

The stability of house dust mite allergens in glycerinated extracts

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Background: Mite allergen vaccines are important diagnostic and immunotherapeutic reagents. Previous studies on mite allergen stability under different storage conditions have yielded contradictory results.

Objective: We sought to compare, over a 12-month period, the stability of mite allergens reconstituted in 50% glycerol and stored at different temperatures and to examine the role of protease inhibitors in enhancing allergen stability.

Methods: Lyophilized allergen extracts were reconstituted in 50% glycerol, with and without protease inhibitors, and stored at -70°C, -20°C, 4°C, or 37°C for 12 months. At 6 and 12 months, the extracts were compared with freshly dissolved extracts by competition ELISA with pooled allergic sera, 2-site ELISA with mite-specific mAbs, and immunoblot analyses.

Results: The overall potencies of the stored extracts measured by competition ELISA were stable at -20°C and 4°C. As determined by means of the immunoblot and 2-site ELISA, Der f 1 levels decreased at 4°C. Levels of Der f 2, Der p 1, and Der p 2 decreased in at least one of the allergen-specific assays. Storage at 37°C led to overall loss of potency and allergen content, whereas storage at -70°C was associated with a moderate loss of potency that increased with multiple freeze-thaw cycles.

Protease inhibitors had no effect on allergen stability.

Conclusion: Although overall potency of the extracts, as measured by competition ELISA, was preserved at -20°C and 4°C, allergen-specific assays indicated loss of allergens. These findings suggest that the competition ELISA is insensitive to decreases in the concentrations of individual allergens. (J Allergy Clin Immunol 2000;105:482-8.)

Key words: Dust mite, monoclonal antibody, allergen, ELISA, immunoblot, stability, allergy, allergen extract

The protein allergens of 2 species of house dust mite, *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*, can cause severe allergic disease in sus-

Abbreviations used

CBER: Center for Biologics Evaluation and Research

CI: Confidence interval

RP: Relative potency

ceptible individuals.¹ Allergen extracts prepared from cultured dust mites have been used to diagnose and, in selected individuals, to treat dust mite allergy.² Because the clinical manifestations of mite allergy can be ameliorated or even cured with mite reduction methods or the administration of specific mite immunotherapy, it is important that available mite extracts be well characterized and potent.

Since 1988, the commercial extracts of these mite cultures have been standardized in the United States by comparison with a reference standard chosen and maintained at the Center for Biologics Evaluation and Research (CBER) at the Food and Drug Administration. Although the initial standardization was performed by skin test titration, these results were subsequently shown to correlate closely with in vitro techniques, such as RAST inhibition and competition ELISA.³

Allergen stability, the persistence of adequate quantities of relevant antigens in an allergen vaccine from the time of initial assay to the time of clinical use, is enhanced by temperature control and by maintaining certain concentrations of protein, glycerol, or both in the vaccine solution.^{4,5} At least 10 allergens have been isolated and characterized from each mite species, and many of the allergens in one species are homologous to the corresponding allergen in another.⁶ It is not certain which of these allergens is most important in maintaining an adequate and safe therapeutic and diagnostic vaccine. Several of these proteins appear to be highly immunoreactive in human subjects and animals, with evidence of specific IgE to the individual allergens in up to 80% of individuals allergic to mites.⁷ Four of these allergen groups (1, 3, 6, and 9) are proteolytic enzymes.

Several investigators have examined the stability of mite allergens in commercial vaccine preparations,^{4,5,8-11} with varying results. In 1998, Liu and Lin¹¹ determined that the relative potency (RP) of glycerinated mite extracts, as determined by competition ELISA, were stable at 26°C compared with those stored at 4°C. However, the specific content of the group 1 and group 2 allergens, as measured by an mAb-based 2-site ELISA, was highly variable. In their investigations the extracts stored at 4°C served as the controls. We initiated the following studies to determine the RP and allergen content of mite

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extracts stored at varying temperatures over a period of 12 months compared with lyophilized controls that were placed in solution a few hours before the assay. In addition, because protease activity is one likely mechanism for allergen degradation,¹²⁻¹⁴ we investigated the effect of protease inhibitors on the stability of RP and specific allergen content.

