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A two-site monoclonal antibody ELISA for the quantification of the major *Dermatophagoides* spp. allergens, *Der p* I and *Der f* I

Christina M. Luczynska¹, L. Karla Arruda¹, Thomas A.E. Platts-Mills¹, Jeffrey D. Miller², Manuel Lopez³ and Martin D. Chapman¹

¹ Division of Allergy and Clinical Immunology, Department of Internal Medicine, University of Virginia, Charlottesville, VA, U.S.A., ² Danbury, CT, U.S.A., and ³ Department of Medicine, Tulane University, New Orleans, LA, U.S.A.

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A two-site monoclonal antibody (Mab) ELISA was developed to measure the Group I allergens from *Dermatophagoides* spp., *Der p* I from *D. pteronyssinus* and *Der f* I from *D. farinae*. Species-specific Mabs were used to coat microtiter plates which were then incubated with allergen or house dust extracts. Bound allergen was detected using a biotinylated Mab which recognized a common epitope on both *Der p* I and *Der f* I, followed by the addition of streptavidin-peroxidase and ABTS/H₂O₂ substrate. The assay had low non-specific binding (~0.08 absorbance units) and had a sensitivity of 5 ng/nl for aqueous allergen extracts (equivalent to 0.1 µg allergen/g dust). 53 dust samples were assayed using the Mab ELISA and an RIA previously described using ¹²⁵I-labelled Mab. The results showed a very good quantitative correlation between the assays ($r = 0.96$, $p < 0.001$ for *Der p* I; $r = 0.92$, $P < 0.001$ for *Der f* I). A further 132 dust samples from a different geographical areas were also assayed by both methods and gave correlation coefficients of 0.90 ($P < 0.001$) and 0.86 ($P < 0.001$) for *Der p* I and *Der f* I, respectively. The Mab ELISA will be useful in epidemiological studies of allergic asthma, both in the assessment of levels of dust mite allergen present in houses and the efficacy of allergen avoidance regimes.

Key words: Allergen; *Dermatophagoides* spp.; Immunoassay; Monoclonal antibody

Correspondence to: C.M. Luczynska, Division of Allergy and Clinical Immunology, Box 225, University of Virginia Medical Center, Charlottesville, VA 22908, U.S.A.

Abbreviations: ab, antibody; ABTS, 2,2'-azinobis-(3-ethylbenzthiazoline sulfonic acid); AgP₁Eq, antigen P₁ equivalent; BBS, borate-buffered saline, pH 8.0; BSA, bovine serum albumin; CV, coefficient of variation; *Der f* I, *Der p* I and *Der m* I, 25 kDa major allergens from *D. farinae*, *D. pteronyssinus* and *D. microceras*, respectively; Mab, monoclonal antibody; PBS, phosphate-buffered saline, pH 7.4; RAST, radioallergo-sorbent test; RIA, radioimmunoassay; SAS, saturated ammonium sulfate; UVA, University of Virginia; WHO/IUIS, World Health Organization/Internal Union of Immunological Societies.

Introduction

House dust mites (*Dermatophagoides* spp.) are among the most ubiquitous worldwide allergens. Epidemiological studies of mite allergy require quantification of mite allergens present in house dust samples and in allergen extracts used for diagnosis and treatment. Previous quantification methods include mite counts (Voorhorst et al., 1967; Tovey and Vandenberg, 1979; Arlian et al., 1982), RAST inhibition (Swanson et al., 1985), and assays for *Der p* I, a major allergen from

Dermatophagoides pteronyssinus (e.g., using counter immunoelectrophoresis, inhibition radioimmunoassay (RIA) or ELISA) (Chapman and Platts-Mills, 1980; Lind et al., 1979; Tovey et al., 1981; Lind, 1986; Wood et al., 1988). *Der p* I assays give good assessments of mite allergen exposure in areas where *D. pteronyssinus* is the predominant mite species, but may underestimate allergen levels in samples containing *D. farinae* because there is only partial cross-reactivity between *Der p* I and *Der f* I, the homologous Group I allergen from *D. farinae* (Dandeu et al., 1982; Heymann et al., 1986). To overcome this problem, we developed an RIA to measure common antigenic determinants (termed antigen P₁ equivalent [AgP₁Eq]) present on both *Der p* I and *Der f* I (Platts-Mills et al., 1986).

