

Mite allergen content in commercial extracts and in bed dust determined by radioallergosorbent tests

EUAN TOVEY and RUSSELL VANDENBERG*

*Respiratory Investigation Unit, Royal North Shore Hospital, Sydney, Australia and Respiratory Investigation Unit, Royal North Shore Hospital, St. Leonards N.S.W., Australia, 2065

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Summary

Radioallergosorbent (RAST) direct binding and inhibition type assays were used to quantitate the mite (*Dermatophagoides pteronyssinus*) allergen content of four commercial mite extracts and a laboratory prepared extract from freeze-dried mites. The content of mite allergen in extracts prepared from twenty samples of dust vacuumed from bedding was measured by RAST inhibition assay. The four commercial mite extracts designated A, B, C and D, and the laboratory extract, designated L, contained 52, 265, 108, 1.5 and 581 arbitrary units of allergen/ml for the direct binding assay and 128, 111, 217, ~1 and 1083 arbitrary, but different, units of allergen/ml for the inhibition assay respectively. Qualitative differences between at least two extracts were suggested by the different slopes obtained when allergen binding of anti IgE was plotted against the volume of extract used in the direct binding assay. Differences in slope between the two extracts were also apparent when they were used in the inhibition assay. The quantities of mite allergen/gm of bed dust expressed in arbitrary units for the inhibition assay were 24 to 457 (mean 129) units. These quantities are similar to and sometimes greater than the quantity in 1 ml of mite extract and so confirm bed dust as a potent source of mite allergen. There was no significant correlation between the weight of dust, the numbers of dead and live mites and the allergen content of dust.

Introduction

Mites of the genus *Dermatophagoides* produce some of the most potent and common allergens in the world (Voorhorst *et al.*, 1967; Pepys, Chan & Hargreave, 1968). At the present time there is no consensus between different manufacturers in the strength of mite allergen extracts, and each prepares them to his own standard of concentration. Of four samples of mite extract available, one is expressed in protein nitrogen units/ml, one as a percentage of weight/volume, and two as units/ml. It has been previously

Correspondence: Dr R. A. Vandenberg, Respiratory Investigation Unit, Royal North Shore Hospital, St. Leonards N.S.W., Australia 2065.

demonstrated that there is little relationship between the allergen and total protein content of extracts (Gleich *et al.*, 1974).

The availability of RAST inhibition and direct binding allergen assays (Ceska, Eriksson & Varga, 1972; Foucard *et al.*, 1972) has provided an opportunity to compare the allergen content of different commercial extracts with a standard laboratory extract using both assay systems.

Studies of mite distribution, population fluctuation and control usually include tabulated figures of mite numbers, implying a relationship between these numbers and quantity of allergen present. Mites occur in association with other allergens in house dust such as animal danders, fungi, and wind borne pollens. Previously, the assay of allergen by skin test only allowed the total allergen content of the dust to be related to the numbers of contained mites (Voorhorst *et al.*, 1967; Miyamoto *et al.*, 1970). Recent studies of the RAST inhibition assay (Tovey & Vandenberg, 1978) suggest that the specificity is determined by the allergen(s) bound to the solid phase. The presence of other proteins or allergens, not cross reacting with or identical to the solid phase bound allergen, will not interfere with the quantitation of a specified allergen at concentrations of total protein normally encountered in an allergen assay. Thus it has been possible to measure mite allergen content of vacuum samples of bedding dust and explore the relationship between allergen content, weight of dust and numbers of mites.

Materials and methods

Bed dust extracts

Dust was collected by vacuuming the blankets and mattresses of beds from twenty homes. The fine dust was separated from fluff by passage through a 425 μ sieve. With the first ten samples two thirds of the dust (ranging from 0.256 to 1.087 g) was extracted overnight in 20 ml of 0.01 M NH_4HCO_3 with constant rotation. The extracts were then centrifuged at 10,000 g for 10 min and the supernatants millipore filtered (pore size 0.22 μ). Protein was estimated by the Folin Coicalteau method after phosphotungstic acid (PTA) precipitation (William & Chase, 1968). Some extracts required concentration by overnight ultrafiltration at 4°C. With the second ten samples two thirds of the dust (ranging from 0.278 to 1.27 g) was extracted with 0.01 M NH_4HCO_3 on a weight/volume basis (2 ml of 0.01 M NH_4HCO_3 per 0.1 g of dust) to eliminate the need for concentration. The millipore filtering step was removed as sterility was not required. Any white precipitate which formed on overnight storage was removed by centrifugation at 3000 g for 5 min. Units of allergen in the extract were finally related to the weight of dust used.

