J Trop Med Parasitol 2007;30:55-63.

ORIGINAL ARTICLE

Available online at www.ptat.thaigov.net

Comparative Study of Siriraj In-house Mite Extracts and Commercially Available Extracts

Vanna Mahakittikun[1], Sirichit Wongkamchai[1], Pattama Ekpo[2], John J Boitano[3], Chulaluk Komoltri[4], Chaiyaporn Manochnon[5], Kunda Kasetsinsombat[5], Pakit Vichyanond[6], Chaweewan Bunnag[7]

[1] Department of Parasitology, [2] Department of Immunology, [3] Stratford, Connecticut , USA, [4] Department of Research Development, [5] Department of Pharmacology, [6] Department of Pediatrics, [7] Department of Oto-rhinolaryngology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

Abstract

This study compared the quality of allergenic extracts produced from pure mite bodies (PMBs) and spent mite medium (SMM) of *Dermatophagoides pteronyssinus* (Dp) and *D. farinae* (Df), with commercially available extracts (FDA, Greer, CSL). It also evaluated the use of glycerine as a preservative, the stability of these extracts under various temperatures, and the reactions of allergy patients to skin-prick testing with these extracts. The concentrations of in-house extracts were determined by ELISA. The extracts were kept at 4°C and their concentrations measured for one year. Another batch of in-house extracts, stored with or without glycerine, was exposed to multiple refrigeration in-out cycles. PMBs yielded significantly greater concentrations of allergen than SMM, while in-house Der f1 was greater than Der p1 in allergenic concentration, regardless of time, equal to the FDA extract and significantly greater than the Greer extract. Glycerine was largely ineffective. There were insignificant differences in allergy patients' reactions to skin-prick tests. It may be concluded that PMBs, rather than SMM, should be used as source material for generating mite allergen extracts.

Keywords: mite extracts, pure mite bodies, spent mite medium, Der f, Der p

Introduction

Mite allergenic extracts are crucial reagents used in the diagnosis, and in selected individuals, the treatment of dust-mite allergy. Batch-to-batch uniformity of extract composition and potency is especially important for immuno-therapeutic purposes. Several factors are involved in the quality and efficacy of extracts, including the source of raw materials [1] and storage conditions [2,5]. Different portions used in house-dust-mite culture (whole-mite culture, spent mite medium, pure mite bodies) give rise to different allergen compositions, due to natural variations in their cultivation and harvesting. Spent mite medium is often used in Europe, whereas in the United States, whole mite bodies of purported 99% purity have been exclusively used as source materials for mite extracts [6]. Furthermore, all mite extracts

Correspondence:

Vanna Mahakittikun, Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.

Tel / Fax: +66(0) 2418 1040

Email: sivmh@mahidol.ac.th

used in allergen vaccines in the US must fall within the 98% confidence interval of recognized reference standards, as established by the Center for Biologics Evaluation and Research (CBER) at the Food and Drug Administration (USFDA), based on three criteria, *ie*, therapeutic, diagnostic, and safety equivalence [7,8].

The current study sought to determine the specific mite-allergen contents of in-house extracts stored at varying temperatures over a period of time. The major objectives of this research were 1) to evaluate the most efficacious part of the mite to be cultured for mite allergenic extracts; 2) to study the composition of the major and minor allergens in: i) in-house extracts, ii) commercially available extracts, and iii) the reference extracts of the USFDA; 3) to evaluate, under various temperatures; multiple refrigeration in-out cycles, room temperatures, and a constant temperature of 4°C, the glycerine preservative used in storage, and the stability of the extracts over the long (1 year) term; and 4) to determine the relative effectiveness of the extracts used in skin-prick tests of 36 allergic patients.

Materials and Methods

Subjects

Thirty-six patients (22 females, 14 males; age range 12-66 years) were recruited into the study. The inclusion criteria were as follows: individuals diagnosed with allergic rhinitis, with or without asthma, testing positive by skin-prick test to *Dermatophagoides pteronyssinus* and *D. farinae* at the Allergy Clinic, Department of Oto-rhinolaryngology, Siriraj Hospital, Mahidol University, Bangkok, Thailand, during the period 30 January-30 April, 2003. Participants were thoroughly informed about the study objectives and provided written informed consent. The Ethical Clearance Committee on Human Rights Related to Research involving Human Subjects, Faculty of Medicine Siriraj Hospital, approved the study.