Subsequently, two-site, species-specific, monoclonal antibody (Mab) based RIAs for either *Der p* I or *Der f* I were developed which correlated well with the AgP₁Eq assay (Chapman et al., 1984, 1987a,b). These Mab RIAs were specific for the Group I allergens and did not cross-react with the Group II allergens recently purified from *Dermatophagoides* spp. (Dandeu et al., 1982; Lind, 1985; Holck et al., 1986; Yasueda et al., 1986; Platts-Mills and Chapman, 1987; Heymann et al., 1989).

This paper describes the development of a two-site Mab ELISA for measuring *Dermatophagoides* spp. Group I allergens, using a single biotinylated Mab and peroxidase-conjugated streptavidin for detection. The Mab ELISA has low background binding and comparable sensitivity to RIA (ng range). It has the advantage of being a non-isotopic immunoassay which can be used in routine clinical laboratories and should be extremely useful in epidemiologic studies for measuring allergen levels in the houses of patients with allergic disease.

Materials and methods

Reagents

Reagents were purchased from Sigma Chemical Co., St. Louis, MO, unless otherwise stated. Enzotin biotinylating reagent (*N*-biotinyl- ω -aminocaproic acid-*N*-hydroxy succinimide ester) (EAB-

406) was obtained from Enzobiochem, NY. Immulon II microtiter plates (flat bottom) were from Dynatech, Alexandria, VA.

Mite extracts

D. pteronyssinus reference extract, UVA 87/03, was prepared from waste fraction (culture from which mite bodies had been removed) (Hollister-Stier Laboratories, Spokane, WA) and contained 2.5 μ g/ml *Der p* I, substandardized against the WHO/IUIS reference preparation of *D. pteronyssinus* (NIBSC code 82/518) (Ford et al., 1985a,b).

D. farinae reference extract, UVA 87/02, was prepared in the same way from *D. farinae* waste fraction (Hollister-Stier). As no WHO/IUIS standard is available, the extract was substandardized against the *D. pteronyssinus* reference (NIBSC code 82/518) using the AgP₁Eq assay (Platts-Mills et al., 1986) and contained 2.5 μ g/ml *Der f* I.

House dust extracts

Dust extracts, obtained from 18 houses in Danbury, CT, over a 3 week period, were prepared as previously described (Tovey et al., 1981; Chapman et al., 1987b). The extracts were 5% sieved dust in BBS (100 mg in 2 ml) and were stored at -20°C until tested, unless otherwise specified. 132 house dust samples were obtained from 44 houses in New Orleans, LA, and 5% extracts of these dusts were also prepared. For the investigation of the effects of 50% glycerol in the assay, house dusts were extracted in 50% glycerol/BBS and diluted in 50% glycerol/PBS or PBS alone.

Monoclonal antibodies

The production and characterization of monoclonal antibodies to *Der p* I and *Der f* I have been described previously (Chapman et al., 1984; Heymann et al., 1986). Mabs 10B9 and 5H8 are specific for *Der p* I; Mab 6A8 is specific for *Der f* I; Mab 4C1 binds to a cross-reacting epitope present on both *Der p* I and *Der f* I and can be used to detect either of the Group I allergens.

