

Biological characterization of glutaraldehyde-modified *Parietaria judaica* pollen extracts

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Summary

Background Allergoids are widely used in specific immunotherapy (SIT) for the treatment of IgE-mediated allergic diseases, but all techniques for standardization of conventional allergic extracts may not be appropriate for standardization of a glutaraldehyde (GA)-modified extract because of the unique characteristics of these extracts.

Objective To assess an accurate methodology for standardization of chemically modified extracts.

Methods GA-modified extracts from *Parietaria judaica* pollen were purified by diafiltration. Biochemical properties were investigated by determination of amino groups, chromatography, and SDS-PAGE. The IgE-binding activity was determined by skin prick test, enzyme allergosorbent test inhibition, basophil activation, and histamine release tests. Peripheral blood mononuclear cells (PBMCs) from *P. judaica* pollen-allergic subjects were stimulated with either native or allergoid extracts, and proliferation was measured.

Results Biochemical data indicated a high degree of allergen polymerization resulting in extract components higher than 100 kDa. IgE-binding activity, both *in vivo* and *in vitro*, was reduced by more than 99.8%. Both allergen and allergoid induced PBMC proliferation and synthesis of blocking IgG antibodies at similar rates. Moreover, no evidence of introduction of new determinants by chemical modification was found.

Conclusions The preparation of GA-modified extracts by diafiltration is faster and more reliable than previous chromatographic methods. These modified extracts have drastically reduced their allergenicity while maintaining their immunogenicity, and therefore they can be used in safer and shortened schedules of SIT.

Keywords allergoids, basophil activation, chemical modification, chromatography, histamine release test, lymphocyte proliferation, *Parietaria judaica*, skin prick test

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Introduction

IgE-mediated allergy is a global problem affecting more than 40% of the population in industrialized countries [1]. In contrast to the merely symptomatic treatments, specific immunotherapy (SIT) is the only prophylactic desensitizing therapy for allergy [2, 3]. On the other hand, the potential for local and systemic reactions has forced to improve the traditional use of conventional allergen extracts. First of all, standardized extracts have improved the reliability of SIT. Another safe approach was the change of the extracts' administration route (i.e. sublingual). Finally, a different approach involves the chemical modification of allergens in order to reduce their allergenicity (ability to elicit IgE-mediated reactions) by destroying conformational B cell allergen epitopes while maintaining their immunogenicity

(induction of IgG antibodies and modulation of the immunological response) by preserving linear T cell allergen epitopes.

Glutaraldehyde (GA) is a bifunctional aldehyde that acts as a protein cross-linker producing high-molecular-weight allergen polymers. The allergenicity of polymerized extracts diminishes because larger molecules diffuse more slowly and contain more concealed antigenic determinants, which are preferentially processed by phagocytic cells (monocytes, macrophages and dendritic cells) and could still transmit complete immunological information to antibody-producing cells. Finally, polymerized allergens have a lower molar concentration than the same weight of native proteins and thus a decreased opportunity to bridge IgE on the surface of the mast cells and cause mediator release [4]. Moreover, modified allergens lacking B cell epitopes prevent IgE binding and effective cell cross-linking and do not employ IgE-mediated antigen presentation. For example, GA-polymerized ovalbumin selectively induced a Th1-like cytokine

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synthesis pattern in mice [5]. As a result of polymerization, the proteolytic rate of polymerized allergens diminished compared with that of the monomer, and therefore provided a slowly degraded depot antigen [6, 7]. Thus, since polymerized allergen extracts are safer, they can be administered with fewer injections over a shorter time period with a lower risk of systemic reactions, and decreasing the cost and inconvenience to the patient in terms of physician visits, and therefore improving patient compliance. The efficacy of SIT with chemically modified allergens has been verified by numerous clinical trials [8–12].

Parietaria is a genus of dicotyledonous weeds of the Urticaceae family. Its pollen is recognized as an important cause of allergy, particularly in the Mediterranean area, and also in central and southern Europe and western USA. *Parietaria* allergic symptoms arise with great severity at pollen counts of half the value of grass pollen. The major *Parietaria* allergens, Par j 1 and Par j 2, are proteins of 10–14 kDa that have a prevalence of 80–90% and are responsible for more than 80% of the IgE binding [13].

In this paper, we describe the production of a standardized polymerized *Parietaria judaica* extract by a reliable technique and with a quality control based on the molecular weight characterization, free amino group determination, and enzyme allergosorbent test (EAST) inhibition. The polymerized standard has also been tested by *in vitro* and *in vivo* methods including high-performance gel filtration chromatography, antigenic response in rabbits, skin prick test (SPT), basophil activation test, histamine release test (HRT), and peripheral blood mononuclear cells (PBMCs) proliferation capacity.