Skin-prick test

All patients were requested to discontinue for 7 days any medication that might interfere with skin-test results, *eg,* antihistamines or oral decongestants, prior to the skin-prick test. The positive control skin-prick was 10% histamine hydrochloride (w/v), while the negative control was buffered saline. The concentration for all positive skin-pricks was 10,000 PNU/ml. All 12 injections, including 10 allergen extracts, were administered in random sequence at one time, 3 cm apart on the surface of the volar forearm. Skin-prick tests were performed between 9:00 am and 12:00 noon to minimize Circadian variations in the results [9]. If erythema was discerned 15 min post-administration with wheal ≥ 3 mm and flare, it was counted as positive.

Mite cultures

D. pteryonyssinus and *D. farinae* were produced separately in large quantities under the House Dust Mite and Allergic Diseases' Research Project's Quality Assurance Program. Stringent control measures were adopted to assure that the house-dust mite (HDM) raw materials were mono-species and virtually free of contamination from other mites or molds. To confirm specific species, random samples were examined during culture and at the final stage. Using our own apparatus and patented method [10], the cultures were divided into two parts, *ie*, pure mite bodies (PMB) with a maximum 1% carryover of culture media, and spent mite medium (SMM), which typically consisted of all stages of dead mites, skin debris, and culture medium. From whole culture, PMB was extricated and the remainder was considered SMM.

Mite extracts

In-house mite allergen extracts were prepared from 10 g samples of PMB and SMM, according to the methods in [11,12]. The samples were dehydrated by lyophilization and defatted with diethyl ether for 2 weeks. The supernatant was further dialyzed using a cellulose dialysis membrane with an 8,000 kDa cutoff, then sterilized by filtration via a 0.2 µm millipore membrane, after which standardization and pyrogen tests were performed. These in-house extracts were prepared at a concentration of 10,000 PNU/ml, either with or without a 50% glycerine

preservative. All in-house extracts were compared with reference extracts obtained from the USFDA and two commercial laboratories (Greer USA and CSL) and the results were expressed as AU/ml units. PNU/ml units were used for the in-house extracts, as these were the standard units of measurement when the study started.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations (µg/ml) of Der p and Der f groups 1 and 2 were determined by two-site monoclonal-based ELISA and compared with those from two commercially available extracts and the reference extract from the FDA at a concentration of 10,000 AU/ml. The assays were performed according to the protocols provided by the supplier (Indoor Biotechnology, Charlottesville, VA, USA) and available at www.inbio.com.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein concentrations of the extracts from PMB and SMM of Dp and Df were determined according to Bradford's method [13]. The protein profiles were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A 3.5% stacking and a 12% separating acrylamide gel were used. The samples were solubilized under reducing conditions. Electrophoresis was performed at a constant current of 170 mA and the bands were visualized by staining with Coomassie blue K-250. The molecular weight (MW) of each protein band was calculated by a gel documentation computer program (Quantity One, University Hood II from Bio-Rad).

Study design

All in-house and ready-made extracts were kept under various temperature and storage conditions, *ie*, 1) those kept at 4°C for one year; 2) those subjected to multiple refrigeration in-out cycles (4°C refrigeration for 21 hours/day and room temperature, 22 ± 2 °C for 3 hours/day) 5 days/week, with or without glycerine for one year; 3) those maintained at room temperature 24 hours/day for 5 weeks, with or without glycerine; and 4) those major and minor in-house and non-in-house extracts kept at 4°C and measured at baseline, and at 6 and 12 months. The 12-month extracts were used to skin-test the 36 allergic patients. Statistical data were analyzed by computer using Abstat™ (Anderson-Bell Corp, 1998). Analysis of variance procedures were employed throughout, and where appropriate, Scheffe's post-hoc tests were used for subsequent for multiple-comparison analysis. The z test was used to contrast proportions among the skin-prick-test results, to test the significance of the differences between the protein and allergen data, and to compare percentage decline as a result of temperature variability. All results were considered significant when $p < 0.05$.