Biotinylation of 4C1 Mab

The cross-reacting Mab, 4C1, was purified from ascites by flat-bed preparative isoelectric focusing, pH 5-9 (Chapman et al., 1987a) and was dialyzed

against 2 liters 0.1 M sodium bicarbonate, pH 8.4, overnight at 4°C . 5.25 mg 4C1 were distributed in a volume of 1.5 ml (3.5 mg/ml), mixed with 0.24 ml of Enzotin biotinylating reagent (4 mg/ml in dimethyl sulfoxide) and left without stirring for 4 h at room temperature. Unreacted Enzotin was removed by dialysis against 2 liters PBS overnight at 4°C . The final concentration of biotinylated 4C1 after dialysis was 1.6 mg/ml as judged by absorbance at 280 nm.

The biotinylated 4C1 was titrated using *D. pteronyssinus* extract (UVA 87/03) and *D. farinae* extract (UVA 87/02) to determine the optimum concentration for binding in the ELISA. For this titration, the ELISA was carried out using a fixed quantity (120 ng/ml) of *Der p* I or *Der f* I on the solid phase, followed by serial dilutions of biotinylated 4C1. The optimum dilution of biotinylated 4C1 was 1/10000. A stock solution (1/10 in 50% glycerol/PBS containing 0.01% thimerosal, stored at 4°C) was prepared and used at a dilution of 1/1000 in the assay. We investigated the effect of pH on the binding of biotinylated 4C1 using buffers at different pH in the ELISA: (i) 0.07 M citrate-phosphate buffer, pH 4.2, (ii) 0.2 M phosphate buffer, pH 7.5, and (iii) 0.05 M carbonate-bicarbonate buffer, pH 9.6.

ELISA procedure

Microtiter plates were coated with 100 μ l/well of a 50% SAS cut of 5H8 (anti-*Der p* I) or 6A8 (anti-*Der f* I) ascites (protein concentration 10 μ g/ml) in 0.05 M carbonate-bicarbonate buffer, pH 9.6 overnight at 4°C . All subsequent incubations were at room temperature. The plates were washed twice with PBS-Tween (PBS containing 0.05% Tween 20) and incubated with 1% BSA in PBS-Tween for 30 min. After a further two washes 100 μ l of *Der p* I (UVA/03) or *Der f* I (UVA/02) standard solution (concentration range 0.05-250 ng/ml), or dilutions of dust extracts (in most cases 1/5 and 1/25) in 1% BSA in PBS-Tween, were added to the wells. Preliminary experiments showed that 1 h was sufficient for maximal binding. The plates were washed five times with PBS-Tween and incubated with 100 μ l of 1/1000 biotinylated-4C1 (16 ng/well) in 0.2 M phosphate buffer, pH 7.5 for 1 h. After a further five washes, 100 μ l of streptavidin-peroxidase (0.25 μ g/ml)

was added to the wells and incubated for 30 min. The plates were washed a final five times and developed with 100 μ l 0.01 M ABTS in 0.07 M citrate-phosphate buffer, pH 4.2, containing 0.03% H_2O_2 added immediately before use. The color reaction was stopped with 100 μ l of 0.002 M sodium azide and the plates read at 405 nm using a Titertek Multiscan Plus Mk II plate reader (Flow Laboratories, Maclean, VA).

Radioimmunoassays

The monoclonal RIAs for *Der p* I and *Der f* I have been described previously (Chapman et al., 1987b). The assays were modified for the present study by using a single ^{125}I -labelled second antibody (4C1) and the same Mab on solid phase as used in the ELISA. The results obtained for Group I allergens were compared with values for AgP₁Eq measured as described by Platts-Mills et al. (1986).

Results

Development of Mab ELISA

The principle of the two-site Mab ELISA for mite allergens is similar to the Mab RIA described previously. Allergen (*Der p* I or *Der f* I) binds to a specific capture Mab coupled to a microtiter plate. A second biotinylated Mab that recognizes a common epitope on both *Der p* I and *Der f* I is then added. The color reaction is developed by the addition of streptavidin-peroxidase followed by ABTS/ H_2O_2 substrate and read at 405 nm.

