

The epitope stability of Group 1 and Group 2 allergens in mite extracts

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Background: Commercial mite allergenic extracts sold in the US are prepared with whole body mites in buffer solutions containing 50% glycerol. Mite extracts were reported to contain large number of proteolytic enzymes and their structural integrity in aqueous solutions have not been evaluated.

Objective: To evaluate the epitope stabilities of Group 1 and 2 allergens in two commercial mite extracts used by Center for Biologics Evaluation and Research (CBER) as reference extracts, E5-Dp (*Dermatophagoides pteronyssinus*) and E5-Df (*D. farinae*).

Methods: Epitope stability was determined by using monoclonal antibodies in a Sandwich ELISA. Samples were stored at four different temperatures and the amounts of Der p 1, Der p 2, Der f 1, and Der f 2 were determined at different time intervals. The overall stability of mite extracts was evaluated by immunoblot and competition ELISA.

Results: The epitope stability of these allergens varies: Der f 1 was stable for at least 3 years and Der f 2 for 1 year when stored at 4 °C; Der p 1 and 2 were less stable. None of the Group 1 and 2 allergens remained intact when stored at 50 °C. Immunoblot and competition ELISA data also showed similar trend of degradation as compared with extracts stored at 4 °C for same length of time.

Conclusion: With the exception of Der f 1, the amount of detectable epitopes in Group 1 and 2 allergens reduce rapidly after 1 year, especially at elevated temperatures. The changes in allergen composition were also observed by immunoblotting and in relative potency by ELISA competition assay. These findings are highly relevant to the users of CBER's mite extracts as standards.

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INTRODUCTION

House dust mites of genus *Dermatophagoides* are a major cause of indoor IgE-mediated hypersensitivity.^{1,2} Increased incidence of bronchial asthma and allergy, particularly in children, in industrialized countries has been correlated with increased mite-specific IgE contents in patients' sera.³ Two groups of major allergens, Group 1 and Group 2, have been identified and isolated from two species of mites, *Dermatophagoides pteronyssinus* and *D. farinae*, commonly found in the United

States.⁴⁻⁷ A number of monoclonal antibodies specific to each group of major allergens were prepared in a number of laboratories.⁸⁻¹¹ Using these antibodies, sensitive immunoassays were developed to determine the amount of mite allergens in allergenic extracts and indoor house dust.⁹⁻¹³

Recognizing the prevalence and severity of mite allergy worldwide, mite allergenic extracts were among the first extracts selected by the World Health Organization to be standardized. In 1988, reference mite extracts prepared from the whole bodies of *D. pteronyssinus* and *D. farinae* were selected by the Center for Biologics Evaluation and Research (CBER), US Food and Drug Administration to be used as reference standards and a RAST inhibition assay was used to compare the potency of manufacturers'

mite extracts to CBER's reference extract. Recently, a more sensitive and reproducible ELISA competition assay¹⁴ was developed to replace the RAST assay. These assays were based on the assumption that the reference extracts used as standards maintained their immunologic integrity through the course of their shelf-life which was arbitrarily determined to be 3 years. Mite extracts prepared from the whole bodies of mites were known to contain a variety of proteolytic enzymes¹⁵⁻¹⁷ which might cause degradation of proteins during storage. This study describes the assessments of the stability of Group 1 and 2 mite allergens by sandwich ELISA in CBER's reference mite extracts under different storage temperatures. The overall stability of mite extracts evaluated by their reactivities to IgE in a mite allergic serum pool was performed for comparison.