Results

Table 1 shows the major results of this study. It shows the degradation of PMB and SMM for the in-house extracts for protein and allergen concentrations of both species when refrigerated at 4°C for one year. All of the PMB extracts from both species were larger at months 0 and 12 than the corresponding extracts derived from SMM. The decline in Der p1 PMB (10.08%) was significantly less ($p = 0.003$) than its corresponding SMM (40%). Even though the Der f1 PMB decrement (71.73%) was greater than its SMM (56%), the difference was not statistically significant ($p = 0.085$). All four protein concentrations had decrements of between 97 and 99%.

As shown in Fig 1 (top panel), Der f1 was significantly different from Der p1 throughout the entire period of observation ($p < 0.001$), with an average concentration of 163.6 µg/ml when contrasted with the mean concentration of 73.7 µg/ml for Der p1. For both species, glycerine was inconsequential. The time variable was also significant ($p < 0.001$), as both species showed a marked decline from baseline. For the minor in-house extracts (bottom panel), the only statistically significant difference was between species ($p < 0.001$), but unlike the major allergens, Der p2 (53.3 µg/ml) was on average greater than Der f2 (2.1 µg/ml) over time.

Table 1 Protein and allergen concentrations stored at 4˚C for 12 months from both types of extracts for both species.

In-house Extracts	Protein Concentration (µg/ml)			Allergen Concentration (µg/ml)				
	Month 0	Month 12	% reduction	Month 0		Month 12		% reduction
				Der p1	Der f1	Der p1	Der f1	
Dp-PMB	2,420	56	97.69	357	-	321	-	10.08
Df-PMB	1,750	40	97.71	-	1,132	-	320	71.73
Dp-SMM	872	6	99.31	10	-	6	-	40.00
Df-SMM	225	6	97.33	-	25	-	11	56.00

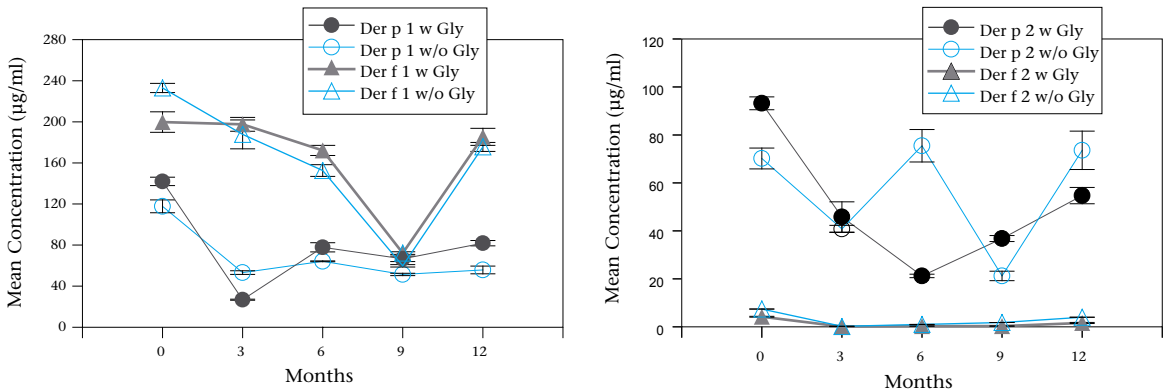

Fig 1 Mean concentrations (& SEM) of the major (top panel) and minor (bottom panel) in-house extracts of allergens treated with (filled symbols) or without (open symbols) glycerine, and kept at room temperature for 3 hours/day for 5 days/ week for 1 year.

