

Quantitation and thermal stability of the mite allergen Dpt 12 in whole mite extracts

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Twelve whole mite extracts (WME) from seven sources were examined for their content of the allergen Dpt 12, protein content, and the inhibitory capacity in RAST by use of paper discs coupled with either WME or Dpt 12. Linear regression analysis demonstrated that the concentration of Dpt 12 determined either by single radial immunodiffusion or by RAST inhibition did not correlate with either the RAST-inhibition titer with use of discs coupled with WME or with protein content, suggesting that Dpt 12 content does not reflect the overall potency of WMEs. However, statistically significant correlations were obtained between Dpt 12 content and whole mite-RAST inhibition titers if extracts from the same manufacturer were examined or if data obtained from extracts containing large Dpt 12 to protein concentrations ($>25\%$) were excluded from the analysis. In contrast, statistically significant correlations between protein concentration and WME-RAST inhibition titers ($p = 0.00037$) and between Dpt 12 content and Dpt 12 RAST-inhibition titers ($p = 0.00001$) were found. Thermal stability studies demonstrated that Dpt 12 per se and WMEs were stable at 4°C for up to 3 mo. Storage at 23°C for 3 mo, however, resulted in partial loss of both Dpt 12 and whole-mite activity. Substantial losses occurred at 36°C for both 1-month and 3-month incubation periods. Esterase, esterase lipase, and alkaline phosphatase were found in three WMEs studied. Dpt 12 was biochemically unrelated to these enzymes. (*J ALLERGY CLIN IMMUNOL* 74:848-54, 1984.)

Several techniques have been used to assess the potency of individual allergen extracts obtained from various manufacturers in an attempt to devise suitable protocols for standardizing allergen extracts. Techniques demonstrated to have merit, either alone or in combination, in this task have included RAST-inhibition assays,¹ crossed immunoelectrophoresis,² and the immunochemical quantitation of individual allergens, particularly AgE from ragweed pollen³ and the group 1 allergen from rye grass.⁴

Although most of these techniques have been applied to the comparison of extracts from allergens such as ragweed and rye grass pollen, recent studies

Abbreviations used

PBS: Phosphate-buffered saline
WME: Whole mite extract

have described the use of these and other techniques in comparing the potency of extracts prepared from both molds^{5, 6} and mites,^{6, 7} two significant sources of aeroallergens. In this regard, the present article describes studies aimed at comparing the potency of several extracts prepared from the house dust mite *Dermatophagoides pteronyssinus* from different sources in RAST-inhibition assays by use of discs coupled with either WME or purified Dpt 12.⁸

In addition, we have quantitated the amount of a major mite allergen Dpt 12, antigenically identical to P₁ and Dp 42,⁹ in each of these extracts to determine whether the concentration of this allergen correlated with the RAST-inhibition titer of the WME as has been reported for AgE and rye group 1 allergens.^{3, 4} Furthermore, we report on the thermal lability of this allergen in a selected WME over prolonged incubation periods.

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MATERIAL AND METHODS

WMEs

WMEs were obtained from several sources. Five extracts were kindly provided by Ms. A. Ford from the National Institute for Biological Standards and Control (London, England), the characteristics of which have been described elsewhere.¹⁰ Two of these extracts were identical (extracts 1 and 3). Other extracts were obtained from Commonwealth Serum Laboratories (Parkville, Australia; 0.01 mg of nitrogen per milliliter and 0.1 mg of nitrogen per milliliter) and from Abello (Madrid, Spain) (100 biological units per milliliter). Two extracts were prepared in the authors' laboratory as described previously,¹¹ one extract from a mixture of mites and growth medium (kindly provided by Beecham Pty. Ltd., Melbourne, Australia) and the other extract from mites free of growth medium (kindly provided by Commonwealth Serum Laboratories). The latter extract was used in the preparation of the anti-whole-mite antiserum and in the preparation of discs for the RAST-inhibition assay.

