

Estimation of *Der p* and *Der f* I quantities in the reference preparations of *Dermatophagoides* mite extracts

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Summary

A monoclonal antibody-based enzyme-linked immunosorbent assay (MoAb-ELISA) was developed to measure the major *Dermatophagoides* mite allergens, *Der p* I and *Der f* I. The assay was highly species-specific and sensitive. Using this assay system, the absolute mass unit of *Der p* I and *Der f* I in the reference preparations of the extracts was estimated. The primary standards used were the purified *Der p* I and *Der f* I preparations. The reference preparations of the *D. pteronyssinus* and *D. farinae* extracts (92-*Dp* and 92-*Df*), which had been prepared from the same amount of mite bodies of both species, were found to contain the same levels of the *Der* I allergens, 10.1 µg/ml of *Der p* I and 10.0 µg/ml of *Der f* I, respectively. A histamine release assay with leucocytes from mite-allergic donors showed that the total allergenic potency of 92-*Dp* and 92-*Df* was comparable. This result indicates that the estimated *Der* I levels in these extracts seem to be valid, at least, in the balance between the two species, although further comparisons of the absolute quantities by several different laboratories are needed. The *Der* I levels in the WHO/IUIS international reference preparation of *D. pteronyssinus* and the CBER standard mite extracts, E4-*Dp* and E5-*Df*, were also estimated using this assay system. They were found to contain 4.4 µg/vial and 13.3 µg/ml of *Der p* I and 9.5 µg/ml of *Der f* I, respectively.

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Introduction

Measurement of major allergens by an immunochemical assay is a useful method for the standardization of allergenic extracts and for the assessment of environmental allergen exposure [1–4]. To express the allergen level with an absolute mass unit, reference preparations of the extracts with known allergen quantities are required.

Dermatophagoides mites, *D. pteronyssinus* and *D. farinae*, are the most significant allergen source in house dust. Allergy to these mite allergens is a very common worldwide phenomenon [5,6]. It is recognized that the *Dermatophagoides* mites have two groups of major allergens, group I (*Der* I) and group II (*Der* II)

allergens [7–10]. Immunoassays to measure the *Der* I allergens are commonly used in epidemiological studies of mite allergy and in studies of mite avoidance measures [11,12]. The *Der* I quantities in micrograms per gram of dust are regarded as the best validated and objective index of mite allergen exposure in houses [5,6]. Thus, the *Der p* I and *Der f* I quantities in the reference preparations of *D. pteronyssinus* and *D. farinae* extracts in particular should be well-characterized.

The availability of allergen-specific monoclonal antibodies (MoAbs) has facilitated the development of specific and simple immunoassays for allergens [13–17]. In the present study, we have produced panels of MoAbs to *Der p* I and *Der f* I and developed an MoAb-based enzyme-linked immunosorbent assay (MoAb-ELISA) to measure these allergens.

Using this assay system, the validity of the estimated values of the *Der p* I and *Der f* I quantities in the

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reference preparations of the mite extracts were assessed.

Materials and methods

Purified mite allergens

The mite allergens, *Der p I* and *Der f I*, were purified from whole cultures of *D. pteronyssinus* and *D. farinae* as previously described [9,10]. The concentrations of the purified allergens were determined by using their extinction coefficient values, $E_{1\text{cm}}^{1\%} = 17.0$ for *Der p I* and 15.6 for *Der f I* [6,10].

Reference preparations of *D. pteronyssinus* and *D. farinae* extracts

The reference preparations of *D. pteronyssinus* and *D. farinae*, named 92-Dp and 92-Df, respectively, were produced from isolated mite bodies of both species. The defatted and crushed mite bodies (4 g) of either *D. pteronyssinus* or *D. farinae* were extracted with 200 ml of 0.125 M NH_4HCO_3 (1:50 w/v) at 4°C overnight. After centrifugation ($30\,000 \times g$, 20 min), 150 ml of the supernatant was dialysed against 5 mM NH_4HCO_3 and then lyophilized. The lyophilized extract was dissolved in 30 ml of 5 mM NH_4HCO_3 , filtered through a 0.45 μm membrane (Millex HA, Millipore, Bedford, MA, USA), aliquotted in polypropylene vials (0.2 ml/vial), and lyophilized. The lyophilized extracts were stored at -20°C and reconstituted in 1 ml of phosphate-buffered saline (PBS) prior to use.