The four panels of Fig 2 present the major and minor mean allergen concentrations of the in-house and ready-made extracts kept at 4°C and assayed over one year. For Der f1 (upper left), ANOVA revealed a significant difference ($p = 0.02$) among all the extracts. The subsequent Scheffe test pinpointed the major source of variance to comparisons between the in-house and Greer ($p < 0.05$), and FDA and Greer ($p < 0.05$) as being significantly different. No other comparisons were significant. For Der p1 (upper right), the only significance was over time ($p = 0.001$), with baseline average concentration (147.4 µg/ml) being significantly greater than the mean concentration (54.7 µg/ml) at month 12 ($p < 0.05$). For the minor allergens (two bottom panels), the only statistically significant result was the month's variable for Der f2 (lower left) as the average baseline concentration of 5.4 µg/ml decreased to 2.03 µg/ml over the one-year period ($p = 0.005$). Fig 3 presents the percentage of positive skin reactions among 36 allergic patients to the two control and 10 allergen inoculations, which were kept for one year at 4°C before patient administration. Aside from the controls, the range of allergen injections extended from 58.3% (21/36 patients responded positively to the in-house Df-PMB with glycerine and Greer's Dp and Df treatments) to 38.9% (14 patients exhibited positive reactions to the in-house Dp-SMM

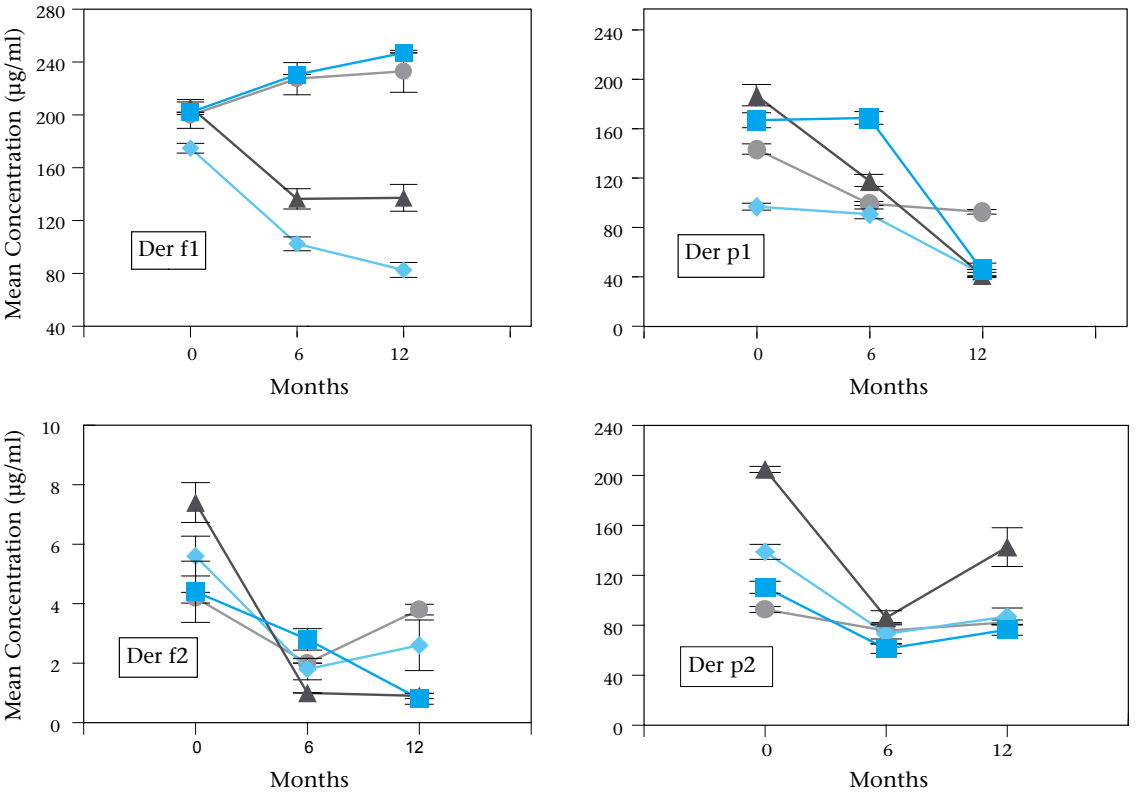

Fig 2 Mean allergen concentrations (& SEM) for the major and minor in-house and ready-made extracts kept at 4˚C for 1 year, where ■ stands for FDA, ▲ for CSL, ● for In-house and ◆ for Greer extracts.

injection). Since the z test between these two extreme proportions was insignificant (z = 1.68, p > 0.10), all other comparisons among the allergen injections were also statistically insignificant.