The protein content of each extract was determined by use of Coomassie blue G 250 assay.¹²

Preparation of Dpt 12

The allergen Dpt 12 was isolated by chromatofocusing from spent-growth medium, kindly provided by Commonwealth Serum Laboratories in a manner similar to that described earlier.⁸ However, the ion exchange matrix and the buffer used in this study were the anion exchanger DE53 (Whatman Ltd., Maidstone, England) and the chromatofocusing buffer system described by Hearn and Lyttle.¹³ A crude Dpt 12 allergen preparation was obtained from an aqueous solution of spent-growth medium by precipitation with 50% saturated ammonium sulphate. The Dpt 12 allergen was then partially purified by gel filtration on Sephacryl S-300 (Pharmacia South Seas Pty. Ltd., Sydney, Australia). Those fractions containing the allergen were pooled, concentrated, and dialyzed exhaustively against 0.005M of Tris buffer, pH 7.5. This preparation was then applied to a DE53 column, preequilibrated with the same buffer. After the removal of nonbinding material, the allergen was eluted by use of the chromatofocusing buffer adjusted to pH 5.0 with 1.0M HCl. Those fractions containing the allergen (pI 6.9 to 5.8) were pooled, dialyzed against 0.05M ammonium bicarbonate, pH 8.0, and freeze-dried. The allergen preparation was finally purified by gel filtration on a P-60 column (Bio Rad Laboratories Pty. Ltd., Sydney, Australia) equilibrated with 0.05M ammonium bicarbonate, pH 8.0. The fractions containing the allergen were pooled and freeze-dried.

Antisera

The anti-WME antiserum was produced in sheep. Briefly, each of three sheep was immunized subcutaneously with 2 mg of WME dissolved in 1 ml of 0.01M phosphate buffer, pH 7.2, containing 0.15M NaCl (PBS) and emulsified with Freund's complete adjuvant. A secondary injection

of WME (2 mg) in Freund's incomplete adjuvant was administered 28 days later, and the sheep were bled 10 days after this and subsequent booster injections. Sera from each sheep were pooled and stored frozen at -20°C until required.

The anti-Dpt 12 antiserum was produced in rabbits. Each of three rabbits was immunized subcutaneously with 500 μg of purified Dpt 12 in 0.5 ml of PBS emulsified with an equal volume of Freund's complete adjuvant. The same amount of material emulsified in Freund's incomplete adjuvant was administered 26 days later, and the rabbits were bled 10 days after this and subsequent booster injections. Sera from each rabbit were pooled and stored frozen until required.

Single radial immunodiffusion

The concentration of Dpt 12 in each extract was determined by single radial immunodiffusion¹⁴ by use of the anti-Dpt 12 antiserum at a concentration of 2.5% (v/v). Plates containing both standard preparations of Dpt 12 (range 1 to 20 $\mu\text{g}/\text{ml}$) and individual extracts were incubated at 4°C for 72 hr. After incubation, the plates were pressed and washed three times with PBS. The plates were again pressed and washed in distilled water. Once dried the plates were stained with Coomassie blue R 250, and the size of the precipitin ring was measured. The concentration of Dpt 12 in the extract was determined by reference to a standard curve constructed by plotting the square of the diameter against concentration of Dpt 12. The concentration of Dpt 12 in the standards was determined by use of the Coomassie blue G 250 assay.¹²

RAST-inhibition

RAST-inhibition assays were performed essentially as described by Wide et al.¹⁵ with the use of ^{125}I -labeled anti-human IgE obtained from Pharmacia (South Seas Pty. Ltd.). WME and Dpt 12 were optimally coupled to cyanogen bromide-activated paper discs as described by Ceska et al.¹⁶ The mite-allergic human serum was a pool prepared from 26 sera from individuals previously demonstrated to be highly mite allergic by RAST. This pool did not react with growth medium supplied by Commonwealth Serum Laboratories in either direct RAST or RAST-inhibition assays. The appropriate dilution of serum to be used in the WME- and Dpt 12 RAST-inhibition assays was determined by performing preliminary serum-dilution experiments with the appropriate discs. RAST-inhibition results were expressed as the number of microliters of extract required to produce 50% inhibition in the RAST assay. When experiments were repeated, several previously analyzed extracts were included, and results were normalized.