MATERIALS AND METHODS

Mite Extracts and Purified Allergens

All mite extracts used for CBER reference standards (E4-Dp, E5-Dp, and E5-Df) in this study were purchased from commercial manufacturers of allergenic extracts. These extracts were prepared from whole bodies of cultured mites in Coca's buffer (0.086 M NaCl, 0.033 M NaHCO₃) containing 50% glycerol. The Center for Biologics Evaluation and Research reference standards were selected based on their immunoreactivities to serum from allergic patients. Purified Der f 1 and Der f 2 were gifts from Dr Mei-chang Kuo (ImmuLogic Pharmaceutical Corp, Cambridge, MA) and Dr Y Okumura¹⁸ (Asahi Breweries, LTD, Japan), respectively (Table 1). Der f 1 was purified from whole bodies of mites by ion-exchange chromatography; Der f 2 was purified from a cDNA clone ex-

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Table 1. Allergens and Monoclonal Antibodies (mAb) for Sandwich ELISA

Allergen	Natural/Recombinant	Name of mAb	Specificity of mAb
Der f 1	Natural*	6A8-B10 † 4C1-B8-3F8‡	Der f 1 Der f 1 & p 1
Der f 2	Recomb§	15E11 13A4	Der f 2 Der f 2, p 2
Der p 1	Natural	P1A03 P1A01	Der p 1 Der p 1
Der p 2	Natural	1D8-C4-D8 7A1	Der p 2 & f 2 Der p 2 & f 2

* Isolated from cultured mites.

† mAb in bold was used as capturing antibody.

‡ mAb in italic was used as detecting antibody.

§ Prepared by recombinant DNA technology.

pressed in *E. coli*.¹⁸ Der p 1 and Der p 2 are gifts from Dr H Yasueda (National Sagamihara Hospital, Japan). These proteins were also purified from whole body extracts of mites by ammonium sulfate precipitation and ion-exchange and sizing chromatography.⁶ Protein concentrations of these allergens were determined by quantitative amino acid analysis.¹⁹

For ELISA competition assay and immunoblot, CBER reference serum pool was used. The Center for Biologics Evaluation and Research mite serum pool (CBER reference serum S3-Dpf, provided by allergenic Product Testing Lab, CBER, FDA) was collected from a group of individuals who are highly allergic to mites.

Monoclonal Antibodies and Biotinylation of Monoclonal Antibodies

Der f 1 quantitation kit and monoclonal antibodies for quantitation of Der p 2 were purchased from Health Science

Center of the University of Virginia.^{9,20} Der p 1 monoclonal antibodies were gifts from Dr H Yasueda (National Sagamihara Hospital, Japan)¹⁰ and Der f 2 monoclonal antibodies were gifts from Dr Y Okumura (Asahi Breweries, LTD, Japan).¹¹ The source and specificities of the monoclonal antibodies are listed in Table 1. With the exception of Table 2, the monoclonal antibodies listed in Table 1 were used for the entire study.

Biotinylation of the second antibody for Der f 2 (13A4) was performed using Sulfo-NHS-Biotin according to the supplier's instruction (Pierce Chemicals). Briefly, 16 µg of biotin was added to 1 mg of antibody in 50 mM sodium bicarbonate buffer, pH 8.5 and incubated at room temperature for one hour. The unreacted biotin was removed by centrifugation using a Centricon-30 Microconcentrator (Amicon) at 1000 g for 30 minutes. The concentrated sample was diluted in 0.1 M

sodium phosphate buffer, pH 7.0, and the process was repeated two more times. The biotinylated monoclonal antibody was in a final buffer of 0.1 M phosphate, pH 7.0, in a concentration of about 20 mg/mL of protein. Other monoclonal antibodies were biotinylated by the suppliers.

Sandwich ELISA

Microtiter plates (Costar E.I.A. plate, Costar) were coated with 2 to 10 µg/mL of monoclonal antibodies overnight at 4 °C. Different amounts of purified mite allergens (for standard curve) or mite allergenic extracts were added to monoclonal antibody-coated microtiter plates, incubated for at least one hour at room temperature, then washed with phosphate buffered saline containing 0.05% Tween-20 (PBS-T), and incubated with biotinylated second monoclonal antibody for another hour. After washing and a final one hour incubation, at room temperature, with streptavidin-labeled peroxidase (KPL, Gaithersburg, MD), the substrate (2,2'-azino-di-[3-ethyl-benzthiazoline sulfonate], KPL) was added for color development. The reaction was stopped by adding 1% sodium dodecyl sulfate (SDS) and the absorbance at 410 µm was measured. The amounts of mite allergens in the allergenic extracts were calculated by comparison to the standard curve.