Fig. 1A shows typical titration results for biotinylated 4C1. The dilution required for maximal binding to either *Der p* I or *Der f* I was 1/10000 (16 ng/well). The biotinylated 4C1 has remained stable at 4°C for at least a year and given similar dose-response curves with different batches of streptavidin-peroxidase. Control curves for *Der p* I with two different combinations of Mabs are shown in Fig. 1B. The original Mab RIA was carried out using two *Der p* I specific Mabs, 10B9 and ^{125}I -labelled 5H8. Similar dose-response curves were obtained when this combination was compared with biotinylated 4C1 and solid-phase 5H8 in ELISA. As 4C1 recognizes a cross-reacting epitope on *Der p* I and *Der f* I, it can be used as a

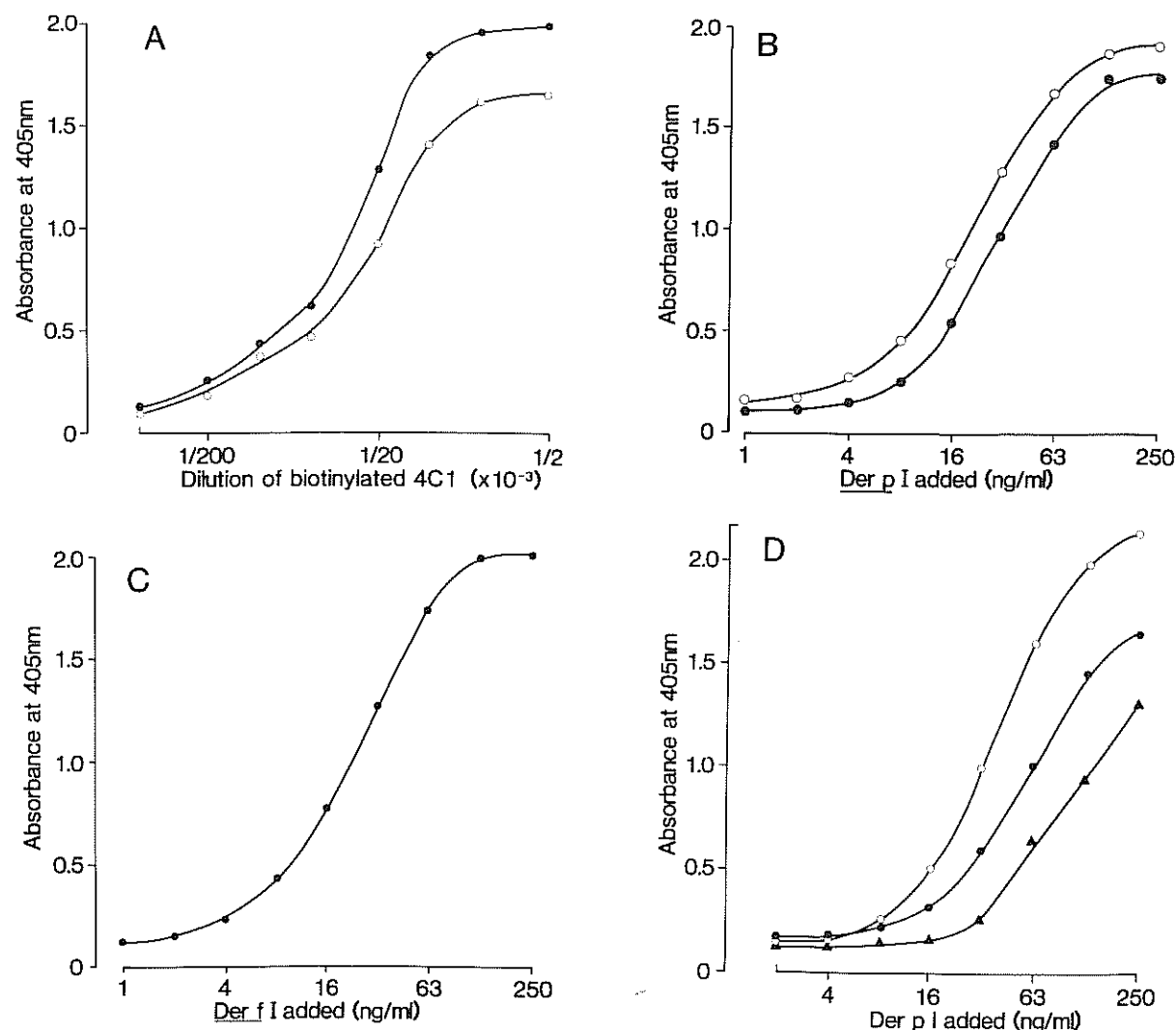


Fig. 1. A: Titration of biotinylated 4C1 against 120 ng/ml *Der p* I (solid circles) and 120 ng/ml *Der f* I (open circles) using 0.25 μ g/ml streptavidin-peroxidase: titer-1/10000 of 1.6 mg/ml biotinylated 4C1 (16 ng/well). B: Control curve for *Der p* I using two combinations of Mabs. Solid circles: 5H8 on plate, biotinylated 4C1; open circles: 10B9 on plate, biotinylated 5H8. C: Control curve for *Der f* I using 6A8 on plate and biotinylated 4C1. D: Binding of 4C1 to *Der p* I at different pH. Open circles: pH 7.5; solid circles: pH 4.2; triangles: pH 9.6.