The WHO international standard for house dust mite (*D. pteronyssinus*) extract (82/518) was obtained from the National Institute for Biological Standards and Control (London, UK) [18]. The CBER standard mite extracts, E4-Dp and E5-Df, were provided by Dr Y. DeVries, Centre for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA.

House dust extracts

House dust samples were obtained from household vacuum cleaner bags of each house and were sieved through a 0.3 mm mesh screen. The fine dust was extracted with 1:100 w/v of PBS containing 0.2% Tween 20, 0.2% bovine serum albumin and 0.05% NaN_3 (PBS-T-BSA) overnight at 4°C. The extracts were centrifuged and the supernatants were stored at -20°C.

MoAbs to *Der p I* and *Der f I*

Mouse MoAbs to *Der p I* and *Der f I* were obtained from hybridoma cell lines established by the fusion using Sp2/0-

Ag14 or P3X63-Ag8.653 myeloma cells and spleen cells from BALB/c or A/J mice which had been immunized with *Der p I* or *Der f I* [19]. Antibody producing hybridoma were screened by an ELISA with microtitre plates coated with purified *Der p I* or *Der f I* and peroxidase conjugated goat anti-mouse IgG. Antibody-positive hybridoma were cloned at least twice by limiting dilution. Ascites was produced by injecting 10^7 cells i.p. into either BALB/c or CAF-1 mice which had been previously treated with pristane. MoAbs were purified from each ascites by affinity chromatography on a protein A column (Ampure PA kit, Amersham Japan, Tokyo, Japan).

The MoAbs (5H8, 6A8 and 4C1) used for the ELISA system [15] were provided by Dr M. Chapman, University of Virginia, Charlottesville, VA, USA.

Biotinylation of MoAbs

One milligram of MoAb in 1 ml of 0.1 M NaHCO_3 was mixed with 0.1 mg of *N*-hydroxysuccinimidobiotin (Sigma Chemical Co., St Louis, MO, USA) in 0.1 ml of dimethylformamide and incubated at 25°C for 4 h. Biotinylated MoAbs were separated from unreacted biotin through a Sephadex G-50 (Pharmacia Biochemistry, Uppsala, Sweden) column.

MoAb-ELISA for *Der p I* and *Der f I*

Polystyrene microtitre plates (MaxiSorp, Nunc, Denmark) were coated with 200 ng of P1A03 (anti-*Der p I*) or F1B01 (anti-*Der f I*) in 100 μl of 0.1 M carbonate-bicarbonate buffer, pH 9.6, overnight at 4°C. The plate were washed three times with PBS containing 0.1% Tween 20 (PBS-T) and incubated with 1% bovine serum albumin in PBS for 1 h at 37°C. After washing twice with PBS-T, 100 μl of *Der p I* or *Der f I* standard solution (32–0.25 ng/ml), or dilutions of mite or house dust extracts in PBS-T-BSA was added to the wells and incubated at 37°C for 2 h. The plates were washed five times with PBS-T and then incubated with 100 ng of biotinylated P1A01 (anti-*Der p I*) or biotinylated F1A05 (anti-*Der f I*) in 100 μl of PBS-T-BSA at 37°C for 2 h. After washing five times with PBS-T, the plates were incubated with 100 μl of streptavidin- β -D-galactosidase conjugate (Boehrings Mannheim, Germany) diluted 1:1000 in PBS-T-BSA at 37°C for 1 h and then washed five times. Finally, 100 μl of 5 mM o-nitrophenyl- β -D-galactopyranoside in 0.02 M sodium phosphate containing 0.1 M NaCl, 1 mM MgCl_2 , 0.2% BSA and 0.05 M NaN_3 , pH 7.2, was added to the wells and incubated at 37°C for 60 min. After the enzyme reaction was stopped with 100 μl of 0.1 M Na_2CO_3 , the absorbance at 415 nm