Thermal stability of Dpt 12 in whole-mite extracts

The thermal stabilities of both Dpt 12 in a WME and the WME per se were determined by RAST-inhibition studies by use of a WME (0.1 mg of nitrogen per milliliter) dissolved in Coca's solution (0.5% w/v of sodium chloride,

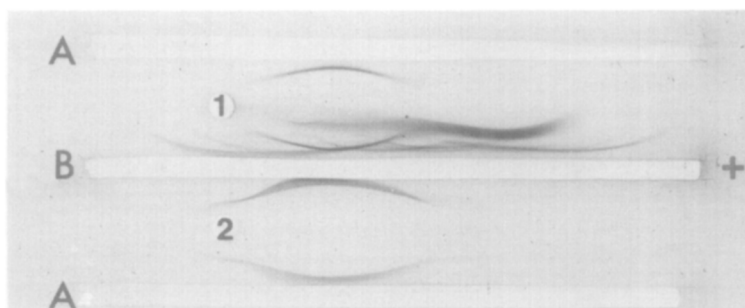


FIG. 1. Agarose immunoelectrophoretic analysis to demonstrate the specificity of the rabbit anti-Dpt 12 antiserum used in the quantitation of the allergen Dpt 12: rabbit anti-Dpt 12 (A); sheep anti-WME (B); WME (1) at 20 mg/ml; Dpt 12 at 500 μ g/ml (2).

TABLE I. Summary of RAST inhibition data and Dpt 12 content of several WMEs

Extract	Type of extract	Dpt 12* (μ g/ml)	Protein† (μ g/ml)	Ratio	Dpt 12‡	RAST-inhibitions	
					Total protein	WME	Dpt 12
1	F	14.6	44.7		32.7	9.2	0.124
2	F	1.4	21.9		6.4	7.8	2.4
3	F	14.8	46.4		31.9	8.6	0.118
4	F	16.1	149.2		10.8	0.4	0.076
5	G	50.2	87.8		57.1	1.2	0.025
6	G	36.2	48.0		75.4	0.92	0.050
7	A	5.3	77.6		6.8	2.8	0.62
8	A	7.0	100.1		6.9	1.0	0.40
9	A	11.3	210.0		5.4	0.39	0.18
10	A	9.0	141.8		6.3	0.54	0.09
11	A	5.1	117.2		4.3	1.6	0.35
12	F	27.7	134.5		20.5	0.52	0.064

F = freeze dried; G = glycerinated; A = aqueous.

*Dpt 12 content measured by single radial immunodiffusion assay.

†Protein determined by use of Coomassie blue G 250 dye-binding assay.

‡Ratio expressed as micrograms of Dpt 12 per micrograms of total protein times 100%.

§Results are expressed as the number of microliters of extract required to produce 50% inhibition in a RAST assay by use of a human mite-allergic serum pool.

||Extracts obtained from the same manufacturer.

0.275% w/v of sodium bicarbonate, and 0.5% w/v of phenol). Portions of the extract were incubated at 4° C, 23° C, and 36° C for 0, 1, and 3 mo. The samples were assayed by RAST inhibition after incubation by use of a reference mite extract as standard. The data were normalized and expressed as a percentage of the inhibitory capacity of the zero times RAST-inhibition titer. The extracts were also examined for Dpt 12 content by single radial immunodiffusion.

