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Original scientific paper

Stability evaluation of house dust mite vaccines for sublingual immunotherapy

LIDIJA BURAZER^{1*}, KATARINA MILOVANOVIĆ¹, TANJA ČIRKOVIĆ-VELIČKOVIĆ^{2#} and MARIJA GAVROVIĆ-JANKULOVIĆ^{2#}

¹*Institute of Virology, Vaccines and Sera – Torlak, Vojvode Stepe 458, Belgrade and*
²*Faculty of Chemistry, Department of Biochemistry, Studentski trg 16, Belgrade, Serbia*

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Abstract: Allergen-specific immunotherapy with house dust mite (HDM) allergen extracts can effectively alleviate the symptoms of allergic rhinitis and asthma. The efficacy of the immunotherapeutic treatment is highly dependent on the quality of house dust mite vaccines. This study was performed to assess the stability of house dust mite allergen vaccines prepared for sublingual immunotherapy. Lyophilized *Dermatophagoides pteronyssinus* (Dpt) mite bodies were the starting material for the production of sublingual vaccines in four therapeutic concentrations. The stability of the extract for vaccine production, which was stored below 4 °C for one month, showed consistence in the protein profile in SDS PAGE. ELISA-inhibition showed that the potencies of Dpt vaccines during a 12 month period were to 65–80 % preserved at all analyzed therapeutic concentrations. This study showed that glycerinated Dpt vaccines stored at 4 °C preserved their IgE-binding potential during a 12 month period, implying their suitability for sublingual immunotherapeutic treatment of HDM allergy.

Keywords: *Dermatophagoides pteronyssinus*; allergen extract; vaccines; ELISA inhibition; stability.

INTRODUCTION

The protein allergens of house dust mite *Dermatophagoides pteronyssinus* can cause severe allergic disease in susceptible individuals.¹ Allergen extract prepared from cultured dust mites has been used for diagnosis as well as for sublingual-swallow immunotherapy. The beneficial effect of immunotherapy (IT) with crude extract or partly purified allergen had been demonstrated in certain IgE-mediated disorders, such as seasonal allergic rhinitis and asthma.^{2,3}

Allergen stability, *i.e.*, persistence of adequate quantities of relevant antigens in an allergen vaccine from the time of initial assay to the time of clinical use, is

* Corresponding author. E-mail: lili9ili@yahoo.com

Serbian Chemical Society member.

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enhanced by addition of glycerol to the vaccine solution.^{4,5} Mite extracts contain at least 20 well-characterized allergens. Several of these proteins appear to be highly immunoreactive in humans with evidence of specific IgE to the individual allergens in up to 80 % of individuals allergic to mites.⁵ Four of these allergen groups (1, 3, 6 and 9) are proteolytic enzymes and, due to their biological activity, may compromise the stability of extracts. Several investigators evaluated the stability of mite allergens in commercially available vaccine preparations.^{4,6-9} Due to the presence of proteolytic enzymes, glycerol has been considered as a stabilizing agent in house dust mite allergen extracts,¹⁰ since protease inhibitors did not contribute to the stability of a mite extract.¹¹ However, in addition to optimization of the procedure for the preparation of a potent allergen vaccine, evaluation of its stability from the time of preparation to the intended clinical use is of major importance for reliable and effective immunotherapeutic treatment.

The aim of this work was to investigate the relative potency of a mite sublingual-swallow immunotherapeutic vaccine stabilized with 50 % glycerol, stored at 4 °C (producer recommendation) over a 12-month period. The stability of a pure mite extract was also examined over a 1-month storage period at the same temperature as used for the preparation of vaccines.

EXPERIMENTAL

Allergen extract preparation

The house dust mite extract was prepared from dried house dust mite bodies of *Dermaphagoides pteronyssinus*. The certificate of analysis for the starting material declared 95 % purity and 5 % medium particles. The extraction procedure was realized according to the manufacturer's recommendation.¹² In brief, the extraction was performed overnight at 4-8 °C using a 1 % solution in 0.15 M phosphate-buffered saline (PBS) pH 7.6. The extract was clarified by centrifugation at 2000 rpm for 30 min and subsequently filtered through a 0.22 µm membrane disc filter (Pall Europe Limited, Portsmouth, UK). The protein content was quantified according to the Kjeldal method.¹³

Allergen vaccines preparation

The *D. pteronyssinus* protein extract was used for the preparation of sublingual-swallow vaccines in PBS with 50 % glycerol. The vaccine marked as "3", with a concentration of 1000 PNU (protein nitrogen units), was used for the preparation of three serial dilutions designated as "2", "1", and "0", containing 125, 16 and 2 PNU, respectively. All vaccines were stored at 4 °C during the investigation period.