Table 1. Characterization of anti-*Der p* I and anti-*Der f* I MoAbs used for MoAb-ELISA

MoAb*	Antigen	Heavy chain isotype	Mouse strain
P1A01	<i>Der p</i> I	IgG1	A/J
P1A03	<i>Der p</i> I	IgG2a	A/J
F1B01	<i>Der f</i> I	IgG1	BALB/c
F1A05	<i>Der f</i> I	IgG1	A/J

*P1A03 and F1B01 were used routinely as 'capture' antibodies on the solid phase and P1A01 and F1A05 as biotinylated second antibodies in the assay.

was measured with a BIO-RAD 3550 microplate reader (Bio-Rad Laboratories, Richmond, CA, USA).

Histamine release assay

Histamine release experiments from washed leucocytes were carried out as previously described [9]. Briefly, washed leucocytes obtained from the venous blood of mite-allergic donors were incubated with a series of concentrations of the allergen preparations at 37°C for 45 min. The histamine released was measured by automated fluorometry [20].

Results

We obtained a total of 10 MoAbs to *Der p* I and 11

Table 2. Estimated *Der p* I/*Der f* I quantities in the reference preparations of mite extracts

Extracts	$\mu\text{g/ml}^*$	
	<i>Der p</i> I	<i>Der f</i> I
92- <i>Dp</i>	10.1	—
92- <i>Df</i> [†]	—	10.0
<i>Dp</i> -IS [†]	4.4 [§]	—
E4- <i>Dp</i> [‡]	13.3	—
E5- <i>Df</i> [‡]	—	9.5

*The purified *Der p* I and *Der f* I preparations were used as the primary standards for the assay.

[†]WHO international standard for *D. pteronyssinus* extract.

[‡]CBER standard mite extracts.

[§]Microgram per vial.

MoAbs to *Der f* I. Two *Der p* I specific MoAbs (P1A01 and P1A03) and two *Der f* I specific MoAbs (F1B01 and F1A05), which recognized the different epitopes on each of the *Der p* I and *Der f* I molecules, were selected for use in the MoAb-ELISA (Table 1). Figure 1 shows typical dose-response curves of the purified allergen preparations. Both assays for *Der p* I and *Der f* I were highly species-specific and sensitive. The same figure shows that the dilution curves of the reference preparations of the