METHODS

Mite extracts

The mite extracts used in this study included two lots of *D pteronyssinus* (JE 321 and JE 343) and two lots of *D farinae* (JE 318 and JE 312). These extracts were prepared from freeze-dried whole-body mites and were provided by the Bayer Corporation for these investigations. *D pteronyssinus* JE 321 and *D farinae* JE 312 were extracted in 50 mmol/L ammonium bicarbonate; *D pteronyssinus* JE 343 and *D farinae* JE 318 were extracted in glycerinated Coca's solution (43 mmol/L sodium chloride, 15 mmol/L sodium bicarbonate, 0.2% wt/vol phenol, and 50% vol/vol glycerol). All of the samples were dialyzed and concentrated by diafiltration by using an Amicon CH2PRS apparatus equipped with 3000-d cut-off, hollow fiber cartridges and lyophilized in the presence of 2% (wt/vol) mannitol. *D pteronyssinus* JE 343 was defatted before extraction.

Storage conditions of extracts

On receipt of the lyophilized extracts, representative vials from each lot were either stored at -70°C or were reconstituted in 50% (vol/vol) glycerol. The reconstituted samples were placed in aliquots and stored in sealed cryotubes (Nunc), with or without added protease inhibitors (Complete Mini Protease Inhibitor Cocktail, Boehringer Mannheim), at -70°C , -20°C , 4°C , and at 37°C . Individual aliquots were prepared for each timed sample at each storage temperature.

Competition ELISA for the determination of relative potency

Lyophilized extracts were reconstituted in 50% (vol/vol) glycerol and allowed to go into solution overnight at 4°C . The CBER competition ELISA protocol was used to determine RP (Laboratory of Immunobiochemistry-Standard Operating Procedure-7, revised November 1998). CBER reference serum S3-Dpf was used as the detection serum. A standard curve was generated by using the freshly reconstituted lyophilized allergen.

Immunoblots developed with the allergic human serum pool

The proteins in the allergen extracts were separated by means of SDS-PAGE by using precast 16% gels (Novex) after denaturation in sample buffer containing 5% (vol/vol) β -mercaptoethanol. The proteins in the gel were transferred to a nitrocellulose membrane (Protran, Schleicher & Schull) in the 6-month study and to a polyvinylidene difluoride membrane (ProBlott, Applied Biosystems) in the 12-month study. The membranes were blocked, incubated in sequence in S3-Dpf and phosphatase-labeled goat anti-human IgE (1 $\mu\text{g/mL}$ in PBS with Tween-20; Kirkegaard & Perry), and developed with 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazoleum (Kirkegaard & Perry).

Two-site ELISA for group 1 and group 2 mite allergens

The mAbs and allergen standards used in this assay were purchased from Indoor Biotechnologies, and the assay was performed

according to the manufacturer's instructions. The group 2 allergens were captured with mAb 1D8, and the group 1 allergens were captured with mAbs 5H8 and 6A8 for Der p 1 and Der f 1, respectively. Antigens were detected with biotinylated 7A1 (group 2 allergens) or biotinylated 4C1 (group 1 allergens). The data are expressed as micrograms per milliliter of allergen by comparison with the standard or as allergen-specific RP. The allergen-specific content of each extract was compared with the content of the freshly dissolved sample. The quotient of the allergen content of an incubated extract and its freshly dissolved lyophilized control is the allergen-specific RP, and these were compared with the RPs that were determined with the pooled allergic sera.

Immunoblots developed with mAbs

The mAb immunoblot was performed in the same manner as the allergic human serum pool immunoblot. The antibody used to detect group 2 allergens was biotinylated 7A1 (1:1000 vol/vol in 1% BSA/PBS), and the antibody used to detect group 1 allergens was biotinylated 4C1 (1:1000 vol/vol in 1% BSA/PBS). These antibodies were then detected with phosphatase-labeled streptavidin (0.5 $\mu\text{g/mL}$ in 1% BSA/PBS; Kirkegaard & Perry) and developed with 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazoleum.