Human sera

The sera from 10 patients allergic to house dust mites with a documented clinical history of HDM allergy and without a record on immunotherapy to this allergen were used for the serological analysis.

SDS PAGE and Western blot

SDS PAGE was performed according to Laemmli.¹⁴ The gel was either stained with Coomassie Brilliant Blue R-250 (CBB) to visualize the separated proteins or the resolved components were blotted by a semi-dry electrotransfer onto a nitrocellulose membrane (0.45

μm , Serva, Heidelberg, Germany). The membrane was blocked in 20 mM Tris-buffered saline (TBS) containing 1 % BSA and 0.1 % Tween 20 for 1 h and dried until development.

IgE detection

IgE-reactive proteins were detected in Western blots with 5-fold diluted individual patient's sera in TBS-buffer with 0.1 % BSA. Alkaline phosphatase labeled monoclonal anti-human IgE (1:1000, Sigma Chemical Co., St Louis MO, USA) was used as the secondary antibody. The binding patterns were visualized with a substrate solution of 1.5 mg BCIP (5-bromo-4-chloro-3-indolyl phosphate, Serva, Heidelberg, Germany) and 3 mg NBT (nitro blue tetrazolium, Serva, Heidelberg, Germany) in 10 mL of 100 mM Tris buffer, containing 150 mM NaCl and 5 mM MgCl_2 , pH 9.6, according to Harlow & Lane.¹⁵

Evaluation of vaccine potency by ELISA-inhibition

The potency of each of the four vaccines was investigated by ELISA-inhibition with a pool of the sera from the allergic persons. Vaccine sampling has been performed after zero, three and 12 months of storage, and the potency was titrated in 6 dilutions. Microtiter plates (Nunc-Immuno Plate, Maxisorp, Inter med, Denmark) were coated with 100 μl /well (10 $\mu\text{g}/\text{mL}$ protein concentration) of *D. pteronyssinus* extract in 0.06 M carbonate buffer, pH 9.6, overnight at 4 °C. The plate was washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 0.1% bovine serum albumin dissolved in PBS-T (PBS-T-B) for 1 h at room temperature. The subsequent steps were performed using PBS-T-B as diluent, and washings in PBS-T were realized between the following steps: the plate was incubated with 100 μl of the pool of patients' sera (CAP class 1 to 6; diluted 1:5 in PBS-T-B) previously incubated with decreasing concentrations of the vaccine (diluted 1, 5, 10, 100, 500, 1000 and 5000 times) at 37 °C for 2 h. Subsequently, the plate was incubated with 100 μl of anti-human IgE (Sigma) for 2 h at room temperature. The assay was developed by adding 100 μl of the enzyme substrate (pNPP 1 mg/mL in 0.1 M diethanolamine buffer, pH 9.6), and the absorbance was measured at 405 nm using a plate reader (Multiskan) after 60 min of incubation. A serum from a non-allergic person was used as the negative control.

RESULTS AND DISCUSSION

SDS PAGE

Coomassie Brilliant Blue staining of a one-dimensional SDS PAA gel revealed a complex protein pattern for the Dpt extract in the range of about 12–116 kD (Fig. 1). The dominant protein bands were at about 116 kD, 25–30 kD, and 12–18 kD. SDS-PAGE analysis of pure non-glycerinated Dpt extract was employed for the evaluation of the extract quality, expressed through the protein bands detected after one month of storage. Under the employed experimental conditions, storage of the Dpt extract at 4 °C was suitable as the starting material for vaccine production. A longer period of storage (two months) revealed deterioration of the protein extract, suggesting one-month period at 4 °C as the maximum for an extract intended for vaccine preparation.

IgE binding profile of separated mite components

Immunoblots were performed using the sera of allergic patients with specific IgE class 6, and class 3 (Fig. 2). These immunoblots showed that patients' sera

class 6 recognized most of the proteins present in the extract while patients' sera class 3 recognized proteins of about 14 and 25 kD (major allergens) and some of higher molecular mass.

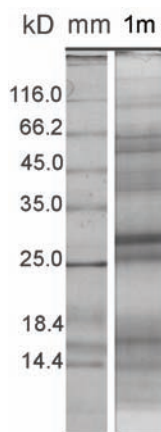


Fig. 1. Separation of house dust mite whole-body extracts by SDS gel electrophoresis on a 12 % PA gel: mm – molecular mass markers, 1m – 1st month.