The SDS-PAGE results for the protein profiles of *D. pteronyssinus* and *D. farinae* PMB and SMM are shown in Fig 4. Overall, there were 18 protein bands with molecular weights (MWs) ranging from 12 to 185 kDa. The composition of these proteins could be divided arbitrarily into 3 groups by MW. The first group is the highest with MWs ranging from 45 to 185 kDa. The second group has MWs extending from 22 to 45 kDa while the third group has MWs ranging from 12 to 22 kDa.

In evaluating the stability of the extracts over time, it was necessary to consider the effects of temperature. Table 2 draws information from Fig 1, and after 5 continuous weeks of no refrigeration, to elucidate the importance of temperature in maintaining concentration stability. Insofar as some allergy clinics routinely combine allergens from two species into one injectable cocktail, the allergen concentrations of Der p1 and Der f1 were combined (for statistical purposes only) with and without the preservative. Since the baseline information was the same for Fig 1 and for the 5-week data, it provided the necessary starting point for comparing the decrements assayed under different temperature conditions. There was a 42.5% decrement over baseline concentration when the two major allergen species were combined and kept in glycerine at room temperature continuously for 5 weeks or 120 hours without refrigeration. When this proportion was contrasted with the month-3 proportion (0.344), where allergens were kept at

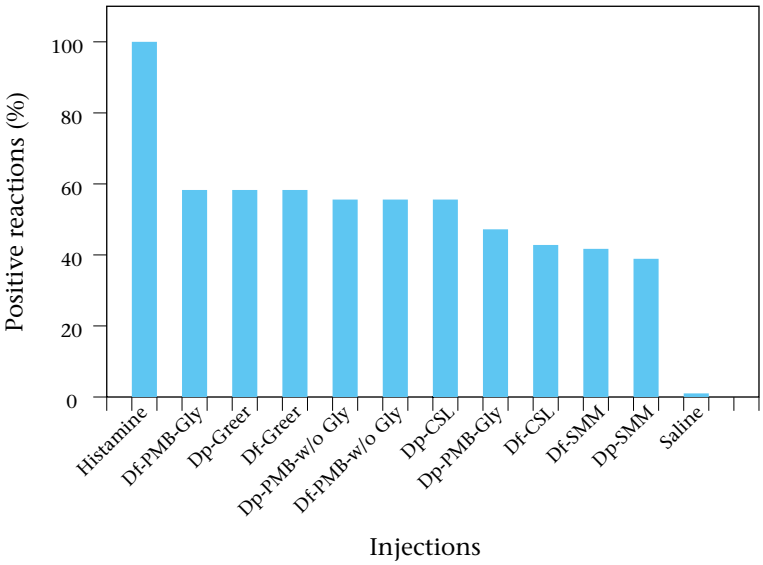

Fig 3 Percentage of positive skin reactions (wheal > 3 cm in diameter) for each of the 10 allergen and 2 control injections preserved at 4°C for 12 months.