Material and methods

Patients

Patients showed *P. judaica* allergy as determined by anamnesis and positive SPT responses to *P. judaica* pollen extract (Bial-Aristegui, Bilbao, Spain). Eighteen patients (12 females and six male patients, aged 13–56 years; mean age 30 years) allergic to *P. judaica* pollen were polysensitized to other pollens as well, olive, Lamb's quarter and grass pollen being the most important ones. Blood from 10 patients allergic to *P. judaica* was used for flow-cytometric cellular allergen stimulation test (FAST) and HRT. Blood was stored at 4 °C, and tests were carried out within 24 h of blood sampling. A full explanation of the procedure was given to all subjects and written consent was obtained.

GA treatment

Lyophilized aqueous extracts from *P. judaica* pollen prepared as previously described [14] were used as bulk material. Pollen extract was dissolved in 5 mg of protein/mL in phosphate-buffered saline (PBS) and cross-linked by the addition of 30 mM GA for 5 h at room temperature. Excess of reagent was neutralized with 50-fold molar excess of glycine. Low-molecular-weight pigments, glycine, and non-cross-linked allergenic proteins were eliminated by exhaustive diafiltration against water through a 500 kDa cut-off ultrafiltration device

(Pellicon XL BIOMAX 500 PE, Millipore Co., Bedford, MA, USA). The final product was freeze-dried and characterized in terms of the net weight and protein content, which was determined by Bradford's method [15] with BSA as the standard. The allergenic activity of the polymerized was calculated by taking into account the process yield and the protein enrichment over the native extract.

SDS-PAGE

Native and polymerized samples were analysed by SDS-PAGE (12.5% polyacrilamide gels) under reducing conditions using Laemmli method [16]. Protein bands were stained with Coomassie Brilliant Blue.

High-performance gel filtration liquid chromatography

Gel filtration analysis was carry out in a Smart System (Amersham Biosciences, Uppsala, Sweden) equipped with a Superdex 75 PC 3.2/30 column equilibrated with PBS at 50 µL/min. The column was previously calibrated with proteins of known molecular mass.

Free amino groups determination

Free amino groups were determined using the TNBS (2,4,6-trinitrobenzenesulfonic acid) method [17]. Optical density at 420 nm was measured and glycine was used as a standard. The degree of polymerization was refereed to the percentage of diminution of free amino groups after GA treatment and diafiltration.

EAST inhibition

The allergenic activity of both *P. judaica* pollen native extract (PNE) and *P. judaica* pollen polymerized extract (PPE) was measured by EAST inhibition using activated paper disks coupled to a native *P. judaica* extract following the procedure described by Ceska et al. [18]. Bound IgE were determined with the Hytec-specific IgE EIA test (Hycor Biomedical Inc., Kassel, Germany) as described by the manufacturer. Allergic activity was calculated on the basis of the 50% inhibition values.

Skin prick test

Skin reactivity was assessed by SPT in 18 *P. judaica*-allergic patients with different concentrations of PNE and PPE, plus a negative (PBS) and a positive control (histamine 10 mg/mL). The weal area was measured by digitized planimetry. For the purposes of analysis, the extrapolated extract dilution giving the same weal area as histamine was calculated.

Flow-cytometric cellular allergen stimulation test (FAST)

FAST of samples from 10 *P. judaica*-allergy patients was performed essentially as previously described [19]. Briefly, cells from the layer on top of the erythrocytes were collected by centrifugation and resuspended in HEPES calcium buffer (20 mM HEPES, 133 mM NaCl, 5 mM KCl, 7 mM CaCl₂, 3.5 mM MgCl₂, 1 mg/mL BSA, pH 7.4) containing IL3 (2 ng/mL) and 10 µL of heparin (5000 UI/mL). Allergens were mixed with the patient's cell suspension, and incubated

at 37 °C for 40 min. The reaction was stopped and the basophils were double labelled with anti-CD63 PE- and anti-IgE FITC-labelled antibodies (Caltag, Burlingame, CA, USA), incubated for 30 min at 4 °C; then 4 mL of an erythrolytic reagent (Ortho Diagnostic System, Madrid, Spain) was added. Flow-cytometric analysis of basophil surface markers was performed at 488 nm on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). FAST responses were considered positive when the stimulation index (% activated basophils with *P. judaica*/ % activated basophils under basal conditions) was equal to or greater than 2 and the allergen-specific activation was greater than 10%.