second antibody for both mite allergen assays, with the appropriate species specific capture antibody. Fig. 1C shows a typical control curve for *Der f* I using 6A8 as capture antibody. The sensitivity of both ELISA assays is approximately 5 ng/ml and there is no detectable cross-reactivity between *Der p* I and *Der f* I over the range of the control curves. A *D. microceras* extract gave results in the *Der p* I and *Der f* I assays of 5% of

the estimated value for *Der m* I (based on AgP₁Eq assay), thus there was some cross-reactivity of both assays with *Der m* I. Fig. 1D shows control curves for *Der p* I at pH 4.2, 7.5 and 9.6, demonstrating that the optimum pH for binding biotinylated 4C1 is pH 7.5 and that the sensitivity is decreased above or below this pH. We did not, however, find raised non-specific binding in the assay at different pH values.

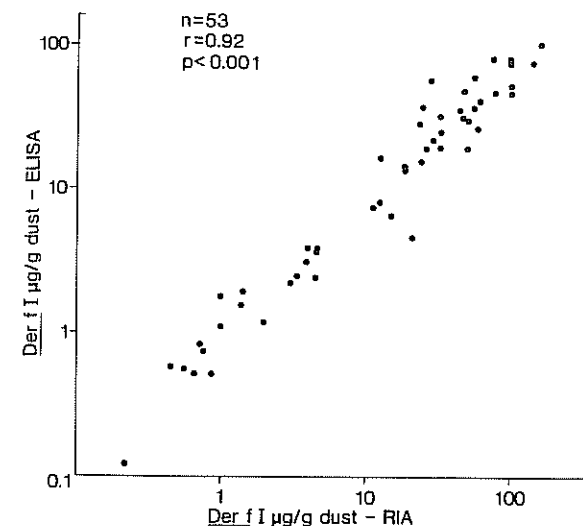


Fig. 2. Correlation between the Mab ELISA and Mab RIA for *Der f* I in 53 house dust extracts collected from 18 houses in Danbury, Connecticut, U.S.A.