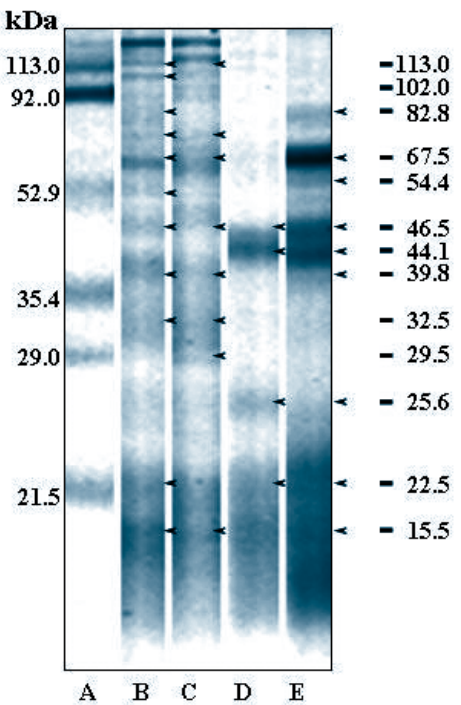

Fig 4 SDS-PAGE of PMB and SMM of *D. pteronyssinus* and *D. farinae* where A = Marker, B = Df-PMB, C = Dp-PMB, D = Df-SMM and E = Dp-SMM.

room temperature for 3 hours/day, 5 days/week for 3 months, the differences were statistically insignificant. However, when compared with the month-12 proportion (0.218), the differences were highly significant ($z = 4.10$, $p < 0.01$). When the allergens were stored without glycerine and refrigeration for 5 weeks, a large 62.9% decrement in concentration resulted. When compared with the proportions from months 3 (0.312) and 12 (0.339), the differences were statistically significant ($p < 0.01$). However, when the 5-weeks-without-glycerine proportion (0.629) was contrasted with the 5-weeks-glycerine proportion (0.425), a z of 3.79 indicated statistically significant differences ($p < 0.01$).

Discussion

This study clearly demonstrated that the most appropriate source of raw material for the preparation of mite allergenic extracts is not SMM, but PMBs, which, because of stringent collecting procedures [10], are free from contamination by the culture medium. Extracts from PMBs have been shown to produce allergens obtained from all stages of mite development (adult stage, larva

Table 2 Mean major allergen concentrations and percentage decrements from baseline for week 5, month 3, and month 12, at different temperatures.

Der p1 + Der f1	Mean allergen concentrations (µg/ml)				Percentage decline
	Baseline	Week 5*	Month 3**	Month 12***	
With Glycerine	-	98.3	-	-	42.5
	170.9	-	112.6	-	34.4
				133.6	21.8
		65.05			62.9
Without Glycerine	175.1		120.5		31.2
				115.7	33.9

* Data after 5 continuous weeks of room temperature (22°C) without refrigeration
** Data from Fig 1: 3 months (15 hours/week) at 22°C and remainder at 4°C
*** Data from Fig 1: 12 months (15 hours/week) at 22°C and remainder at 4°C

and nymph stages, eggs and fecal pellets), whereas SMM-derived allergens consist of a mixture of fecal pellets, all stages of dead mites, exoskeletal debris, eggs and culture media [1,6]. Not only is PMB more plentiful in protein and allergen concentrations immediately after preparation than SMM, but the differences persist for one year if kept at 4°C. The greater initial protein and allergen concentrations for PMBs over SMM are especially relevant, given that equal amounts of raw source material were utilized in their preparation.

SDS-PAGE analysis of the two HDM species revealed that the extracts prepared from both PMB and SMM contained specific allergen groups; viz Dp & Df, groups 1, 2, and 3, with molecular weights of 25, 14, and 28 kDa, respectively. Thus, both may be used as a source of raw materials for the preparation of mite extract. However, a cursory examination of the SDS-PAGE results suggested that PMB might be superior to SMM as a source of raw material, since it contained more protein components and a greater concentration.

The reduction of mite allergens in the extract from SMM might be due to culture media contamination and prolonged processing, resulting in the denaturing of allergens. In this regard, the concentration of the extract from SMM appears less stable over time than that from PMB. Furthermore, there seems to be a clear benefit for using in-house *D. farinae* rather than *D. pteronyssinus* for allergen extraction as this species does not lose its potency after one year (Fig 2) and compares favorably with the reference extract from FDA but not Greer and, to a lesser extent, the extracts from CSL. Thus, the in-house and FDA extracts are very similar and stable over one year. These results are clearly not in agreement with Soldatova *et al* [5], who found that the relative potencies of Df extracts prepared using 2-site ELISA decreased over 6 and 12 months, which was not the case with the Dp extracts. At present, the cause of the discrepancy between the present findings and those of Soldatova *et al* [5] are unclear. However, these results are in agreement with Liu and Lin [3], who found Der f2 and Der f1 were stable for 1 and 3 years, respectively, when stored at 4°C.