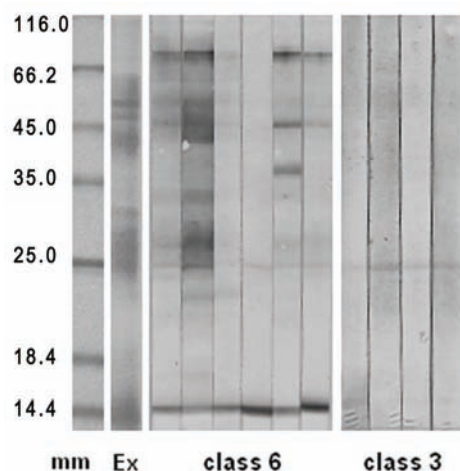


Fig. 2. IgE reactivity of the HDM extract with patients' sera class 6 and class 3, mm – molecular mass markers, Ex – extract.

ELISA-inhibition

Dpt vaccine “0” and “1” showed 100 % inhibition in full concentration after zero and after three months; however, the potency was reduced to 66 %, and 75 %, respectively after 12 months (Figs. 3 and 4). A remarkable decrease in potency of both vaccines was noticed within the first dilution and continued throughout the investigated period. The decrease could be ascribed not only to dilution of the glycerol as the stabilizing agent of the allergen proteins, but also to protein instability in diluted concentrations (2, and 16 PNU). Vaccines “2” and “3” showed 100 % inhibition after zero and after three months period. The high IgE inhibition

potential of these vaccines (> 80 %) was preserved after 12 months (Figs. 5 and 6). Vaccine “3”, with the highest allergen concentration, retained high IgE inhibition potential at almost all dilutions after 12 months storage.

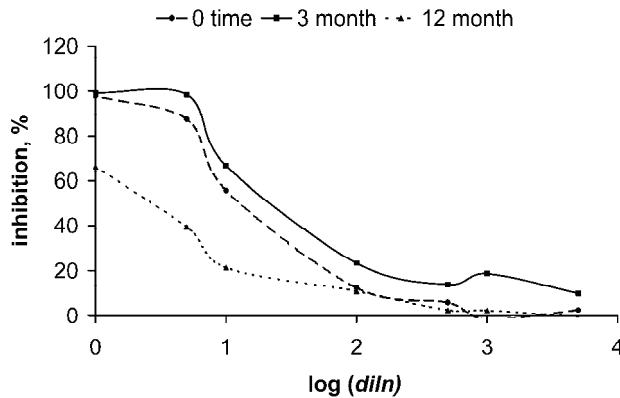


Fig. 3. IgE ELISA-inhibition of vaccine SLIT “0” during 12 months: percentage of inhibition vs. logarithm of dilution, (diln – volumetric ratio of solution and diluent).

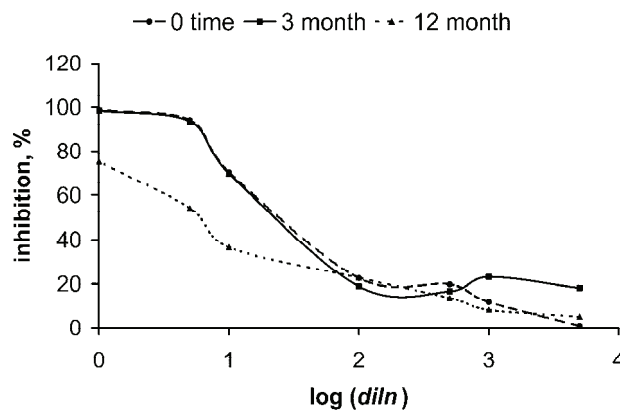


Fig. 4. IgE ELISA-inhibition of vaccine SLIT “1” during 12 months: percentage of inhibition vs. logarithm of dilution, (diln – volumetric ratio of solution and diluent).

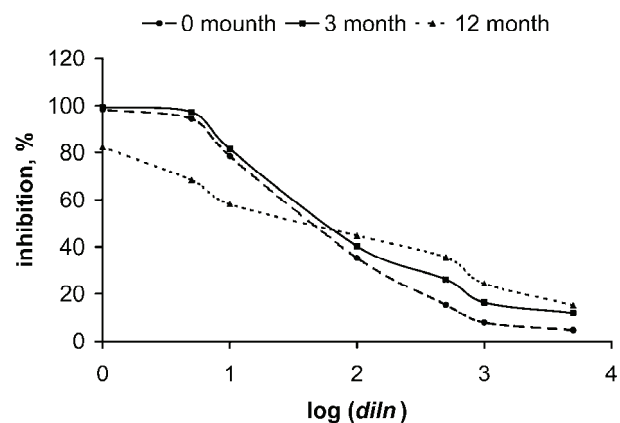


Fig. 5. IgE ELISA-inhibition of vaccine SLIT “2” during 12 months: percentage of inhibition vs. logarithm of dilution, (diln – volumetric ratio of solution and diluent).