Statistical analyses

For the measurements of RP, equivalence to the reference was determined at the 95% confidence level, assuming the previously established SD of the assay ($\sigma = 0.1375$ in $\log[\text{RP}]$). Consequently, a sample with an RP that bracketed where N is the number of replicates was considered equivalent. N was typically 3, leading to the RP range of 0.699 to 1.43. For determinations of potency by using the 2-site ELISA, the 95% confidence intervals (CIs; $x \pm 1.96 \sigma/\sqrt{N}$) were determined by using a pooled σ value of 17 $\mu\text{g/mL}$. For comparisons of the RP determined by using the 2-site ELISA with the RPs measured with the pooled allergic sera, significance was tested by using *t* tests with equal and unequal variance and by using the Wilcoxon-Mann-Whitney ranks test.¹⁵

RESULTS

Competition ELISA for the determination of relative potency

As shown in Fig 1, the RPs of each of the 4 mite extracts stored for 6 and 12 months at -20°C and 4°C were statistically equivalent to 1.0 (Fig 1). When the RPs of the *D pteronyssinus* extracts are pooled and compared with the corresponding RPs of the *D farinae* extracts, the RPs are statistically indistinguishable from each other (95% CIs of 0.59-1.21 compared with 0.73-1.50 for -20°C and 0.53-1.09 compared with 0.71-1.46 for 4°C). In contrast, the RPs of each of the same 4 extracts stored at 37°C significantly decreased over the 12-month study in comparison with the lyophilized counterparts, with RPs ranging from 0.009 (95% CI, 0.006-0.012) to 0.374 (95% CI, 0.262-0.535). The RPs of samples stored at -70°C were determined at 12 months for all 4 extracts, but the RP of one extract (JE 312) was determined at 6 months as well. For those extracts tested only at 12 months, the RPs were either not significantly different (JE 318) or slightly decreased (JE 321 and JE 343) from those of the lyophilized control. On the other hand, the -70°C sample of JE 312, which was thawed and measured at both the 6- and 12-month time points, had an RP

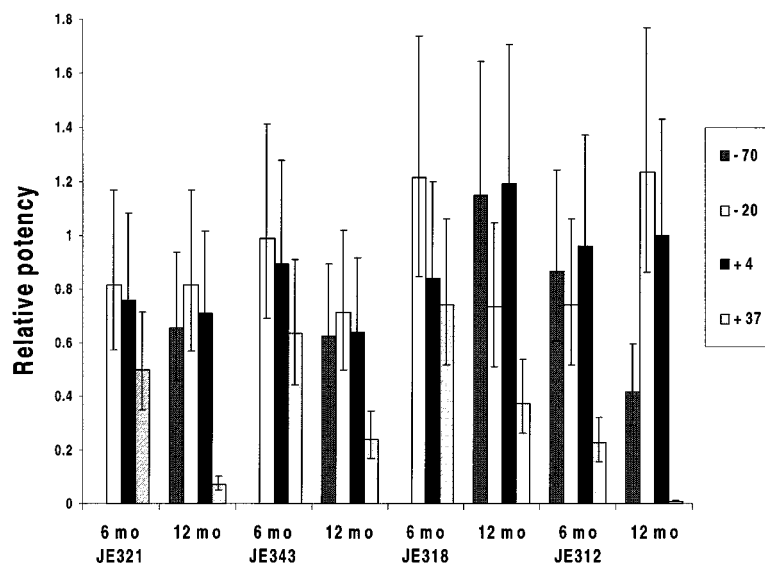


FIG 1. The RPs of stored mite extracts were determined by means of competition ELISA with pooled allergic sera, comparing the stored extracts to freshly reconstituted reference material (RP, 1.0). The storage temperatures were -70°C , -20°C , 4°C , and 37°C . Bars represent the average values of RP for 3 parallel ELISA plates. Error bars indicate 95% CIs of each set of data. The 95% CI of reference extract ranges from 0.699 to 1.43 when $N = 3$.