A logical extension of this argument begs the question: is there an advantage to using PMBs only as the source material for immunotherapeutic treatment as opposed to SMM? The results of the skin-prick test (Fig 3) suggest cautious ambivalence. None of the 10 extract injections indicated a clear superiority over another. However, a sample cohort of 36 patients is small; all injections were administered at one time, and the results were simply classified as positive if the diameter of the wheal was ≥ 3 mm, or negative if it was not.

Further tests are necessary to clarify this issue, especially with a larger number of patients, having multiple injections spaced over time and using the $ID_{50}EAL$ method for determining bioequivalent allergy units [6], tor compare the allergenicity of different source extracts. Not everyone agrees that PMBs are superior to SMM: "Our results do not support the hypothesis that the use of pure PMBs as sources for an allergen extract might increase the immunologic efficacy of immunotherapy, *D. pteronyssinus* extract" [1].

However, there is a decided advantage in using PMBs rather than SMM as a source material for extracts, *ie* a greater yield of allergens from PMBs, since the production process takes longer due to defatting and removal of mite media. There is essentially no difference in the quality of the extract from PMB or SMM; *eg*, Der p1 from PMBs is the same as Der p1 from SMM, and reacts similarly in sensitized patients. A greater yield from PMBs results in a greater dilution ratio, which is not the case with SMM. The stock from PMBs has a higher concentration than SMM, and therefore results in a greater amount.

Regarding the stability of the allergens at various intervals, temperature had a major impact concentration. Mite-allergen extract is normally kept in a refrigerator at 4-10°C. Even at this temperature, it was found that the composition of the extracts gradually decreased over a year, regardless of the presence of preservatives. On a daily basis, the ambient temperature surrounding the extracts varied due to their being removed and subsequently returned to the cold environment. If however, the extracts were not refrigerated 24 hours/day for up to 5 weeks, the loss in concentration was quite noticeable, regardless of storage with or without preservative. In those instances, when the extracts were kept in a cold environment most of the time, and exposed to room temperature only minimally for 3 and 12 months, the decline in concentration was much less. These results are related to those of Bousquet *et al* [4], who found a significant decay in prick-test activity for Dp lyophilized extracts after 2 months of storage in glycerol. Therefore, this study supports the contention that to prolong the shelf-life of extracts, refrigeration at 4°C should be maintained as much as possible and refrigeration in-out cycles minimized. However, even though the overall potency of the mite allergens may be better preserved at 4°C than 22°C, further assays for specific allergens might reveal degradation, since competition ELISA has been shown to be relatively insensitive to decreases in the concentrations of individual allergens [5].

In this study, the use of glycerine as a preservative was mostly ineffective. In only one instance did it retard the loss of extract concentration and that was when the extracts were continuously exposed to room temperature for a full 5 weeks. Sridhara *et al* [14] found that the addition of glycerol did not change the stability of wheat dust allergenic extract at temperatures ranging from 4-55°C for 15-60 days. Liu and Lin [3] found that the allergen content of the mite extracts used for CBER reference standards did decrease "at a relatively fast pace in the presence of 50% glycerol", although Der f1 was stable for 3 years when stored at 4°C, while Der p1 was not stable at all. Other studies have indicated that 50% glycerine is an effective preservative in a variety of extracts [15,17], has a longer shelf-life, and is routinely employed in dust-mite extracts produced by various commercial laboratories, such as Greer, Hollister-Stier, and ALK. CBER's dating period is 3 years after the extract leaves the manufacturer's storage, if kept at 2-8°C with 50% or more glycerine. With < 50% glycerine, the dating period of the extract in final containers is 18 months. As regards the present study, a study period contrasting glycerine- and non-glycerine-treated extracts of more than one year is preferable. Efforts are ongoing to further refine the quality of the allergen concentrations derived from extracts of the pure mite bodies of either species.