SDS-PAGE and Immunoblot

Novex precast 16% acrylamide gels were used for gel electrophoresis. Samples were treated with SDS and β-mercaptoethanol prior to loading onto the gel.

Table 2. Comparison of Group 1 and 2 Allergens in CBER's Mite Reference by Different Laboratories Using Different Monoclonal Antibodies*

	Der f 1 (µg/mL)		Der p 1 (µg/mL)		Der f 2 (µg/mL)		Der p 2 (µg/mL)		
	F1B01	6A8-B10	4C1	P1A03	15E11	F2A41	1D8	7A1	P2A24
E5 Df	9.5†	10.1 ± 1.0§			7.0 ± 0.7§	7.1‡			
E4 Dp			12†	11.0 ± 1.1§			40.0 ± 5.0§	13.6 ± 1.3§	9.0 ± 0.9§
E5 Dp			24†	20.7 ± 1.5§			65.0 ± 10.1§	21.0 ± 3.2§	11.4 ± 1.5§

* Source of monoclonal antibodies: 6A8-B10, 4C1, 1D8 and 7A1 from Dr. Chapman, University of Virginia; F1B01, P1A03, F2A41, and P2A24 from Dr. Yasueda, Japan. 15E11 from Dr. Okumura, Japan.

† Published data from other laboratories: Der f 1, Ref. #12, Der p 1, Ref. #13.

‡ Dr. H. Yasueda's personal communication.

§ Values from our laboratory are average of at least three determinations.

Proteins were transferred onto polyvinylidene difluoride membranes (Pharmacia) in 3-(cyclohexylamino)-1-propanesulfonic acid buffer at 35V for one and one-half hours. Membranes were first blocked with 1% bovine serum albumin in PBS-T for one hour and incubated in a serum pool collected from mite allergic patients (1:10 in PBS-T) at 4 °C overnight. After washing three times in PBS-T, phosphatase-labeled, goat anti-human IgE (KPL) was added. Incubation was continued at room temperature for one hour. Membranes were washed 3 times with alkaline phosphatase buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl₂) and reacted with phosphatase substrate 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue-tetrazolium (KPL) until reactive bands were clearly visible.

Competition ELISA for Relative Potency Determinations

Relative potency determinations were performed according to CBER ELISA Competition Assay protocol¹⁴ using CBER's Reference Extracts and serum pool as standard reagents.

Stability of CBER Reference Mite Extracts

The stability of Group 1 and 2 mite allergens in CBER Reference Extracts, E5-Dp (*D. pteronyssinus*) and E5-Df (*D. farinae*) was determined by sandwich ELISA using the monoclonal antibodies listed in Table 1. At time zero, CBER reference, E5-Df, contains 10.1 µg/mL and 7.0 µg/mL of Der f 1 and f 2 respectively; E5-Dp, contains 20.7 µg/mL and 85.5 µg/mL of Der p 1 and

p 2 respectively. Mite extracts were stored at 4 °C and room temperature (average 26 °C), 37 °C and 50 °C. The two higher storage temperatures 37 °C and 50 °C, were included for accelerated degradation studies. Der f 1, Der f 2 or Der p 1, Der p 2 contents at different time intervals were determined with Sandwich ELISA. A comparison of allergen contents was made for E5-Dp stored at 4 °C and stored at -20 °C.