Der p I and *Der f* I levels in house dust extracts

53 dust extracts were assayed for *Der p* I and *Der f* I using both the Mab ELISA and Mab RIA. The results showed a very good quantitative correlation between the assays for both *Der f* I ($r = 0.92$, $p < 0.001$, Fig. 2) and *Der p* I ($r = 0.96$, $p < 0.001$, data not shown). The mean within assay coefficient of variation (CV) for three separate

Der p I and *Der f* I assays was 4.8% and 8.7% ($n = 144$) and the between assay CV on two assays was 17.8% and 13.1%, respectively. The correlation coefficient between Mab ELISA and Mab RIA for 132 house dust extracts collected in New Orleans, many of which contained low or undetectable levels of mite allergens (range < 0.1 – 65μ g/g), was also very good: $r = 0.90$ ($P < 0.001$) for *Der p* I and $r = 0.86$ ($P < 0.001$) for *Der f* I. The correlation between the combined concentrations of *Der p* I and *Der f* I determined by the Mab ELISA and total Group I allergen content (measured as AgP₁Eq by RIA) was 0.85 ($n = 132$, $P < 0.001$).

We compared the effect of different extraction procedures and freeze-thawing on the stability of both Group I allergens in house dust extracts. Table I shows the results for the *Der p* I content of five house dust samples extracted in BBS or in 50% glycerol/BBS and either kept frozen for 8 days or freeze-thawed four times at -20°C or -70°C . The geometric mean *Der p* I content of the BBS extracts was significantly lower after freeze-thawing at -20°C ($t = 10.63$, $P < 0.005$) or -70°C ($t = 15.48$, $P < 0.001$). Extracts prepared in 50% glycerol/BBS showed no significant difference in *Der p* I levels after repeated freeze-thawing at -20°C , but they had lower levels

TABLE I

EFFECT OF REPEATED FREEZE-THAWING AND STORAGE TEMPERATURE ON THE *Der p* I CONTENT OF FIVE HOUSE DUST EXTRACTS^a

Extract	50% glycerol/BBS				BBS			
	-20°C	-70°C	-20°C^b	-70°C^b	-20°C	-70°C	-20°C^b	-70°C^b
1	405	340	265	185	170	150	125	105
2	4060	3010	6125	2460	2435	1960	1785	1060
3	3540	2975	3310	2100	2520	4015	1820	1350
4	335	285	260	180	175	155	125	115
5	260	260	145	140	165	145	90	80
GM ^c	871	741	724	479	501	479	339	269
t^d	–	3.46	1.07	12.43	6.09	3.13	10.63	15.48
p^e	–	0.01	0.17	< 0.001	0.002	0.02	0.002	< 0.001

^a *Der p* I concentration (ng/ml) of five house dust samples extracted in either 50% glycerol/BBS or BBS and stored for 8 days at different temperatures.

^b Freeze-thawed four times in 8 days.

^c GM geometric mean.

^d t , one-sided Student's t -test comparing GM *Der p* I values with GM *Der p* I in 50% glycerol/BBS at -20°C .

^e P , percentage value of significance.

TABLE II
STABILITY OF *Der p* I OVER 3 MONTHS STORAGE AT DIFFERENT TEMPERATURES

Der p I levels in ng/ml in two house dust extracts stored at different temperatures in either BBS or 50% glycerol/BBS

		<i>Der p</i> I (ng/ml)			
		+20 °C	+40 °C	-20 °C	-70 °C
BBS extracts	A	1100	2250	2250	2200
	B	2300	3700	4050	4100
50% glycerol/BBS	A	3650	4150	3400	3300
	B	4350	5000	5850	4950

after freeze-thawing at -70°C ($t = 12.43$, $P < 0.001$). The results suggest that 50% glycerol not only extracts slightly more Group I protein but also protects the allergen from denaturation by freeze-thawing presumably because it does not freeze at -20°C . In order to investigate this further, two samples were extracted either in BBS or 50% glycerol/BBS, stored at various temperatures for 3 months, and then assayed for *Der p* I. The results confirm that extracts prepared in 50% glycerol are stable over a wide temperature range and that the stability of *Der p* I at room temperature is improved by storage in 50% glycerol/BBS (Table II).