Mite extracts

A standard extract (designated L) was prepared in the laboratory from freeze-dried mites as described previously (Tovey & Vandenberg, 1978). Commercial extracts were obtained from four manufacturers. These have been designated A, B, C and D (names available on request). The protein concentration of these extracts and of extract L measured by Folin Coicalteau method, was 0.065, 0.30, 0.40, 0.055 and 1.54 mg/ml respectively. Glycerol, used as a preservative in A and B but not C and D, was removed by overnight dialysis against 0.9% NaCl at 4°C. Extract L, containing no glycerol, was also dialysed as a control.

Atopic serum

Pooled serum, previously described (Tovey & Vandenberg, 1978) was prepared from blood collected from subjects with strong skin test reaction to mite. Sera with low mite specific IgE detected by RAST specific antibody assay or high mite precipitating antibodies detected by counter immunoelectrophoresis were not used.

Anti-IgE

^{125}I -Anti-IgE was purchased as the isotope unit for the RAST assay (Phadebas RAST®, Pharmacia Diagnostics AB, Sweden).

Buffer

The buffer, PDT, supplied with Phadebas RAST isotope unit (Pharmacia Diagnostics AB, Sweden) was used as the incubation medium for the inhibition assay and as the diluent for the serum and anti-IgE.

RAST inhibition assays

These assays were performed as described previously (Tovey & Vandenberg, 1978). Briefly, for the bed dust extracts, fourfold dilutions, in triplicate, of each extract were incubated with 12.5 μl of atopic serum and a paper disk prepared using 1 μl of mite extract L, in a total volume of 150 μl of PDT buffer. For the commercial mite extracts, twofold dilutions were used. The tubes were angle rotated for 12–16 hr at room temperature. After 3 washes, 2 ng of ^{125}I -anti-IgE in 50 μl of PDT buffer was added to quantitate the IgE bound to the disk. After 6–8 hr incubation the tubes were washed 3 times and counted in a gammacounter. The data producing between 15 and 80% inhibition was subject to logit transformation and linear regression analysis to produce a straight line of best fit to the approximately linear portion of the sigmoid relationship between inhibition and log allergen volume. If the coefficient of fit (r) was less than 0.90 the data was discarded. The slope of the relationship and volume of allergen required to produce 50% inhibition of the assay when no allergen was present were then obtained from the logit transformed data (Tovey & Vandenberg, 1978). The quantity of allergen has been expressed in arbitrary units as the reciprocal of the volume in ml required for 50% inhibition. It has previously been demonstrated (Tovey & Vandenberg, 1978) that the reproducibility of this system had a coefficient of variation ($\text{Sd} \times 100/\text{mean}$) on different days of 15% when standard and unknown extracts were simultaneously assayed. The coefficient of variation of the slope of the standard extract was 18% for the four assays and 22% for the slope of a second extract relative to the standard extract assayed simultaneously.

RAST direct binding assays

These assays were performed as described previously (Tovey & Vandenberg, 1978). Briefly, activated paper disks were incubated with twofold dilutions, in triplicate, of the allergen extract in coupling buffer for 3 hr at room temperature. Vacant binding sites were blocked by 2 hr incubation with 0.5 Methanolamine (titrated to pH 8 with HCl) followed by 3 cycles of washing in pH 4, 0.1 M acetate, 0.5 M NaCl and pH 8, 0.1 M phosphate, 0.5 M NaCl buffers. Quantitation of the bound allergen was by overnight incubation with 12.5 μl of atopic serum followed by 6–8 hours incubation with 2 ng of ^{125}I -anti IgE, both in 50 μl of PDT buffer. For calculation of slope, points lying between 15 and 80% of the maximum binding occurring in the assay were