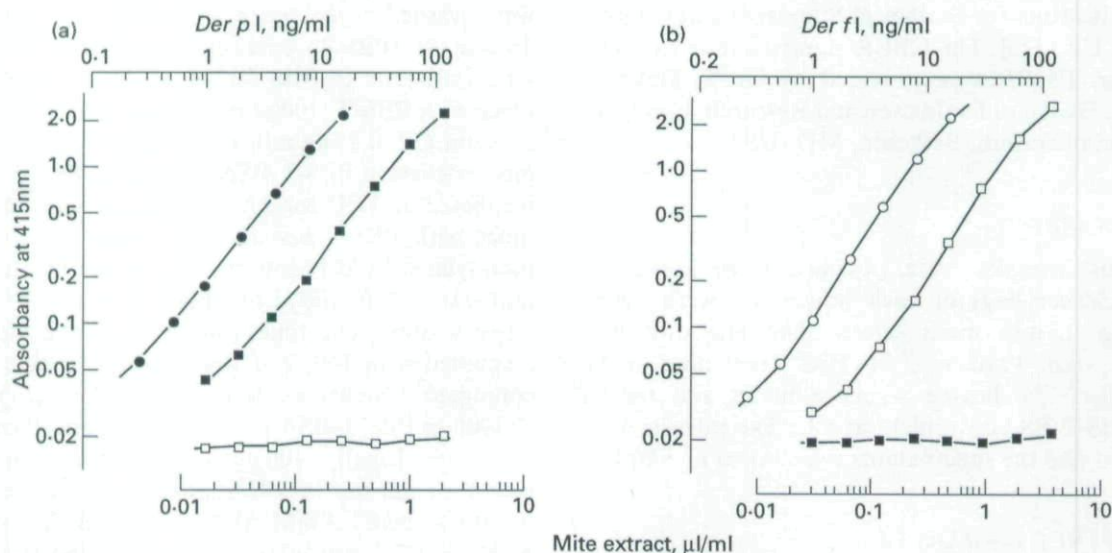


Fig. 1. Dose-response curves of the purified *Der p* I (●) and *Der f* I (○) and the reference preparations of the extracts, 92-*Dp* (■) and 92-*Df* (□), in the MoAb-ELISA for *Der p* I (a) and *Der f* I (b).

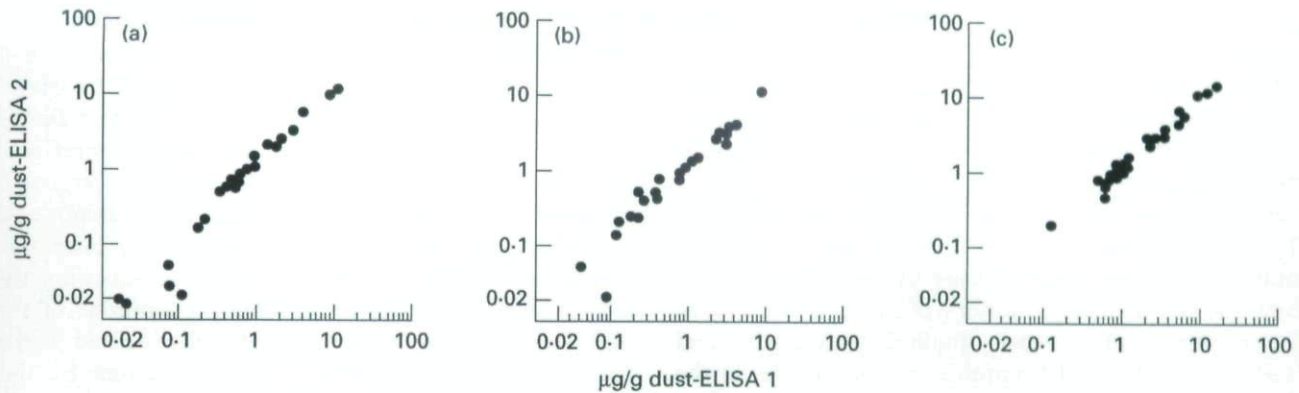


Fig. 2. Comparison of the *Der* I levels as measured by two different MoAb-ELISA systems. The concentration of *Der p* I and *Der f* I was determined by the ELISA described by Luczynska *et al.* [15] (ELISA 1) and by the ELISA developed in this study (ELISA 2). (a) *Der p* I, $r = 0.975$. (b) *Der f* I, $r = 0.946$. (c) *Der p* I and *Der f* I, $r = 0.995$.

D. pteronyssinus and *D. farinae* extracts (92-Dp and 92-Df) are parallel to those of the purified allergen preparations. Parallel dilution curves were also obtained with the house dust extracts (data not shown). Twenty-three dust

extracts were assayed for *Der p* I and *Der f* I using the two different MoAb-ELISA systems, i.e. the ELISA developed in the present study and that described by Luczynska *et al.* [15]. The results demonstrated an