Enzyme activity

The enzyme content of several WMEs and a sample of Dpt 12 was determined by use of the Api Zym system according to the manufacturer's instructions (API system, Montalier Vercieu, France). Nineteen enzymes were assayed for: acid and alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase,

cystine arylamidase, trypsin, chymotrypsin, phosphoamidase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, and α -fucosidase. To each well of the Api Zym tray containing the appropriate freeze-dried buffer and naphthol-coupled substrate were added either 66 μ l of each mite extract (0.1 mg of nitrogen per milliliter), Dpt 12 (1 mg/ml), growth medium (1 mg/ml), saline control, or Coca's solution control, and the tray was incubated at 37° C for 4 hr. After incubation, the free naphthol released from the substrate was detected by adding equal volumes (33 μ l) of fast blue BB (0.35% w/v in 2-methoxyethanol) and 2M of Tris buffer, pH 7.6, containing 10% w/v of sodium dodecyl sulphate. The amount of product formed was semi-quantitatively assessed by use of the color chart provided (see Table IV).

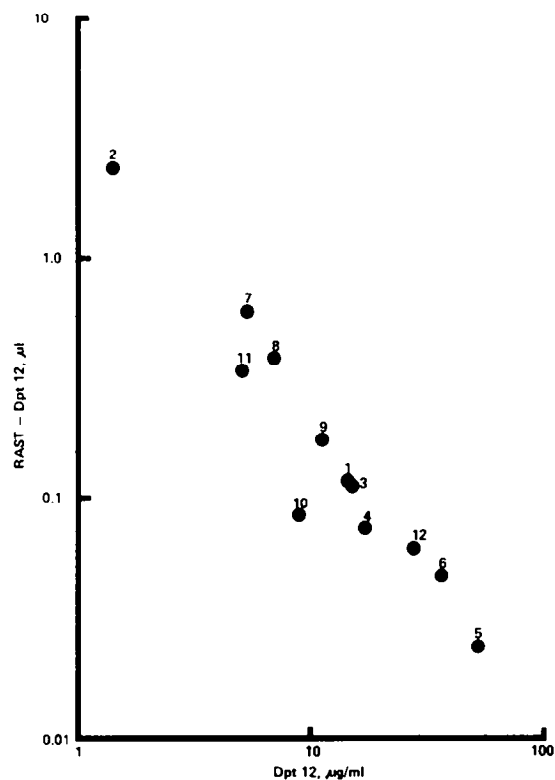


FIG. 2. Scatter diagram to demonstrate the relationship between the content of Dpt 12 in various WMEs as measured by single radial immunodiffusion and the number of microliters of extract required to produce 50% inhibition in a RAST assay by use of Dpt 12 coupled discs. The numbers associated with each point correlate with the extracts described in Table I.

RESULTS

Single radial immunodiffusion studies

The concentration of Dpt 12 in 12 WMEs was determined by use of the single radial immunodiffusion technique. The specificity of the anti-Dpt 12 antiserum and the purity of the allergen used in the assay are demonstrated in Fig. 1. These experiments demonstrated that the concentration of Dpt 12 varied between extracts and ranged from 1.4 $\mu\text{g/ml}$ to 50.2 $\mu\text{g/ml}$ (Table I). The ratio of Dpt 12 to protein concentration also varied, ranging from 4.3% to 75.4%.

RAST-inhibition studies

The potency of the WMEs with regard to both Dpt 12 content and the whole spectrum of mite allergens was determined by their capacity to inhibit the reactivity of a mite-allergic serum pool in a RAST-inhibition assay by use of discs coupled with either WME or Dpt 12. By use of WME discs, there was a 24-fold variation in potency between the extracts, whereas the variation in potency increased to 96-fold when Dpt 12 discs were examined (Table I).