subject to linear regression analysis as they were approximately linear over this range of values. If on linear regression analysis the coefficient of fit was less than 0.90 the data was discarded. Volumes of allergen producing 50% of the greatest mean counts bound by any allergen concentration in the assay (50% maximum binding) were considered to contain equal volumes of allergen. The allergen content has been expressed in arbitrary units as the reciprocal of the volume in ml required for 50% maximum binding. Such units apply only to this assay and not to the inhibition assay. The coefficient of variation of this system on different days when a standard extract and an unknown were simultaneously assayed was 24%. The coefficient of variation of the slope of the standard extract was 15% for the four assays and 18.5% for the slope of a second extract relative to the standard extract assayed simultaneously.

The mites were separated from the dust and counted by the method of Shamiyeh *et al.* (1971), as modified by Tovey, Guinan & Vandenberg (1975). Live specimens and dead specimens in a good state of preservation were mounted in Hoyer's modification of Berleses' mounting solution for further study by phase contrast microscopy. The specimens were identified using the criteria of Fain (1966, 1967) for *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, and Blythe, Williams & Morrison (1974) for *Euroglyphus maynei*.

Results

Typical inhibition and direct binding assays have been published previously (Tovey & Vandenberg, 1978) and the method of calculation of allergen content of an extract in arbitrary allergen units demonstrated. Both quantitative and qualitative differences

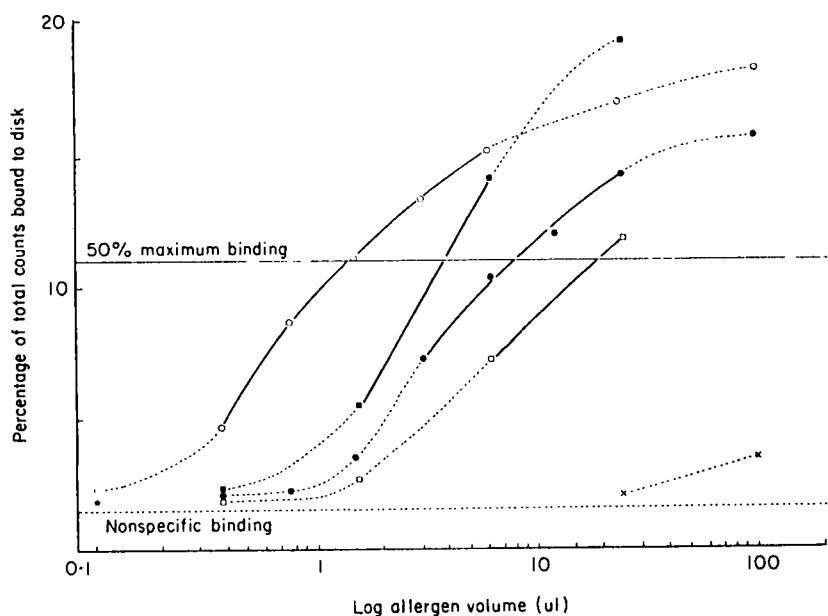


Fig. 1. Assay of the five mite extracts by the RAST direct binding assay. Each point on the graph is the mean of triplicate assays. The solid line joins those points approximating a straight line which were subject to linear regression analysis to determine allergen content and slope. The remaining points were not used in the calculations. Extracts are as follows: $\square$ A, $\blacksquare$ B, $\bullet$ C, $\times$ D, $\circ$ L.