RESULTS

Comparison of Different mAbs Used for Allergen Content Determinations

Table 2 compares the allergen contents in mite extracts (E4-Dp, E5-Dp and E5-Df) as determined by our laboratory and other laboratories using dif-

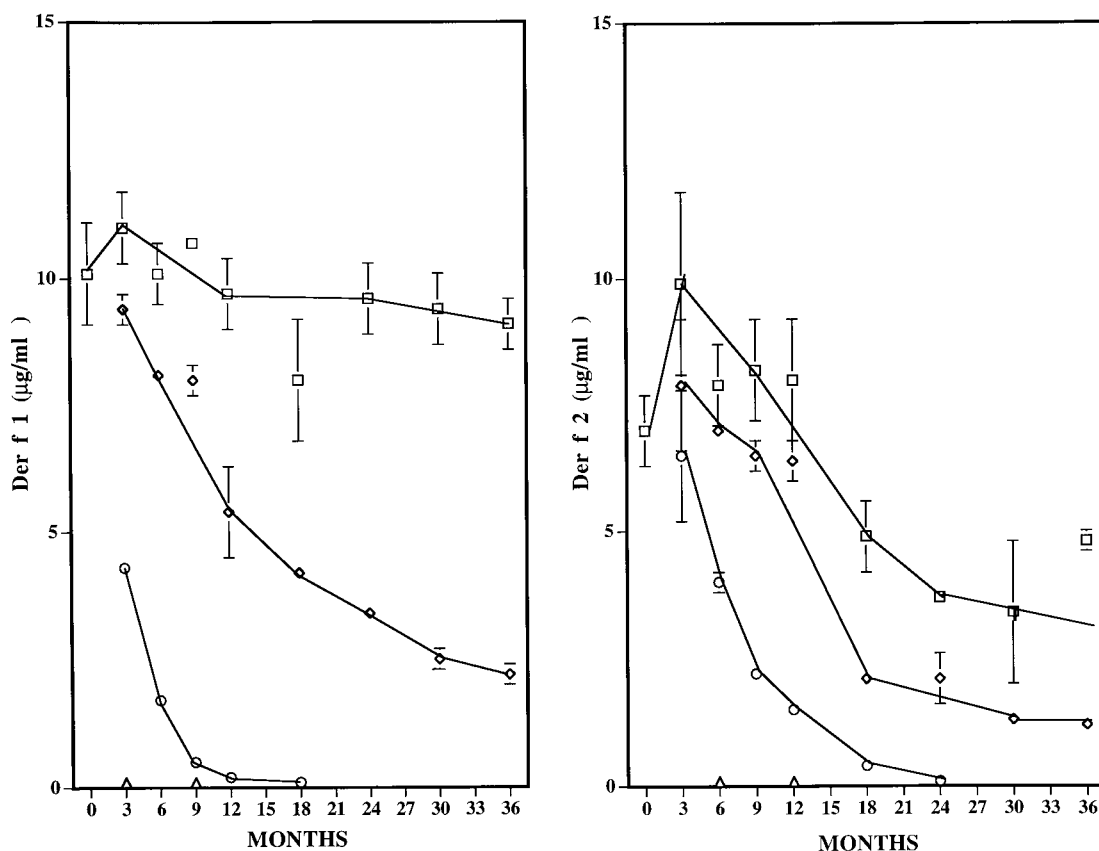


Figure 1. Group 1 and 2 allergen contents in CBER reference extract of *Dermatophagoides farinae* (E5-Df). Extract was stored at four different temperatures for up to 36 months. The amounts of Der f 1 and Der f 2 were determined by sandwich ELISA using purified allergens (as standards) and monoclonal antibodies listed on Table 1. At zero time, Der f 1 and Der f 2 were determined to be 10.1 ± 1.0 µg/mL (n = 30) and 7.0 ± 0.7 µg/mL (n = 40). (□), 4 °C; (◇), 26 °C; (○), 37 °C; and (△) 50 °C.