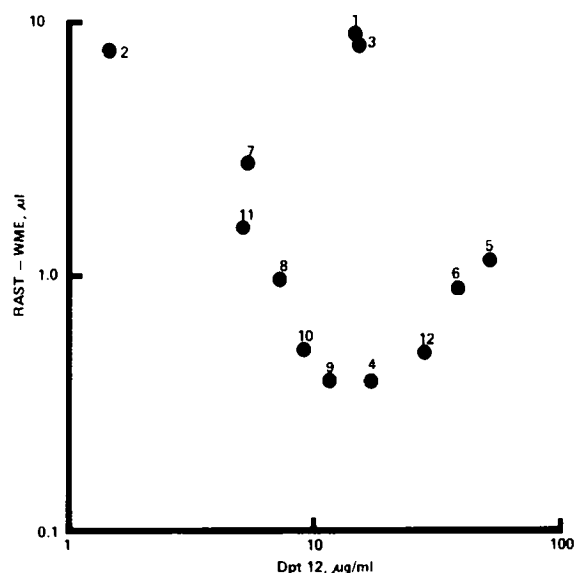


FIG. 3. Scatter diagram to demonstrate the relationship between the content of Dpt 12 in various WMEs as measured by single radial immunodiffusion and the number of microliters of extract required to produce 50% inhibition in a RAST assay by use of WME coupled discs. The numbers associated with each point correlate with the extracts described in Table I.

The relationship between the various parameters studied was determined by linear regression analysis. These studies demonstrated significant correlations between Dpt 12 RAST-inhibition titers and Dpt 12 concentrations (Fig. 2, Table II) but not between WME RAST-inhibition titers and Dpt 12 concentrations (Fig. 3, Table II). A significant correlation was obtained between WME RAST-inhibition titer and protein concentration (Table II). Correlations between Dpt 12 RAST-inhibition titer and protein concentration and Dpt 12 RAST-inhibition titer and WME RAST-inhibition titers were not significant (Table II). In regard to the latter relationship, however, significant correlations (Table II) were obtained if only extracts from the same manufacturer were analyzed (extracts 7 to 11) or alternatively, if extracts with large Dpt 12 to total protein concentration ratios (arbitrarily defined as being $>25\%$) were excluded (extracts 1, 3, 5, and 6). Similar results were obtained when the relationship between Dpt 12 concentration (determined by single radial immunodiffusion) and WME RAST-inhibition titers were studied by use of the same restrictions (Table II).

Thermal stability of Dpt 12 in WME

The thermal stability of both Dpt 12 in a WME and the WME per se was studied after 1 mo and 3 mo incubation at 4° C, 23° C, and 36° C. These studies (Table III) demonstrated that the WMEs and Dpt 12

TABLE II. Summary of linear regression data

Relationship	Correlation coefficient	Probability (=)
Dpt 12 concentration vs Dpt 12 RAST-inhibition titer	0.96	0.00001
Dpt 12 concentration vs WME RAST-inhibition titer	0.38	0.10 (NS)
Dpt 12 concentration vs WME RAST-inhibition titer*	0.95	0.005
Dpt 12 concentration vs WME RAST-inhibition titer†	0.90	0.001
Dpt 12 RAST-inhibition titer vs protein concentration	0.26	0.21 (NS)
WME RAST-inhibition titer vs protein concentration	0.83	0.00037
Dpt 12 RAST-inhibition titer vs WME RAST-inhibition titer	0.45	0.07 (NS)
Dpt 12 RAST-inhibition titer vs WME RAST-inhibition titer*	0.84	0.036
Dpt 12 RAST-inhibition titer vs WME RAST-inhibition titer†	0.93	0.0003

NS = not significant.

*Correlation coefficient determined on data from extract obtained from the same manufacturer only, i.e., 7, 8, 9, 10, and 11.

†Correlation coefficient determined on data from extracts 2, 4, 7, 8, 9, 10, 11, and 12, i.e., excluding data from those extracts with large Dpt 12 concentration to protein concentration ratios, namely, 1, 3, 5, and 6. With the exception of the protein concentration variable, all relationships were determined by use of log transformed data.