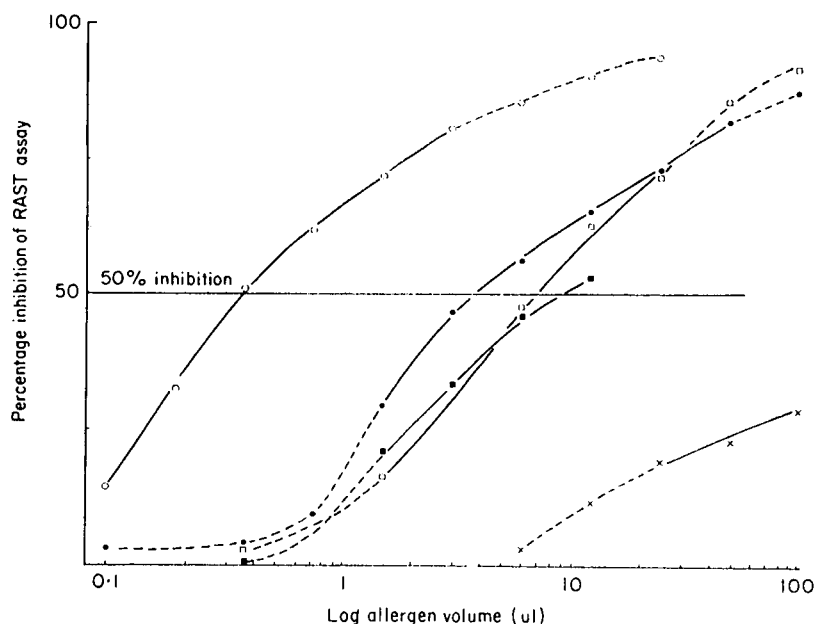


Fig. 2. Assay of the five mite extracts by the RAST inhibition assay. Each point on the graph is the mean of triplicate assays. The solid line joins those points subject to logit transformation and linear regression analysis which were used to determine allergen content and slope. The remaining points were not used in the calculations. Extracts are as above.

were found between the five mite extracts when their allergen contents were assayed by the two types of RAST allergen assay.

The direct binding and inhibition assay curves for the extracts are shown in Figs 1 and 2, the quantities of allergen expressed in arbitrary units and the slopes of the curves are shown in Table 1. Extract D contained virtually no allergen by either method. The qualitative differences are demonstrated in several ways. The allergen content of extract L was much greater relative to the commercial extracts A B C in the inhibition assay than in the direct binding assay. In the inhibition assay the slopes of the portions of the curves subject to logit transformation and linear regression analysis for extracts A and L had steeper slopes than extracts B and C. In the direct binding assay extract B had a steeper slope than the other extracts and increasing quantities of extract B continued to bind anti IgE, although extracts C and L tended to plateau horizontally at lower levels of anti IgE binding.

Each assay system responded differently to the dialysis of extracts for removal of glycerol. Inhibition assay curves generally became flatter and there was an apparent loss of allergen activity (Table 2). This flattening of the curves was most pronounced at higher levels of allergen.

In two parallel control experiments where glycerol was added to an inhibition assay for extract L, the glycerol increased the inhibition of the assay rather than decreased the effect of the inhibitor when present as 33% of the incubation volume. When the glycerol comprised 8% of the incubation volume, its effect on the assay was negligible. There was little change in the slope of either assay.

In the direct binding assay there was an apparent loss of allergen activity by the

Table 1. Allergen content and linear slope from the analysis of mite extracts as determined by two methods of assay

Mite extracts	Inhibition assay			Direct binding assay		Protein concentration mg/ml
	Arbitrary units/ml*	Quantity of allergen relative to extract 1	Slope†	Arbitrary units/ml‡	Quality of allergen relative to extract L	
L	2083	100	0.87	581	100	1.54
A	128	6.1	0.87	52	9.0	0.065
B	111	5.3	0.71	265	45.6	0.30
C	217	10.4	0.72	108	18.6	0.40
D	~1	—	~0.47	<1	—	0.055

* Reciprocal of the volume of extract in ml required to produce 50% inhibition of uptake of anti-IgE by the solid phase allergen bound to disk.

† Slope of the straight line obtained by linear regression analysis of the logit transformed inhibition assay data.

‡ Reciprocal of the volume of extract in ml required to produce 50% of the maximum uptake of anti-IgE by the solid phase allergen.

§ Slope of the straight line obtained by regression analysis of uptake of anti-IgE plotted against log allergen volume.

Table 2. Allergen content and linear slope from the analysis of dialysed mite extracts as determined by two methods of assay

Extract	Inhibition assay		Direct binding assay	
	Arbitrary units/ml*	Slope†	Arbitrary units/ml‡	Slope§
L	909	0.90	515	11.2
A	65	0.70	59	10.2
B	71	0.38	244	15.7

* Reciprocal of the volume of extract in ml required to produce 50% inhibition of uptake of anti-IgE by the solid phase allergen bound to disk.