In carrying out these investigations we noticed that samples with relatively low mite allergen content ($< 10 \mu\text{g/g}$) had substantially lower (by approximately 85%) values of *Der p* I in 50% glycerol extracts when they were assayed undiluted. We

TABLE III
INTERFERENCE OF GLYCEROL IN ASSAY FOR MITE ALLERGENS

Mean percent inhibition of binding of 125 ng/ml *Der p* I and *Der f* I in the Mab ELISA ($n = 24$) by increasing concentrations of glycerol

% glycerol added	equivalent dilution of extract in assay	Mean % inhibition of binding	
		<i>Der p</i> I	<i>Der f</i> I
1	1/50	0	0
5	1/10	1	0
10	1/5	7	13
25	1/2	36	26
50	neat	90	54

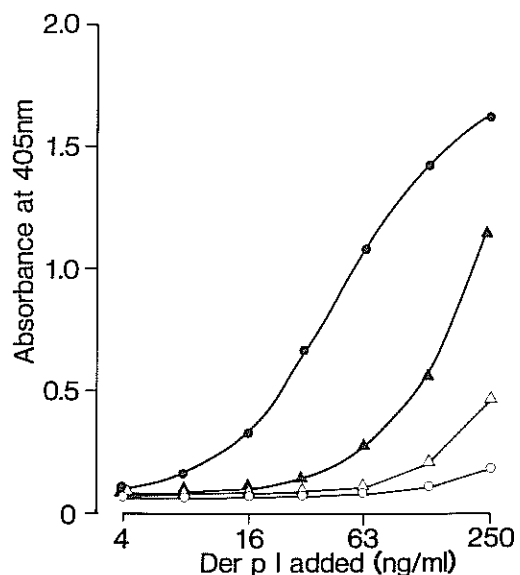


Fig. 3. Effect of different incubation times on *Der p* I control curves. Solid circles: *Der p* I in PBS, 1 h; solid triangles: *Der p* I in 50% glycerol/PBS, 1 h; open triangles: *Der p* I in 50% glycerol/PBS, 4 h; open circles: *Der p* I in 50% glycerol/PBS, overnight.

suspected that high concentrations of glycerol reduced binding of mite allergen to the Mab. This was confirmed by comparing the binding of 125 ng/ml solution of *Der p* I or *Der f* I prepared in different concentrations of glycerol in the ELISA. The results showed that $> 10\%$ glycerol (v/v) caused a marked inhibition of binding of mite allergen to the capture Mab: up to 90% and 50% for *Der p* I and *Der f* I, respectively (Table III). Fig. 3 shows control curves for *Der p* I in PBS and in 50% glycerol/PBS, incubated for various times with the capture Mab in the ELISA. In the presence of 50% glycerol, longer incubation times (up to 16 hours) gave greater but never maximal binding of *Der p* I.

Discussion

The two-site Mab ELISA for *Der p* I and *Der f* I shares the advantages of the Mab RIA previously described in that it has similar sensitivity and does not require the use of purified allergen. However, non-isotopic immunoassays have several advantages over RIA and are more widely used in

clinical laboratories. Biotinylation of Mab is easier than enzyme labelling and the biotinylated antibody is stable for long periods, without the problems of radioactive decay. Despite extra steps in the method, the ELISA has shorter incubation times than the RIA and results are obtained more quickly using a microplate reader. The Mab ELISA for the Group I mite allergens reported here correlates well with the Mab RIA previously described (Chapman et al., 1987b). The specificity of the assays for *Der f* I is the same (i.e., cross-reactivity using *D. pteronyssinus* extracts is $\sim 0.1\%$), however, the *Der p* I ELISA uses a different combination of Mab than the RIA and in this format the level of cross-reactivity with *D. farinae* extracts is slightly higher (1–2% as compared to $< 0.1\%$ using the Mab combination previously used in RIA). The ELISA format was adopted because it permits a single biotinylated Mab to be used in assays for both allergens and for most applications, particularly assays of house dust extracts, this degree of cross-reactivity is acceptable. Where absolute specificity is required, the ELISA can be carried out using the original Mab combination, with comparable results to the RIA (see control curves, Fig. 1B).