TABLE III. Thermal stability of Dpt 12 in a WME

Incubation period	Temperature (°C)	RAST-inhibition*		DPT 12* concentration
		WME	Dpt 12	
1 mo	4	103	100	100
	23	133	77	112
	36	25	14	65
3 mo	4	93	88	92
	23	70	75	76
	36	14	10	48

*Data expressed as a percentage of the values obtained with the zero time extract.

were stable as judged by Dpt 12 RAST inhibition, WME RAST inhibition, and Dpt 12 content in aqueous extracts at 4° C for at least 3 mo. At 23° C there was a 25% reduction in Dpt 12 RAST-inhibition titer at both 1-month and 3-month incubation. This reduction in Dpt 12 content was also detected by single radial immunodiffusion after incubation for 3 mo at 23° C but not at 1 mo. Marked changes in Dpt 12 content (as judged by Dpt 12 RAST inhibition and single radial immunodiffusion) and in WME activity (as judged by WME RAST inhibition) were observed after incubation at 36° C for both 1 and 3 mo.

Enzyme content of WME

Certain of the WMEs, including that used in the thermal stability studies, were examined for enzymes by use of the Api Zym system to determine whether enzymes could account for the observed losses in allergenic activity. These studies (Table IV) demonstrated that three of the extracts contained alkaline phosphatase, esterase, and esterase lipase in similar

quantities. Two of these extracts contained leucine arylamidase, and one extract contained acid phosphatase. None of these enzymes (except for a trace of leucine arylamidase) was found in extracts of the medium on which the mites were grown. Nonspecific phosphoamidase activity was detected in all the extracts, growth-medium extracts, and extracting fluid. The purified allergen Dpt 12 was also analyzed in the system and demonstrated not to be one of the enzymes detected in the WMEs.

DISCUSSION

The results obtained in this study demonstrated that both the allergenic potency of WMEs from several sources and the amount of Dpt 12 therein varied considerably. However, as none of the extracts had been standardized by any criterion before analysis, these results are hardly surprising. Of interest, however, was the observation that the amount of Dpt 12, expressed either gravimetrically or as a Dpt 12 RAST-inhibition titer within each extract, did not

TABLE IV. Enzymatic analysis of Dpt 12 and WMEs

Enzyme	Dpt	Saline	Extract 8	Extract 9	Extract 11	Growth medium	Coca's solution
Control	—	—	—	—	—	—	—
Alkaline phosphatase	—	—	5+	5+	5+	—	—
Esterase	—	—	3+	3+	3+	—	—
Esterase lipase	—	—	5+	5+	5+	—	—
Lipase	—	—	—	—	—	—	—
Leucine arylamidase	—	—	3+	—	1+	tr	—
Valine arylamidase	tr	tr	—	—	—	—	—
Cystine arylamidase	tr	tr	—	—	—	—	—
Trypsin	—	—	—	—	—	tr	—
Chymotrypsin	—	—	—	—	—	—	—
Acid phosphatase	—	—	5+	—	—	1	—
Phosphoamidase	tr	tr	1+	1+	1+	1+	1+
α -Galactosidase	—	—	—	—	—	—	—
β -Galactosidase	—	—	—	—	—	—	—
β -Glucuronidase	—	—	—	—	—	—	—
α -Glucosidase	—	—	—	—	—	—	—
β -Glucosidase	—	—	—	—	—	—	—
N-acetyl- β -glucosaminidase	—	—	—	—	—	—	—
α -Mannosidase	—	—	—	—	—	—	—
α -Fucosidase	—	—	—	—	—	—	—

5+ = >40 nanomoles of hydrolyzed substrate; 3+ = 20 nanomoles of hydrolyzed substrate; 1+ = 5 nanomoles of hydrolyzed substrate; tr = <5 nanomoles of hydrolyzed substrate. Extracts 8, 9, and 11 at 0.1 mg of nitrogen per milliliter in Coca's solution; growth medium at 0.3 mg of nitrogen per milliliter in Coca's solution; Dpt 12 at 1 mg/ml in saline.

correlate with total protein and that the ratio of Dpt 12 to total protein varied between the extracts, ranging from 4.3% to 75.4%. These data suggested that the extracts were qualitatively and quantitatively different; further evidence for which was provided by an examination (data not shown) of the WME RAST-inhibition curves where it was demonstrated that extracts 1 and 3 had slightly shallower slopes, whereas extracts 6 and 12 had slightly steeper slopes than did the remaining extracts.