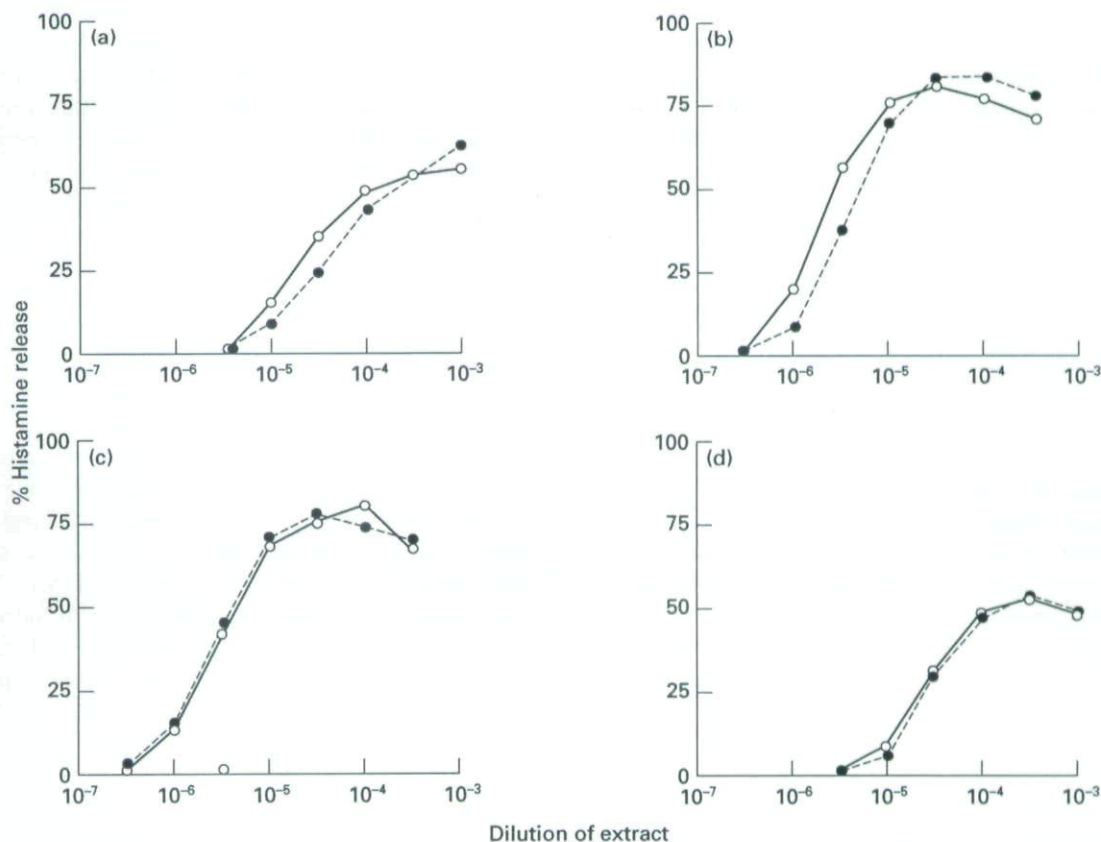


Fig. 3. Histamine release from washed leucocytes of four mite-allergic donors to compare the allergenic activity of the reference preparations of the extracts, 92-Dp (●) and 92-Df (○). (a) Donor 1. (b) Donor 2. (c) Donor 3. (d) Donor 4.

excellent quantitative correlation between the two assay systems (Fig. 2).

The reference preparations of the extracts, 92-*Dp* and 92-*Df*, were found to contain the same levels of the *Der* I allergens, 10.1 µg/ml of *Der p* I and 10.0 µg/ml of *Der f* I, respectively, when the purified *Der p* I and *Der f* I preparations were used as the primary standards (Table 2). The allergenic activity of 92-*Dp* and 92-*Df* was compared by a histamine release assay with leucocytes from four mite-allergic donors (Fig. 3). Very similar dose-response curves were obtained with 92-*Dp* and 92-*Df* in the release of histamine from leucocytes of the donors. In all four donors, a difference between the cell sensitivity (the concentration required to cause 50% histamine release) to 92-*Dp* and 92-*Df* was less than twofold, indicating that the total allergenic potency of 92-*Dp* and 92-*Df* was almost comparable. The *Der p* I and *Der f* I levels in the WHO/IUIS international reference preparations of *D. pteronyssinus* and the CBER standard mite extracts E4-*Dp* and E5-*Df* were also measured using this assay system. The results are shown in Table 2.