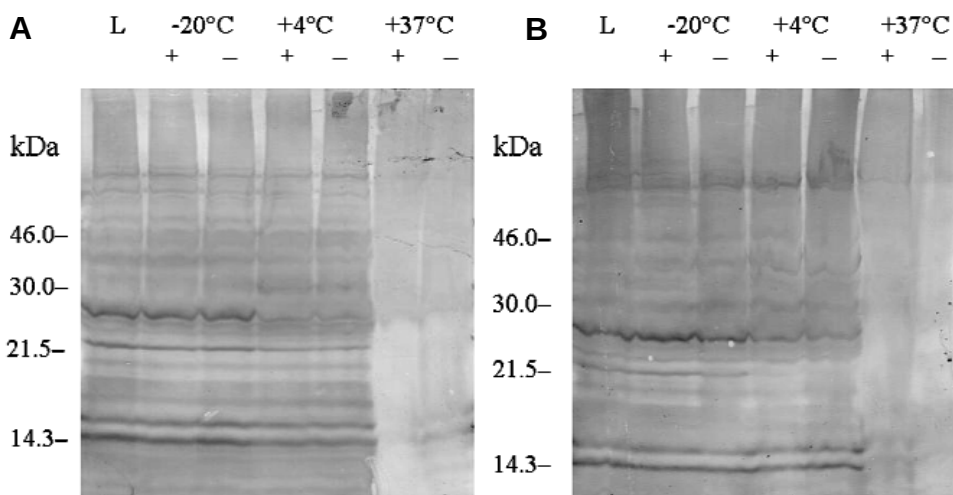


FIG 2. Immunoblots of mite extracts separated by SDS-PAGE on a 16% polyacrylamide gel, transferred to a nitrocellulose membrane, and developed with pooled allergic sera (6-month study). Storage temperatures were -20°C , 4°C , and 37°C . **A** shows extract JE 318, and **B** shows extract JE 312. L, Lyophilized extract; +, extracts with added protease inhibitors; -, extracts without added protease inhibitors.

of 0.416 (95% CI 0.291–0.596), presumably because of the extra freeze-thaw cycle to which it had been subjected.

Immunoblots developed with the allergic human serum pool

Immunoblots of the 4 mite extracts were performed at the 6- and 12-month time points by using the allergic serum pool. Representative immunoblots appear in Fig 2; other data are summarized in Table I. These immunoblots showed that most of the allergens were well preserved at

-20°C but not at 4°C . In particular, the 25-kD bands corresponding to Der f 1 (in JE 312 and JE 318) and Der p 1 (in JE 343) decreased in intensity at 6 months at 4°C (Fig 2 and Table I). In addition, an unidentified *D farinae* 20-kD protein band disappeared at 6 months in JE 312 (Fig 2).

The 12-month study confirmed the selective loss of group 1 allergens at 4°C in 2 of 4 of the extracts examined (JE 312 and JE 343, Table I). In addition, the 12-month study showed instability of Der p 2, as well as 2 other proteins, at about 13 and 18 kD at 4°C in JE 321.