Within the limitations of this experiment, it may be concluded that: 1) PMB rather than SMM should be used as a source of raw material for the generation of extracts for mite allergens, since the production yield is greater; 2) the in-house extracts produced from PMBs and prepared at Siriraj

Hospital, Mahidol University, compared favorably with the commercially prepared extracts, and, in one case, were superior to the commercial extracts and equal to the reference extract from FDA; 3) temperature had an effect on the stability and concentration of mite allergens; 4) glycerine was largely ineffective in reducing extract degradation; 5) there were no differences in any of the reactions for the extracts tested with allergy patients, but further investigation is necessary.

Acknowledgements

This work was supported by the National Research Council of Thailand.

References

1. Wahn U, Schweter C, Lind P, Lewenstein H. Prospective study on immunologic changes induced by two different *Dermatophagoides pteronyssinus* extracts prepared from whole mite culture and mite bodies. J Allergy Clin Immunol. 1988;82:360-70.
2. Niemeijer NR, Kauffman HF, van Hove W, Dubois AEJ, de Monchy JGR. Effect of dilution, temperature, and preservatives on the long-term stability of standardized inhalant allergen extracts. Ann Allergy Asthma Immunol. 1996;76:535-40.
3. Liu T, Lin Y. The epitope stability of group 1 and group 2 allergens in mite extracts. Ann Allergy Asthma Immunol. 1998;80:177-83.
4. Bousquet J, Djoukadar F, Hewitt B, Guerin B, Michel F. Comparison of the stability of a mite and a pollen extract stored in normal conditions of use. Clin Allergy. 1985;15:29-35.
5. Soldatova LN, Paupore EJ, Burk SH, Pastore RW, Slater JE. The stability of house dust mite allergens in glycerinated extracts. J Allergy Clin Immunol. 2000;105:482-8.
6. Lockey RF, Slater JE, Esch RE. Preparation and standardization of allergen extracts. In: Adkinson NE, Yunginger JW, Busse WW, Bochner BS, Holgate ST, Simons, FE, editors. Middleton's allergy principles & practice. 6th ed. Philadelphia: Mosby Inc; 2003. p. 573-84.
7. Guidance for reviewers: potency limits for standardized dust mite and grass allergen vaccines: a revised protocol; 2000. Available from: http://www.fda.gov/cber/guidelines.htm.
8. Slater JE, Pastor RW. The determination of equivalent doses of standardized allergen vaccines. J Allergy Clin Immunol. 2000;105:468-74.
9. Vichyanond P, Nelson HS. Circadian variation of skin test reactivity and allergy skin tests. J Allergy Clin Immunol. 1989;83:1101-6.
10. Mahakittikun V, Pattanachitvilat S, Nochot H, Bunnag C. Innovative method for rapid isolation of live dust mites with high purity. J World Allergy Org. 2003;S1:157.
11. Criep LH. Diagnosis of atopy. In: Criep LH, editor. Allergy and clinical immunology. New York: Grune & Stratton; 1976. p. 161-78.
12. Dhorranintra B. Preparation of allergenic extracts. In: Tuchida M, editor. Allergic diseases. Bangkok: Unity Publication; 1978. p. 526-33.
13. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-54.
14. Sridhara S, Singh BP, Arora N, Verma J, Gangal SV. A study on antigenic and allergenic changes during storage in three different biological extracts. Asian Pac J Allergy Immunol. 1992;10:33-8.
15. Johnson MC, Schlegle AW, Hampton SF. Studies on the optimum concentration of glycerine in the preparation and preservation of ragweed pollen extract. J Allergy Clin Immunol. 1955;26:429-36.
16. Nelson HS. Effect of preservatives and conditions of storage on the potency of allergy extracts. J Allergy Clin Immunol. 1981;67:64-9.
17. Morell F, Codina R, Rodrigo MJ. Increased positivity of skin test and allergenic stability of glycerinated soybean hull extracts. Clin Exp Allergy. 1999;29:288-93.