† Slope of the straight line obtained by linear regression of the logit transformed inhibition assay data.

‡ Reciprocal of the volume of extract in ml required to produce 50% of the maximum uptake of anti-IgE by the solid phase allergen.

§ Slope of the straight line obtained by regression analysis of uptake of anti-IgE plotted against log allergen volume.

dialysed extracts evident at low levels of allergen, but the binding curves showed a steeper slope than their undialysed counterparts. The product of these two factors was little changed in apparent allergen content when determined at 50% maximum binding (Table 2).

The allergen content/gram of dust did not correlate with the numbers of dead or live mites/gram of dust, as shown in Figure 3. The units of mite allergen in the bed extracts when converted to the same arbitrary units as the mite allergen extracts, through their equivalence with mite extract L used in each assay, range from 24 to 457 (mean 129) units/gm dust. Thus it is apparent from Table 1 and Fig. 1 that the mite allergen content of 1 gm of bed dust is comparable to the allergen content of 1 ml of commercial mite extract.

As the 0.1 g of freeze dried mites contains approximately 97,000 mites and yields 10 ml of mite extract, the quantity of allergen per mite can be calculated at 0.2 units/mite.

It was also notable that qualitative differences between mite extract L bound to disc as solid phase allergosorbent in the inhibition assay and the bed dust extracts, were suggested by lower slopes of the dust extract assay curves (mean 0.46) compared with the slope of standard extract L (mean 0.54).

Of the 148 mites mounted for further identification by phase contrast microscopy, *Dermatophagoides pteronyssinus* comprised 94% (56% female, 24% male and 14% juvenile stages), of the balance 3% were *Euroglyphus maynei* and 0.6% Tarsonemidae species. A few dead specimens of the predator mite family Cheyletidae were seen when counting but were not mounted. No *Dermatophagoides farinae* were found.

Discussion

The reproducibility of the inhibition assay (coefficient of variation 15.4%) and the direct binding assay (coefficient of variation 24%) was such that the quantitative differences between the four commercial extracts were significant whichever assay was used. The finding that one extract was virtually useless highlights the need for standardization of allergen content of extracts.

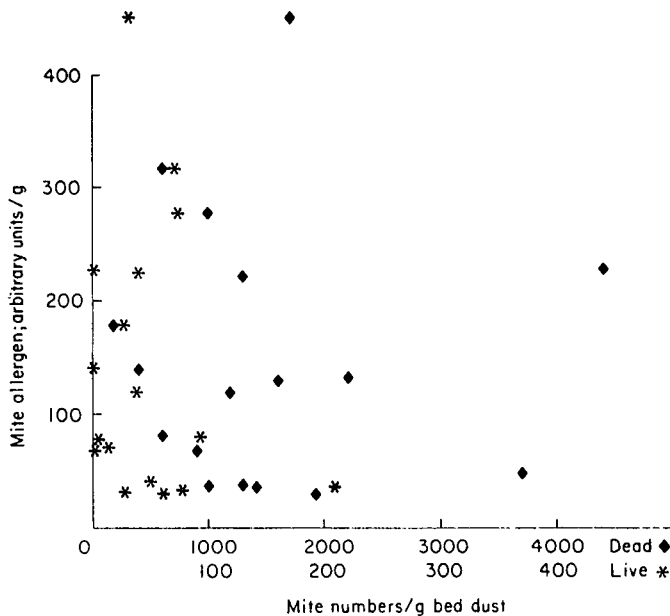


Fig. 3. Relationship between mite allergen content per gram of bed dust, determined by RAST inhibition allergen assay, and mite numbers.

Several qualitative differences between the extracts were suggested by the results. As the direct binding assay utilizes the total allergen content of the extracts reacting with pooled sera from people with marked mite sensitivity, the steeper slope and greater quantity of IgE bound by extract B relative to the other extracts would be consistent with extract B containing mite allergen fractions differing from those in other extracts or containing additional allergens contributed by the mite culture medium. The inhibition assay compares the allergenicity of the unknown with that of the standard extract (in this case extract L) bound to the solid phase. Thus, the superior performance of soluble extract L in the inhibition assay relative to its performance in the direct binding assay is not surprising. Additional factors may be involved as extract A had a similar slope to L in the inhibition assay and performed consistently in both assays. The relationship between flatter slopes and decreasing identity between the solid and soluble phase allergens has been previously demonstrated by Gleich *et al.* (1974).