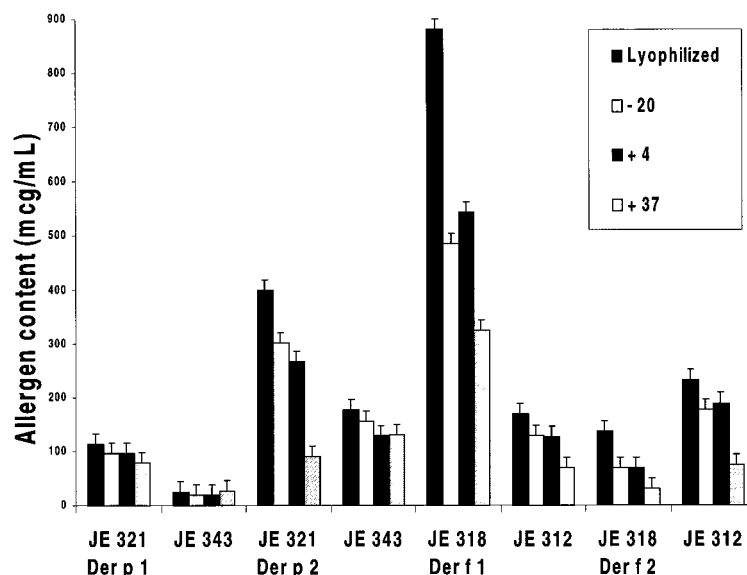


FIG 3. Group 1 and group 2 allergen content of mite extracts was determined by using 2-site ELISA with monoclonal capturing antibodies and biotinylated monoclonal detection antibodies (6-month study). Storage temperatures were -20°C , 4°C , and 37°C . Bars represent the average values of allergen content of extracts incubated at different temperatures in multiple parallel microtiter plates; error bars indicate 95% CIs based on a pooled SD of $17\text{ }\mu\text{g/mL}$.

TABLE I. Degradation of the group 1 and group 2 mite allergens at 4°C detected on immunoblots with human serum pool

	JE 318(Df)		JE 312(Df)		JE 343(Dp)		JE 321(Dp)	
	6 mo	12 mo	6 mo	12 mo	6 mo	12 mo	6 mo	12 mo
Group 1	++	±	++	+	+	++	±	—
Group 2	—	—	—	—	—	—	—	+
Comments and observations	Loss of 20-kd band						Loss of 13, 18, and >46-kd bands	

Df, *D farinae*; Dp, *D pteronyssinus*; ++, significant degradation; +, some degradation; —, no observed degradation.

High molecular weight proteins (>46 kd) also appeared to degrade at 4°C in JE 321 (Table I).

Two-site ELISA for group 1 and group 2 mite allergens

The group 1 and group 2 mite allergen content in each of the 4 different mite extracts was determined by using 2-site ELISA with specific mAbs. At 6 months, significant decreases in the levels of Der f 1, Der f 2, and Der p 2 occurred in some of the samples stored at -20°C and 4°C compared with the lyophilized freshly dissolved sample (Fig 3). The 12-month data (not shown) were consistent with the 6-month data.

The scatterplot in Fig 4 compares the allergen-specific RP with the RPs determined with the pooled allergic sera. This plot contains two striking features. First, there is little correlation between the two quantities. For example, the pooled sera RPs of the *D farinae* extracts range from 0.42 to 1.24, whereas the 2-site RPs only range from 0.5 to 0.8. A linear fit, including all the data, yields

a slope that is not statistically significantly different from zero. Second, the mean RPs (2-site), 0.93 for *D pteronyssinus* and 0.63 for *D farinae*, differ significantly. Statistical significance was established by using *t* tests with equal and unequal variance, and the Wilcoxon-Mann-Whitney ranks test for the 6-month, 12-month, and pooled 6- and 12-month data sets. In no case was the *P* value greater than .05, and in most cases the *P* value was less than .01. Hence Der f 1 and Der f 2 appear to degrade more rapidly than Der p 1 and Der p 2, even though the RPs of the extracts, as measured with pooled allergic sera, degrade at approximately the same rate.

Immunoblots developed with mAbs

To study further the degradation of the individual allergens, immunoblots were performed with group 1- and group 2-specific mAbs. Representative immunoblots appear in Fig 5; additional data are summarized in Table II.