Discussion

We have developed a MoAb-ELISA to measure the major *Dermatophagoides* mite allergens, *Der p* I and *Der f* I. The principle of the MoAb-ELISA is similar to that described by Luczynska *et al.* [15]. In this assay, microtitre plate wells coated with a species-specific 'capture' MoAb were incubated with allergen solutions and then with a biotinylated MoAb directed against a different epitope. The bound allergen-biotinylated MoAb complex was detected by the addition of streptavidin-β-D-galactosidase conjugate followed by a colour reaction. Although the sensitivity of the MoAb-ELISA described in this study (down to 1 ng/ml) is not as high as that of the radioimmunoassay using rabbit polyclonal antibodies (10 pg/ml) [21], it is sensitive enough to assess mite allergen levels in house dust samples and commercial allergenic extracts. MoAbs have a great advantage over rabbit polyclonal antibodies in specificity and reproducibility, and ELISAs are more suitable than radioimmunoassays for general use in routine clinical laboratories.

Using this assay system, absolute quantities of *Der p* I and *Der f* I in the reference preparations of the *Dermatophagoides* mite extracts were estimated. The primary standards used in this assay were the purified *Der p* I and *Der f* I preparations. The concentrations of the purified allergens were determined from their extinction coefficients [10], which were calculated from the number of tryptophan, tyrosine and cystine residues in the

molecule according to the method of Wetlaufer [22]. The accurate number of these amino acid residues is now available from amino acid sequence data translated from the cDNA sequences of *Der p* I [23,24] and *Der f* I [24]. Our estimate of the number from amino acid analysis of the hydrolysates of the purified allergens is in good agreement with that from the amino acid sequence data. We have also estimated the extinction coefficients of *Der p* I and *Der f* I by measuring the absorbancy at 280 nm of salt-free preparations of the samples which had been weighed and dissolved in 6 M guanidinium hydrochloride. The $E_{1\text{cm}}^{1\%}$ values by this method (16.4 for *Der p* I and 15.9 for *Der f* I) are in close agreement with those by the method of Wetlaufer (17.7 for *Der p* I and 15.4 for *Der f* I) [10].

The reference preparations of the *D. pteronyssinus* and *D. farinae* extracts, 92-*Dp* and 92-*Df*, were estimated to have the same level of the *Der* I allergens (Table 2). The extracts were prepared from the isolated mite bodies of both species by the same procedure. The histamine release assay with leucocytes from mite-allergic donors demonstrated that the total allergenic potency of both extracts was nearly comparable (Fig. 3). It would be appropriate to consider that the *D. pteronyssinus* and *D. farinae* extracts prepared from the same source material by the same procedure with a comparable total allergenic potency have a comparable level of the corresponding allergens including the *Der* I and *Der* II allergens. Thus, our estimate of the *Der* I allergen levels in these extracts seems to be valid, at least, in the balance between the two species. Similarly, the *Der* II levels in 92-*Dp* and 92-*Df* as measured by radioimmunoassay [21] were demonstrated to be comparable (data not shown).

The CBER standard mite extract, E5-*Df*, has been claimed to contain 10 µg/ml of *Der f* I (Dr Y. DeVries, personal communication). This is in good agreement with our estimate (Table 2). However, our estimates of the *Der p* I quantity in the WHO/IUIS international reference preparations of *D. pteronyssinus* in this study (4.4 µg) and the previous study (5.2 µg) [21] were two- to threefold less than the value reported (12.3 µg) [25]. The same level of variation was also seen in the cat major allergen, *Fel d* I (1 U *Fel d* I = 1.9–5 µg) [16,17,26]. These variations are not due to the differences in the assays but are due to the difference in the primary standards used in the assays, because the *Der* I allergen levels in the dust samples determined by the two different ELISA systems with the same standard were identical (Fig. 2). Thus, more accurate quantitative comparisons of the data on the *Der* I and *Der* II allergen levels among laboratories will be possible provided that international consensus is established on the absolute quantities of the *Der* I and *Der* II allergens.

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