Biotinylation alters the surface charge and therefore the isoelectric point of antibodies as it substitutes a neutral *N*-hydroxy-succinimidobiotin molecule for a basic lysine residue. If the solubility of the Mab was decreased at or near neutral pH by biotinylation, aggregation of the Mab could occur, resulting in a high background in the ELISA (Wadsley and Watt, 1987). We found that although the sensitivity of the assay was decreased at above or below pH 7.5, no increased non-specific binding was observed. On investigating the use of 50% glycerol for the extraction of house dusts, we found slightly higher levels of *Der p* I or *Der f* I compared with BBS extracts, but these differences were not statistically significant. In addition, the presence of 50% glycerol protected against denaturation of the mite allergens by freeze-thawing or prolonged storage at room temperature, and this may be an important consideration when carrying out studies on the prevalence of house dust mite allergens in under-developed countries or tropical environments. However, we found that glycerol concentrations of $> 10\%$ (v/v)

TABLE IV
TWO-SITE Mab IMMUNOASSAYS FOR MAJOR ALLERGENS

Allergen	Solid phase ab	Second ab	Reference standard	Assay
<i>Der p</i> I	5H8	4C1	<i>D. pteronyssinus</i> UVA 87/03 NIBSC 82/518	RIA, ELISA
<i>Der f</i> I	6A8	4C1	<i>D. farinae</i> UVA 87/02 OBRR E1-Df	RIA, ELISA
<i>Der p</i> II, <i>Der f</i> II	7A1	6D6	Affinity-purified <i>Der f</i> II	RIA
<i>Fel d</i> I Cockroach	6F9 10A6	3E4 Rabbit IgG ab	OBRR Cat E3 UVA CR extract	RIA, ELISA

Key: CR, cockroach; *Fel d* I, *Felis domesticus* allergen I; NIBSC, National Institute for Biological Standards and Control; OBRR, Office of Biologics and Research Resources.

considerably inhibited binding of the Group I allergens to the capture Mab. This needs to be taken into consideration when house dust extracts are assayed at dilutions less than 1/10 and when assaying commercial allergen preparations containing glycerol, e.g. skin prick test solutions.

This Mab ELISA is a simple, specific and sensitive assay for the detection of the major allergens from house dust mite. The use of biotinylated Mab has the advantage that assays for different allergens can use the same enzyme and detection systems. Table IV lists the immunoassays for the measurement of major allergens currently used in our laboratory. Lombardero et al. (1988) recently reported a Mab RIA for the major cat allergen, *Fel d* I, and we subsequently developed a Mab ELISA of comparable specificity and sensitivity. We are currently developing an ELISA for Group II mite allergens and have also developed an ELISA for a major cockroach allergen which has been used to measure cockroach allergen in the houses of asthmatic patients (Pollart et al., 1988).

The Mab ELISA for *Der p* I and *Der f* I will have applications in epidemiologic studies where a standardized assay is required for the quantitative assessment of allergen levels both in dust and airborne in houses of patients with asthma. This

will make it easier to provide information on the relationship between the levels of mite allergen and the prevalence of allergic disease. It has been proposed that a *Der p* I or *Der f* I content of 2 µg/g fine house dust should be regarded as a risk level for sensitization and that 10 µg/g represents a level at which mite allergic individuals are at risk for developing acute asthma (Platts-Mills and De Weck, 1988). Using the Mab ELISA to assay large numbers of house dust mite extracts, it is now possible to test these proposed risk levels and to assess the corresponding airborne allergen levels which constitute risk factors. The assay will also be useful for standardization of allergen extracts used for diagnosis and immunotherapy and for comparing the efficacy of allergen avoidance regimens.

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