dust samples are shown in figure 5. The sum of *Der p I* and *Der f I* assayed separately was considered as the total *Der I* allergens. The geometric mean values for the *Der p I*,

Der f I, *Der I* and *Der II* levels were 2,150, 1,050, 4,640 and 4,300 ng/g fine dust, respectively.

Because both assays for the two *Der I* allergens were highly species-specific, the ratio of *Der p I*:*Der f I* in individual dust samples was considered to reflect the proportions of each mite species in the dust samples. Allergens from both species were detectable in all 47 dust samples analyzed, and the ratio of *Der p I*:*Der f I* in the dust samples from individual houses varied greatly. However, more than 10 times as much *Der f I* as *Der p I* was found in only 4 samples (8.5%), while 11 samples (23.4%) contained more than 10 times as much *Der p I* as *Der f I* (fig. 6a).

Figure 6b shows a comparison of the levels of the two groups of allergens in 47 dust samples. There was a highly significant correlation ($r = 0.899$, $p < 0.001$) between $\log[\text{Der I}]$ and $\log[\text{Der II}]$ levels, and the ratio of *Der I*:*Der II* in most of the dust samples ranged from 2:1 to 1:2 irrespective of either the absolute levels of the allergens or the proportions of the two mite species.

Although the ratio of *Der I*:*Der II* in individual dust samples was almost constant, this ratio in different types of mite extract varied markedly. In the mite body extracts, the levels of the two groups of allergens were nearly equivalent, while the whole culture extracts including the WHO international standard for *D. pteronyssinus* extract (82/518) contained 10- to 20-fold more *Der I* than *Der II* (table 1).

Discussion

The assay system described in this study is advantageous in that individual antigens can be measured without using radiolabeled antigens or antibodies. In this assay system, ^{125}I -labeled protein A serves as a general tracer for measurements of a wide variety of antigens. Radiolabeled protein A with a reproducible reactivity is commercially available, so that radiolabeling at each laboratory is unnecessary. This is of great advantage in routine measurements of a wide variety of antigens. Moreover, this assay system can be readily adapted for enzyme immunoassays without any loss of the assay sensitivity with the use of protein A- β -galactosidase conjugate which is also commercially available [unpublished observ.]. Enzyme immunoassays would be more suitable for general use.

By using this assay system, we have developed radioimmunoassays for the two groups of major allergens of *Dermatophagoides* mites. The major advantages of these mite allergen assays are that: (1) they are very sensitive; (2) assays for the two *Der* I allergens are highly species-specific, and (3) both groups of mite allergens (*Der* I and *Der* II) are measurable. Under the optimal conditions, the lowest detectable concentration of the allergens was 10 pg/ml. There have been no other radio- or enzyme immunoassays that can detect a mite allergen at concentrations of less than 100 pg/ml. The high sensitivity in our assays was achieved by using glass rods as solid-phase immunosorbents and affinity-purified antibodies as the second antibodies of the assay. The use of paper disks as solid-phase immunosorbents or IgG fractions of rabbit antisera as the second antibodies reduced assay sensitivity 10-fold or more because of a high level of nonspecific binding.

Allergens from heterologous mite species demonstrated little or no binding in either the *Der p* I assay or the *Der f* I assay (fig. 1). This shows that the high degree of species specificity of the assay for the *Der* I allergens can be achieved not only with the use of monoclonal antibodies directed against species-specific epitopes [18] but also with the use of polyclonal rabbit antibodies, if they are extensively immunoabsorbed. Because the F(ab')_2 fragments used as the solid-phase antibodies were not immunoabsorbed and consequently were not species-specific, negative interference by heterologous mite allergens could have resulted from competition with homologous allergens for binding to the solid-phase antibodies [17].

However, this type of interference by heterologous allergens at concentrations up to 10 $\mu\text{g/ml}$ did not occur in either the *Der p* I assay or the *Der f* I assay, indicating that F(ab')_2 -fragment-coupled glass rods had sufficient capacity to bind homologous allergens irrespective of the presence of heterologous allergens in the test samples.

By the use of our *Der p* I assay, the WHO international standard for *D. pteronyssinus* extract was found to contain 5.16 μg of *Der p* I per ampoule (table 1), which is 2- to 6-fold less than the previously reported values (10–31 μg of *Der p* I) from four laboratories [16, 20]. This variation probably reflects differences in the internal standards, or the internal methods to determine the concentration of the standards for this allergen used by the laboratories. The absolute quantity of *Der p* II in this international standard has not been demonstrated by other laboratories up to this time. Although there have been a variety of assays for measuring the *Der* I allergens [16–18, 21], only a few assays have been developed for the *Der* II allergens [21, 33]. The availability of separate simultaneous measurements of the two groups of major allergens should provide more detailed information on the allergenic features of mite extracts and house dust samples.

The *Der p* I and *Der f* I assays provide a good index of the distribution of *D. pteronyssinus* and *D. farinae* in individual dust samples [17, 18]. *D. pteronyssinus* was the dominant species in this area, although both mite species were found in all of the houses examined (fig. 5, 6a). This result is consistent with the previous finding that IgE antibodies directed against the species-specific determinants on *Der p* I were found preferentially in sera from mite-allergic patients in this area [13].

An interesting finding is that the relative proportions of the *Der* I and *Der* II allergens varied among the mite body extracts, the whole culture extracts and the dust samples (fig. 6b, table 1). The whole culture extracts contained much more *Der* I than *Der* II, while an almost equal quantity of the *Der* I and *Der* II allergens was found in the mite body extracts. This confirms the previous findings that a large proportion of the *Der* I allergens is derived from fecal particles, while the *Der* II allergens are present in higher concentrations in mite bodies [19–21].

The ratio of *Der* I:*Der* II in the dust samples (2.6:1–1:2.2; fig. 6b) was less than that in the whole culture extracts ($>10:1$), but close to that in the mite

body extracts (~1:1, table 1). One might suppose that house dust contains mite fecal particles, thus has a higher *Der I:Der II* ratio. Although it remains unclear why the ratio of *Der I:Der II* in the dust samples is quite different from that in the whole culture extracts, this may be related to the lability of the *Der I* molecules. They were very labile towards heating, denaturing agents and acid or alkali treatment, while the *Der II* molecules were essentially stable towards these treatments [12, 34].

The radioimmunoassays for mite allergens described above should be useful for assessments of mite allergen exposure as well as the standardization of mite and house dust extracts. Moreover, as these assays are very sensitive, it is possible to use them to measure the level of mite allergens in the air in houses where ordinary domestic life is in progress. Measuring airborne mite allergens with these assays will elucidate the aerodynamic properties of the particles carrying mite allergens. And they should be useful in studying the relationship between the absolute quantities of inhaled mite allergens and the exacerbations of allergic symptoms.

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