The presence of growth medium may influence the ratio of Dpt 12 to total protein, since the medium is known to contain significant amounts of mite feces, a potent source of the allergen Dpt 12.¹⁷ Although the method of preparation of extracts 1 to 6 is unknown, a comparison of the ratio (Table I) obtained with extracts 7 to 11 (where whole mites free of medium were extracted) with that obtained with extract 12 (extracted with medium) suggest that low ratios of the allergen to protein are associated with extracts prepared from whole mites free of medium. Despite the low amounts of Dpt 12, however, these extracts were allergenically potent, indicating that other major allergens exist.

Of further interest was the finding that the amount of Dpt 12 did not correlate with RAST-inhibition

titers by use of discs coupled with WMEs. This finding contrasts with those obtained with rye group 1 and ragweed AgE, where it was demonstrated that the amount of these allergens correlated with both in vivo and in vitro measurements of allergenicity^{3, 4} but is in accord with the findings obtained with the Alt-I allergen from *Alternaria*.⁵ Good correlations were, however, obtained when WMEs from the same source were examined or extracts with large Dpt 12 to protein ratios were excluded from the analysis.

Thermal stability studies demonstrated that at 4°C the aqueous WME and Dpt 12 were stable for up to 3 mo. However, at higher temperatures and longer incubation periods, both the WME and Dpt 12 lost allergenic potency. Such findings are in accord with data from studies on whole extracts and individual allergens from ragweed and timothy grass pollens.^{18, 19, 20} On the basis of these results, it would therefore be of interest to determine the effect of different storage conditions, such as the inclusion of glycerol or storage under nitrogen, on the stability of both Dpt 12 and WMEs. Although Dpt 12, in WMEs, was found to be thermolabile, the observed effects of heating were more pronounced in the Dpt 12 specific RAST-inhibition assay than in the single radial immunodiffusion assay. This observation may reflect the greater sensitivity of

the RAST assay or a differential thermal susceptibility of IgE and IgG-specific epitopes. Further studies are now underway with the use of more sensitive assays to monitor antigenicity.

Several enzymes were found in the WMEs. They included alkaline phosphatase, esterase, and esterase lipase. In addition, two of the extracts contained leucine arylamidase, and one extract contained acid phosphatase. Esterase and esterase lipase, but not alkaline phosphatase, have been found in mites such as *Dermatophagoides farinae* and *Glycyphagus destructor*.²¹ Whether such enzymes are allergenic is not yet known, but data from this study demonstrated that Dpt 12 was not one of the enzymes detected. However, these studies do not preclude Dpt 12 from being an enzyme per se.

It is not known whether these enzymes were responsible for the loss in allergenicity observed with both Dpt 12 and the WMEs in this study. However, accelerated thermal stability studies at 56° C on the purified allergen performed in our laboratory (unpublished observations) and data from other studies⁹ indicate that Dpt 12 per se is heat labile.

In conclusion, these studies demonstrated that quantitation of a single, albeit major allergen, namely Dpt 12, from the house dust mite is an insufficient indicator of the overall potency of individual mite extracts, a finding that may well reflect the use of differing methods of WME preparation. Thus, in attempting to standardize mite extracts, the source of the extract must be taken into consideration, a conclusion that is also expressed by other workers.²² In addition to the above findings, these studies have demonstrated that at least some mite extracts contain enzymes that may contribute to their degradation but that Dpt 12 per se was not one of those enzymes identified. The allergen Dpt 12 was relatively stable in aqueous WMEs at 4° C for prolonged periods but was labile when it was incubated at higher temperatures for long incubation periods.

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