Immunoblots of the *D farinae* mite extracts stored for

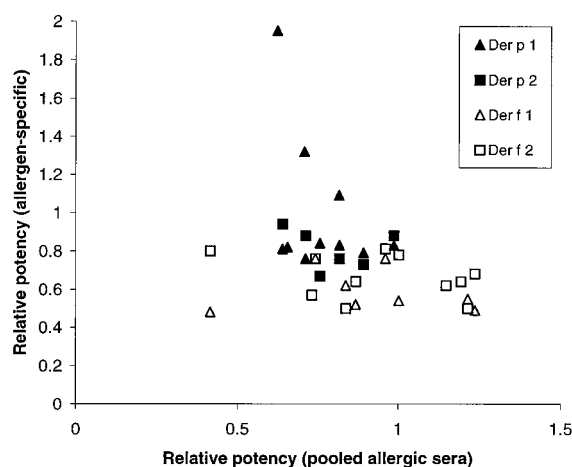


FIG 4. RPs of stored mite extracts were compared with allergen-specific RPs, which were calculated by dividing the allergen content of the stored extract by the allergen content of the freshly reconstituted control. Six-month and 12-month data are shown.

12 months and developed with anti-group 1 mAbs indicate some loss of Der f 1 allergen (migrating with an apparent molecular weight of 22 kd in this experiment) in the JE 312 extract stored at 4°C, which is concurrent with the appearance of a new high molecular weight (>46 kd) protein band (Fig 5, A). In addition, there is loss of a 33-kd protein (presumably group 3 allergen⁶) at 4°C in each of the two *D farinae* extracts JE 312 and JE 318 and the appearance of a new immunoreactive band at approximately 27 kd in JE 312 (Fig 5, A, and Table II). Immunoblots of the same *D farinae* mite extracts developed with anti-group 2 antibodies show no degradation of Der f 2 allergen (at about 15 kd) at 4°C in either extract; however, several new immunoreactive protein bands, migrating at approximately 27 kd and above, appear in extract JE 312 stored at 4°C (Fig 5, B, and Table II).

Immunoblots of the *D pteronyssinus* extracts developed with the anti-group 1 antibodies suggest the degradation, in extract JE 321, of an unidentified immunoreactive protein band at 17 kd after 12 months at 4°C, which is concurrent with the appearance of new protein bands with apparent molecular weights at 33 kd and greater than 46 kd (Table II). In sample JE 343 stored at 4°C, additional immunoreactive bands appear at 17 and 28 kd. When immunostaining is performed with anti-group 2 antibodies, there is no obvious degradation of Der p 2 at 4°C. However, the Der p 1 band decreases at 4°C in JE 321, with the appearance (in JE 321) or loss (in JE 343) of the unidentified immunoreactive bands over 46 kd (Table II).

Effect of protease inhibitors on relative potency and allergen content

To study the potential prevention of allergen degradation in the mite extracts, we added a cocktail with broad protease inhibitor activity to the stored mite extracts. The comparison of the parallel samples of the extracts (with and without the inhibitor cocktail) in the competition

ELISA and in the 2-site ELISA indicate no statistically significant difference in the values of RP or allergen content at 6 or 12 months (data not shown). Furthermore, no differences were noted in the polyclonal immunoblots between the extracts incubated with and without added protease inhibitors (*D farinae* extracts shown in Fig 2).

DISCUSSION

The most striking feature of the studies presented in this report is the difference between the apparent stability of the mite extracts, as determined by different methods. The RPs of the stored allergen vaccines, as measured by competition ELISA, were stable at -20°C and 4°C. However, with the other methods, using either monoclonal or polyclonal antisera, there was measurable allergen degradation, aggregation, or both in the samples stored at 4°C. Storage in 50% glycerol at -20°C appeared to ensure stability of each of the allergen preparations by all of the techniques used, except for the 2-site ELISA.

As determined by both types of immunoblots and the 2-site ELISA, Der f 1 levels decreased over the period of observation. For the other allergens, the different methods suggested different results. Der f 2 decreased in the 2-site ELISA but was stable in the monoclonal and pooled serum immunoblots. Der p 1 decreased in the pooled serum immunoblot but was stable in the 2-site ELISA. Der p 2 decreased in one of the pooled serum immunoblots of the 12-month samples and the 2-site ELISA of the 6-month samples but appeared to be conserved in the 12-month 2-site ELISA (not shown) and the monoclonal immunoblot. Unidentified immunoreactive aggregates, as well as new immunoreactive bands, were noted in several immunoblots, whether developed with pooled sera or mAbs.