The most likely cause of the changes in allergen content and slope after dialysis of extracts is the loss of low molecular weight allergen fractions. Such loss is most evident at low levels of allergen binding in the direct binding assay and at high levels of allergen in the inhibition assay. The loss of low molecular weight allergen or non-allergen fractions may have multiple effects on the functioning of the assays themselves. The loss may decrease the competition for binding sites and facilitate the binding of more mite allergen in the direct binding assay. This would be consistent with the greater slope of dialysed extracts in the direct binding assay. The lower slope of curves in the inhibition assay is consistent with the loss of allergen fractions. In addition it may be that low molecular weight allergen fractions can function as inhibitors in the soluble phase of the inhibition assay but are not capable of binding IgE after becoming bound to the disk in the direct binding assay. A loss of 50% allergen activity as

determined by RAST inhibitor assays has been reported by Dewdney *et al.* (1975), after dialysis of *D. pteronyssinus* extracts.

The existence and nature of the mite allergen fractions is still speculative. While it has been shown (Romagnani *et al.*, 1976) that allergenic activity is associated with fractions over a molecular weight range of 10,000–50,000, it was suggested on the basis of their crossreactivity in RAST inhibition assays, that these fractions contain a single determinant. On the other hand extensive diffusion studies, using antiserum raised in rabbits against mite antigens (Ricci, Biliotti & Romagnani, 1975) and the selective partial absorption of allergenic activity by the lectin tridacnin (Baldo, Turner & Uhlenbruek, 1976) support the concept of distinct mite allergenic determinants. The role of growth media, culture conditions, extraction process and storage on the production and content of possible mite allergen fractions are unknown.

Without a knowledge of the mite allergen fractions it is impossible to prepare a standard mite extract to use as the basis of comparison. Extract L was prepared from visibly clean freeze dried mites in an effort to obtain an extract containing only mite allergens. It is understood that commercial extracts are prepared from mites plus their culture medium. The possible contribution of non-mite allergens by culture medium could not be investigated without the cooperation of the manufacturers.

Although the mite allergen content of dust samples covered a wide range of 24 to 457 units of allergen/g, the mean of 129 units/g was comparable with the commercial mite extracts (mean of A, B and C is 112 units/ml) and confirms dust as a potent source of mite allergen. The quantity of allergen associated with 1 live mite, calculated from the extract prepared from freeze-dried mites, is 0.2 units/mite. This represents the maximum any mite would contain. If this principle is applied to the dust samples, nineteen of the twenty samples contain vastly more allergen than their respective numbers of live mites could contribute. It could be expected that the dead mites contain variable and considerably lower quantities of allergen than the live mites, yet five of the samples contain more allergen than a calculation based on the same contribution of allergen by both live and dead mites. Presumably the vast majority of activity resides invisibly in the dust itself. This is consistent with the experiment of Voorhorst *et al.* (1967) where considerable activity was obtained from a jar of powdered glass after mites which had previously been placed in the jar for several weeks had all been removed.

The lack of correlation in this study between mite numbers and allergen content in bedding dust was not surprising considering the unknown variables in synthesis and breakdown and the uncertainty of representative vacuum samples. However, two previous studies have demonstrated a relationship between the number of mites (dead or live not stated) and the total allergen content of the dust as demonstrated by skin test end point titrations. In the first case (Voorhorst *et al.*, 1967), a large number of samples ($n = 100$) of highly varied activity were involved. The second study (Miyamoto *et al.*, 1970) found a correlation between the total numbers of mites (thirty-six species were counted) in nine bulked dust samples obtained from different cities in Japan and Taiwan and the potency of the extracts prepared from the dust. This is the only reported study where *D. pteronyssinus* allergen itself has been assayed and related to mite numbers in dust containing almost exclusively *D. pteronyssinus* mites.

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