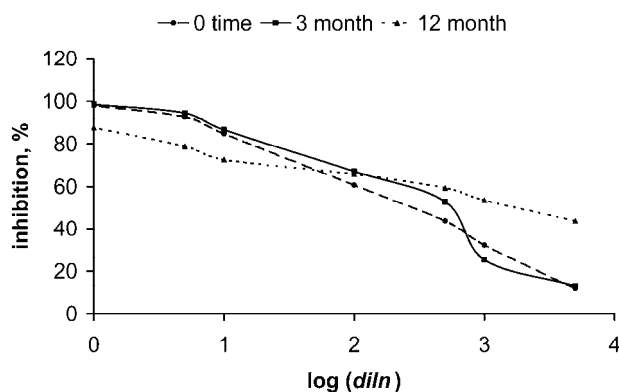


Fig. 6. IgE ELISA-inhibition of vaccine SLIT "3" during 12 months: percentage of inhibition vs. logarithm of dilution, (diln – volumetric ratio of solution and diluent).

The stability of allergen extracts expressed as IgE inhibition potential depends on many factors, including the starting material, the method of manufacture, dilution, stored temperature, *etc.* It was already noticed that the loss of potency was higher in diluted allergen extracts and at higher storage temperatures.¹⁶ Nelson *et al.* explained the loss of potency in dilute extracts by adsorption of protein onto the surface of the vial, which could be decreased by the addition of extra protein, such as human serum albumin, to the extract.¹⁷ Thereafter, the presence of proteases in some allergen extracts, which could break down allergenic proteins, was recognized as an additional cause for the loss of potency. The presence of proteases was confirmed in allergen extracts of fungi,¹⁸ cockroaches and house dust mite.^{19,20}

Allergen manufacturers usually recommend storage of commercial allergen vaccines at 4 °C for a 12-month period.¹² Guided by this recommendation, the stability of Dpt allergen vaccine intended for application in sublingual-swallow immunotherapy was investigated. Four therapeutic concentrations were analyzed: solution "3", with the highest concentration (1000 PNU), was used for the preparation of serial vaccine dilutions in the proportion 1:7. Solution "3" showed the highest stability maintained over 12 months, expressed as more than 80 % preserved inhibition potential. The other solutions also retained a high % of inhibition (more than 65 %) during the investigated period. Glycerol used in the vaccine preparation seems to be a good stabilizer for *D. pteronyssinus* allergens. A protective effect was reported for 25 % glycerol, while 50 % glycerol, which was also found to be effective in the present study, was described as being able to inhibit enzyme activity.¹⁶

CONCLUSIONS

This study showed that allergen vaccines intended for sublingual HDM immunotherapy fulfill the requirement for the estimated potency, derived from an assay of total allergenic activity to be not less than 50 % of the stated potency,²¹

by retaining more than 65 % of inhibition after 12 months storage at 4 °C. All relevant Dpt allergens were presented in the primary house dust mite extract which was used for the preparation of the vaccines. However, the rapid advancement in recombinant DNA technology, peptide synthesis and protein analysis will offer new opportunities for the design and improvements in the standardization of allergen vaccines in the near future.

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ИЗВОД

ИСПИТИВАЊЕ СТАБИЛНОСТИ ВАКЦИНЕ КУЋНЕ ПРАШИНЕ
ЗА СУБЛИНГВАЛНУ ИМУНОТЕРАПИЈУ

ЛИДИЈА БУРАЗЕР¹, КАТАРИНА МИЛОВАНОВИЋ¹, ТАЊА ЋИРКОВИЋ-ВЕЛИЧКОВИЋ²
и МАРИЈА ГАВРОВИЋ-ЈАНКУЛОВИЋ²

¹Институт за вирусологију, вакцине и серуме Торлак, Београд и

²Хемијски факултет, Универзитет у Београду, Београд

Алерген-специфична имунотерапија представља поступак који може да промени ток болести код алергијског ринитиса и астме. Квалитет вакцине припремљене од кућних гриња значајно утиче на ефикасност имунотерапеутског третмана. Испитивање је имало за циљ процену стабилности вакцине кућних гриња намењене за сублингвалну имунотерапију. Тело лиофилованих *Dermatophagoides pteronyssinus* гриња (Дрт-гриња) употребљено је као полазни материјал за производњу сублингвалне вакцине у 4 различита терапеутска разблажења. Потентност Дрт вакцина праћена ELISA-инхибицијом у 4 терапеутска разблажења, након 12 месеци задржан је у свим разблажењима у опсегу 65–80 %. Испитивање је показало да глицеролом стабилизоване Дрт вакцине за сублингвалну имунотерапију, уз чување на 4 °C задржавају алергени потенцијал (> 65 %) након 12 месеци од њихове припреме, и као такве могу се користити за третман алергије на кућне гриње.

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