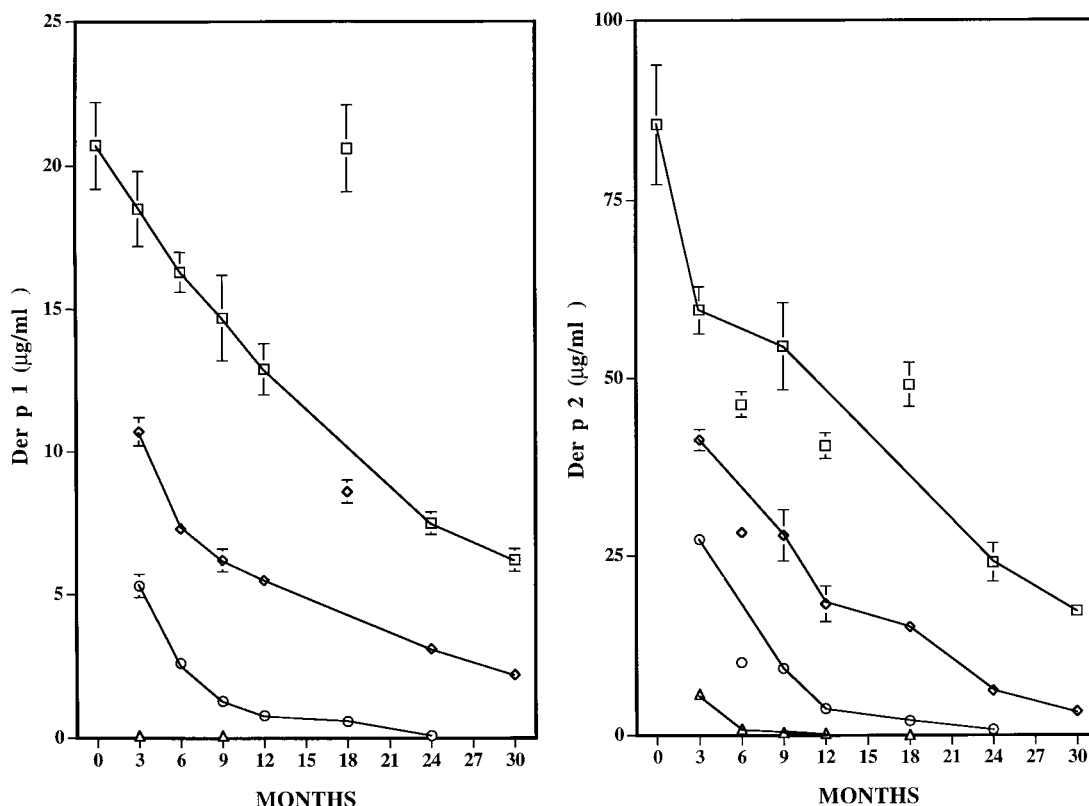


Figure 2. Group 1 and 2 allergen contents in CBER reference extract of *Dermatophagoides pteronyssinus* (E5-Dp). Extract was stored at four different temperatures for up to 30 months. At each time point, the amounts of Der p 1 and Der p 2 were determined by sandwich ELISA using purified allergens (as standards) and monoclonal antibodies listed on Table 1. At zero time, Der p 1 and Der p 2 were determined to be $20.7 \pm 1.5 \mu\text{g/mL}$ and $85.5 \pm 8.3 \mu\text{g/mL}$ respectively. (□), 4 °C; (◇), 26 °C; (○), 37 °C; and (△) 50 °C.

ferent monoclonal antibodies. These results showed that similar results were obtained for Der f 1, Der f 2, and Der p 1 when two different sets of antibodies were used by different laboratories in the sandwich ELISA. For Der p 2, there was a 3-fold difference in the allergen concentration when two mAbs against Der p 2 prepared from the same laboratory were used as capturing antibodies.

Stability of Group 1 and 2 Mite Allergens in CBER Reference Extracts

At different time intervals, the major allergen contents were determined for the reference mite extracts stored at different temperatures (Figs 1 and 2). Using the monoclonal antibodies listed in Table 1, the allergen contents in these extracts decreased at a relatively fast pace in the presence of 50% glyc-

erol. Der f 1 was stable for 3 years and Der f 2 was stable for 1 year when stored at 4 °C. Both Der f 1 and Der f 2 were relatively stable at room temperature for 9 months but started to break down after 12 months. Der p 1 and Der p 2 were not stable at any of the storage conditions, although they degraded more slowly at 4 °C as compared with higher temperatures. None

of the Group 1 and 2 allergens retained their integrity at 50 °C.