These results are consistent with much of the data in the previous report from this laboratory by Liu and Lin.¹¹ The major differences are in the 2-site ELISA determinations. In that study, Der f 1 was stable at 4°C, whereas the other allergens deteriorated. In our hands Der f 1 deteriorated at 4°C in at least one of the *D farinae* extracts examined at 6 and 12 months by each of the methods that we used. These differences are largely due to our expanded use of controls, which allowed us to make important comparisons among different storage conditions. In particular, we used lyophilized extracts, whereas Liu and Lin used commercially obtained glycerinated products. We chose to use lyophilized extracts to address a specific weakness in the design of the previous study, the absence of an adequate nondegrading control. Dialysis was performed to remove glycerol from the extracts before lyophilization, and mannitol was added as a bulk stabilizer. Both of these were considered essential to the production of a stable and potent lyophilized control and are unlikely to have affected the results.

The apparent insensitivity of the competition ELISA to the loss of individual allergen bands may indicate that relevant epitopes are conserved, albeit on peptides with different electrophoretic migration. Although immuno-

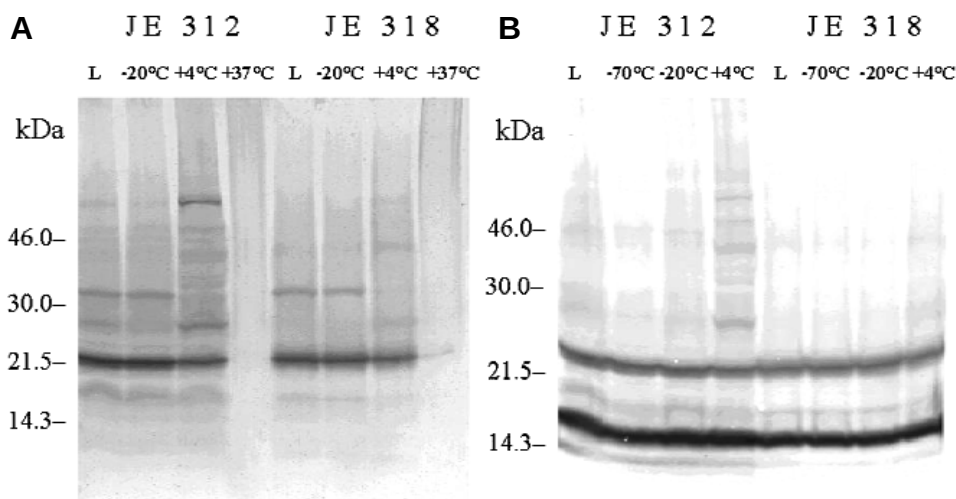


FIG 5. Immunoblots of *D farinae* mite extracts separated by SDS-PAGE on 16% polyacrylamide gel, transferred to a polyvinylene difluoride membrane, and developed with anti-group 1 (**A**) and anti-group 2 (**B**) mAbs (12-month study). Storage temperatures were -70°C , -20°C , 4°C , and 37°C . L, Lyophilized extract.

TABLE II. Degradation of the group 1 and group 2 mite allergens at 4°C detected on immunoblots with anti-group 1 and anti-group 2 mAbs at 12 months

	JE 318(Df)	JE 312(Df)	JE 343(Dp)	JE 321(Dp)
Group 1	±	+	—	+
Group 2	—	—	—	—
Comments and observations	Loss of 33-kD band	Loss of 33-kD band; appearance of new 27-kD and >46-kD bands	Loss of >46-kD band; appearance of new 17- and 28-kD bands	Loss of 17-kD band; appearance of 33-kD and >46-kD bands

Df, *D farinae*; Dp, *D pteronyssinus*; ++, significant degradation; +, some degradation; —, no observed degradation.