A comparison was made for extracts E5-Dp stored at 4 °C and -20 °C (Table 3). After 30 months of storage, degradation of major allergens occurred even at -20 °C. As expected, degradation occurred at a slower rate at -20 °C as compared with that stored at 4 °C.

Table 3. Der p 1 and Der p 2 Contents in E5-Dp After 30 Months of Storage at 4 °C and -20 °C

Reference Extract	Mite Allergen	Allergen Concentration (µg/mL)		
		At Zero Time	After 30 months	
			4 °C	-20 °C
E5-Dp	Der p 1	20.7*	6.3	11.6
	Der p 2	85.4	15.0	30.0

* Average of at least three determinations.

Immunoblot of Mite Allergens Stored at Different Temperatures

Immunoblotting data (Fig 3A and B) showed a degradation pattern similar to that of the sandwich ELISA results. After 9 months at 50 °C, neither Group 1 nor 2 allergens were detectable by immunoblot. Degradation for most proteins began as early as 9 months of storage, especially at higher temperature for E5-Df and E5-Dp. Proteins were more stable at 4 °C, but degradation became obvious when sample was stored at higher temperatures.

Relative Potency of Mite Extracts

The reference extract used for relative potency determination was the sample (E5-Df or E5-Dp) stored at 4 °C for the same length of time. For example, at the 24-month time point, E5-Dp stored at 4 °C for 24 months was used as reference; samples stored at different temperatures for 24 months were compared with the 4 °C sample. The standard deviation of this method is approximately $\pm 30\%$. These results (Table 4) indicated that relative potency decreased with increasing stor-

age temperatures, but the decrease in potency was about the same for samples stored at 4 °C and those stored at 26 °C.

DISCUSSION

Mite extracts contain a number of proteolytic enzymes^{15,16} that could cause degradation of allergens during storage. All mite extracts sold in the US market contained 50% glycerol and were stored at 2 to 8 °C prior to shipping. Each lot of mite extract prepared by manufacturers was tested against a CBER reference extract to determine its relative potency assuming the potency of the CBER reference was 100%. It is thus crucial to monitor the integrity of the allergens in the CBER reference extracts by a most sensitive method available. Group 1 and 2 allergens in mites were shown to react to >90% of mite-allergic patients and accounted for 75% of mite-allergic serum IgE reactivities.⁴⁻⁷ The availability of both purified mite allergens and monoclonal antibodies against these allergens provide the tools to monitor the epitope stability of these allergens in mite extracts. The observation from this study indicated that the integrity of Der p 1 and 2 and Der f 2 in E5-Dp and E5-Df were compromised after 1 year of storage at 4 °C in 50% glycerol. This result was further corroborated by the immunoblots of the extracts which showed the compositional change of the extract upon storage. At this point, there is no clinical data to determine whether the epitope stability of Group 1 and 2 allergens correlates with the clinical effectiveness of these extracts. Relative potency determinations, using allergic serum, seemed to indicate that some degree of correlation exists between the stability of these allergens and the IgE reactivities to the extracts. Relative potency determinations give a global reactivity assessment based on the presence of mite IgE in the serum pool. Such a method is not as sensitive in detecting subtle changes in the integrity of a specific group of epitopes defined by monoclonal antibodies.