blots allow the investigator to assess several antigen-antibody interactions concurrently, the antigens are partially or fully denatured, and the conditions for the assessment of each band should be optimized individually. In addition, the epitopes recognized by human and murine antibodies are often different; this has been shown convincingly for Der p 1 and Der f 1.¹⁶ Monoclonal antibodies recognize specific antigenic sites, and the disappearance of these murine epitopes may be of no consequence for a reagent intended for human use. In particular, murine antibody 7A1, which was used in this study to detect the group 2 antigens, binds to a conformational epitope for which intact disulfide bonds at two loci (21-27 and 73-78) appear to be essential.^{17,18} Thus the binding of antibody 7A1 to the group 2 antigens is extremely sensitive to disruption at multiple noncontiguous sites in the primary sequence. These physical disruptions may be associated with conservation of the human IgE-binding sites (eg, as in a similar study performed with Der f 2¹⁹) and may have no effect on the efficacy or safety of a product used in the diagnosis and treatment of human allergic disease.

On the other hand, because the competition ELISA uses polyclonal antisera to detect multiple antigens, the loss of an important allergen, even one associated with significant immunopathology, may be undetectable.

Examination of Fig 4 suggests one source of this discrepancy. Comparison of the RPs of the extracts with the pooled allergic sera with the RPs with the mAbs indicates that the pooled sera contain adequate amounts of *D pteronyssinus*-specific IgE. However, the amount, avidity, or both of the *D farinae*-specific IgE in the pooled sera is such that a measurable loss of antigen remains undetected by the competition ELISA.

Furthermore, methods that use physicochemical separation of the analyte can also detect specific losses with greater sensitivity than methods such as the 2-site ELISA that do not, regardless of the specificity of the detecting antibodies. For example, although there was no evidence with the 2-site ELISA of allergen loss at 4°C relative to -20°C (Fig 3), the same detecting antibodies indicated allergen loss, aggregation, or both at 4°C relative to -20°C when the allergens were separated electrophoretically and transferred to a solid support first (Fig 5 and Table I).

The loss of specific bands when the human serum pool was used for immunoblot analysis indicates that these changes can be recognized by pooled human IgE and are not merely an artifact associated with the use of murine antibodies or mAbs. Furthermore, the specific loss of individual bands in the immunoblots by using both types of antibodies is evidence of antigen-specific degradation

rather than a global and proportionate decay of heterogeneous proteins over time.

The seroepidemiologic evidence that the group 1 and 2 allergens are major allergens⁷ fails to address the more relevant issue of the immunodominance of these allergens in the pathogenesis of asthma and rhinitis. However, although studies have not yet demonstrated that these allergens are pathogenic, or, conversely, that immunotherapy with these specific allergens is curative, their detection and accurate measurement in allergen vaccines is desirable. The data in this study indicate that further studies on the sensitivity of the polyclonal inhibition ELISA are needed.

Protease inhibitors did not contribute to the stability of the mite extracts. There is no direct evidence that the instability of mite allergens in aqueous solution is due to the activity of proteases, although this has been inferred by several investigators.^{9,13} Because we examined our samples at only two time points, our experiments cannot exclude the possibility that the inhibitors enhanced allergen stability at shorter incubation intervals. However, our data do suggest that allergen loss at 6 and 12 months may be due to intrinsic instability or the loss of native inhibitors²⁰ and that specific protease inhibitors are unlikely to improve the stability of commercial mite allergen vaccines.

In summary, assays that measure specific allergen content contribute to our ability to characterize allergen vaccines and their stability. Because the stability of specific dust mite allergens varies widely under normal storage conditions, the choice of a particular analyte will have to be based on the relative clinical importance of the allergen, as well as the regulatory objectives of the monitoring program. It is significant that we were better able to assess specific allergen loss with an immunoblot with a pooled allergic serum than with immunoblots with mAbs. This may be due to suboptimal sensitivity and specificity (Fig 5) of the mAbs currently available to detect dust mite allergens. If mAbs are to be incorporated into an allergen vaccine standardization program, the extent of cross-reactivity must be identified and minimized through careful clone selection and assay optimization.

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