Table 4 shows that there is no detectable difference in potency between

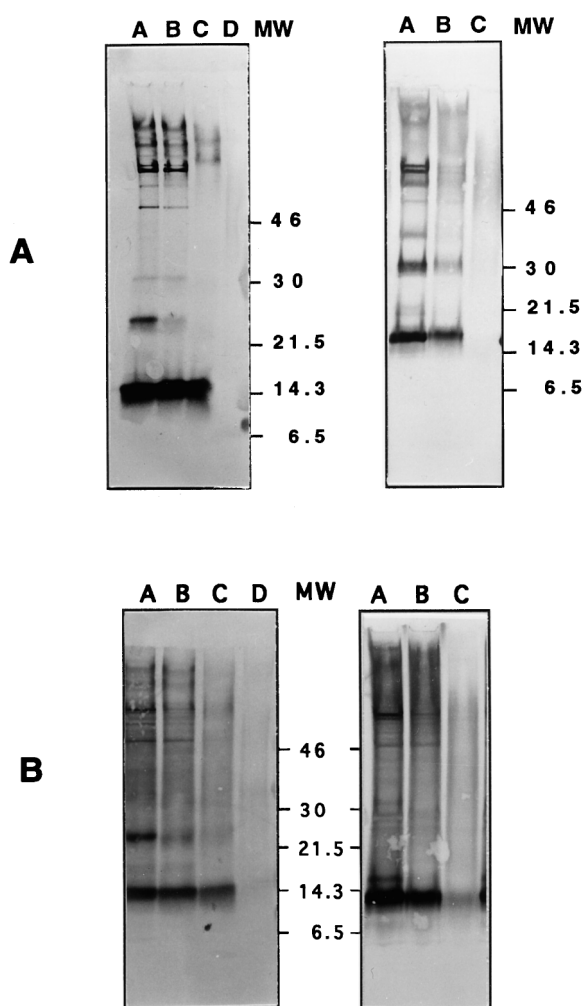


Figure 3A. Immunoblots of CBER mite reference extracts (E5-Df) after storage for 9 (left panel) and 36 (right panel) months at different temperatures. Lane A, 4 °C; Lane B, room temperature; Lane C, 37 °C; and Lane D, 50 °C. (3B) Immunoblots of CBER mite reference extracts (E5-Dp) after storage for 9 (left panel) and 30 (right panel) months at different temperatures. Lane A, 4 °C; Lane B, room temperature; Lane C, 37 °C; and Lane D, 50 °C. Molecular weight for Group 1 allergen is 25 kD and for Group 2 allergen is 14 kD.

Table 4. Relative Potencies of Mite Extracts Stored at Different Temperatures

Extract	Storage	Relative Potency			
		4 °C	26 °C	37 °C	50 °C
E5-Df	6 months	1*	0.9	0.7	0.1
E5-Dp	6 months	1	0.9	0.6	0.1
	24 months	1	1.0	0.3	
	30 months	1	0.9		

* Sample stored at 4 °C was used as reference and assigned as 1.

extracts stored at 4 °C and 26 °C for the same period of time. The degradation, however, was detectable by immunoblotting which also depended upon the reactivity of mite-specific IgE present in the allergic serum pool. Currently, all CBER's mite reference extracts are stored at -20 °C. Storage at -20 °C helped to maintain the reactivities to monoclonal antibodies, but did not completely preserve the integrity of the epitopes in E5-Dp (Table 3). The results of this study suggest that it is essential to store the mite extracts at low temperatures and preferably in small aliquots to slow down the possible degradations.

Due to the amount of available reagents, only two lots of mite extracts used by CBER as reference reagents were used in this study. Nevertheless, two lots used in this study (E5-Dp and E5-Df) were purchased from two different manufacturers and represent two different species of mites. Data from other lots of mite extracts are needed to assess the stability of mite extracts prepared under current manufacturing conditions.

Group 1 and 2 allergen contents determined in this report and those from a number of other laboratories^{12,13} were comparable with the exception of Der p 2 (Table 2). Two monoclonal antibodies against Der p 2, 1D8 and 7A1, were prepared by Health Science Center, University of Virginia. When 7A1 was used as the capturing antibody, a value of 21.0 µg/mL of Der p 2 for E5-Dp was obtained; when 1D8 was used as the capturing antibody, a value of 65.0 µg/mL was obtained. The cause for such a discrepancy remains unclear and needs further investigation.

Interestingly, an early study²¹ indicated that there was no significant reduction in overall activity of patients when Der p 1 was depleted from the extract. With the availability of in vitro assays applicable to determine the composition of allergenic extract, it is essential that their clinical relevance be evaluated. From the standardization point of view, a sensitive method, such as the sandwich ELISA with mAbs, can provide the assurance of the integrity of the reference reagents which are used by the industry to calibrate their production lots.

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