Histamine release test (HRT)

HRT was performed by the method of Siraganian [20], using a Brän-Lubbe autoanalyzer (Bran-Luebbe Inc., Roselle, IL, USA) according to the modifications for whole blood previously described [21]. Polyclonal anti-IgE antibodies (Tago, Caltag) at 4.2 µg/mL were used as a positive control. Histamine release was measured as: % of allergen-induced histamine release = $100 \times (\text{histamine in allergen-stimulated supernatant} - \text{basal value}) / (\text{total histamine} - \text{basal value})$, where basal = spontaneous histamine release in supernatant without stimulus and total = histamine content in blood sample measured after treatment with 2.5% perchloric acid. Values greater than 10% were considered positive.

PBMC proliferation assay

PBMC were isolated from eight *P. judaica*-allergic patients by density gradient centrifugation on lymphocyte separation solution (Lymphoprep, Nycomed, Oslo, Norway). PBMC were resuspended at 1×10^6 viable cells/mL in culture medium (AIM V serum-free Medium, Gibco, Paisley, UK) and tested for viability with 0.25% trypan blue in PBS (Sigma Chemical Co., St Louis, MO, USA). Freshly isolated PBMC (viability greater than 90%) were immediately used for *in vitro* proliferation assays. Cell proliferation was quantified by a colorimetric method according to the manufacturer's instructions (Cell proliferation Reagent WST-1, Roche Diagnostics, Barcelona, Spain). Briefly, microcultures of 2×10^5 PBMC in a final volume of 200 µL medium were performed in flat-bottom microplates (Nunc, NUNC, Roskilde, Denmark). For each sample, triplicate wells were incubated (at 37 °C and humidified atmosphere of 5% CO₂ in air) with native or polymerized antigen at a final concentration of 3 µg/mL. In all cases, triplicate unstimulated cultures were included. After 3 days, 20 µL of Cell Proliferation Reagent WST-1 was added to all wells, and plates were incubated for 4 h. Formazan produced by metabolically active cells was quantified by optical density at 450 nm. The percentage of proliferation was calculated as follows: $(S - C)/C \times 100$, where *S* and *C* represent the absorbance values for antigen-stimulated and control cells, respectively.

Rabbit antiserum production and ELISA-inhibition assays

Polyclonal antibodies were obtained by immunizing New Zealand rabbits with five boosts of 200 µg of PNE or PPE emulsified in complete Freund's adjuvant (Gibco, Detroit,

MI, USA) every 2 weeks. Sera were collected just before inoculation and 2 weeks after each injection, tested by ELISA and stored at -80 °C.

Rabbit serum titre was analysed in plates (Maxisorp, NUNC) coated with 1 µg/well of *P. judaica* extract in PBS, overnight at 4 °C. The coated wells were blocked with 200 µL of PBS containing 1% BSA and 0.05% Tween 20 (PBS-B-T). Afterwards, they were incubated with serial dilutions of rabbit serum followed by peroxidase-labelled goat anti-rabbit IgG (Sigma) diluted 1:2000 in PBS-B-T. All the incubations were carried out for 1 h at 37 °C, with three washes of 200 µL/well PBS-T between successive steps. Finally, detection was performed with a solution of 40 mM 3-dimethylaminobenzoic acid (DMAB), 0.8 mM 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH), 30 mM phosphate buffer, pH 7.0, 0.007% H₂O₂ at room temperature in the dark. The reaction was stopped after 30 min by adding 50 µL of 1 M H₂SO₄ and the absorbance was measured at 600 nm. Titres were determined as the inverse of the greatest dilution of test serum with an optical density greater or equal to 3-fold that of the control serum.

For ELISA-inhibition assay, the rabbit anti-PPE serum (1:100 000) was pre-incubated with increasing amounts of inhibitor (PNE or PPE) for 2 h at 37 °C. The plates were coated overnight at 4 °C with 0.1 µg protein of PPE allergen and the assay was carried out as described above.

Statistical analysis

SPSS 9.0 Software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Statistical analysis of FAST and HRT data was carried out by a non-parametric test for comparison of the means: Mann-Whitney test for independent groups and Wilcoxon test for the paired groups. *P* < 0.05 was considered statistically significant.

Results

Polymerization characterization

After polymerization, *P. judaica* extract appeared in SDS-PAGE as a band of high molecular mass with a 'smear' due to different components not reacting with GA. Diafiltration through a 500 kDa cut-off system eliminated free GA, excess of glycine, non-protein components present in the extract, and monomeric proteins. This preparation, after SDS-PAGE and Coomassie staining, appeared as a unique high molecular mass band (Fig. 1a). The gel permeation chromatogram profile of PPE diafiltration showed that most of the proteins eluted with the void volume pointed to an average molecular mass greater than 100 kDa (Fig. 1b).

Polymerization also carried out an efficient removal of non-protein components from the extract and thus, protein content increases drastically after polymerization. Only 14.4% of the total mass of PNE is protein, whereas PPE has 72.6% of protein. The degree of allergen modification produced by GA was monitored by the measurement of free amino groups that are targets for the cross-linker. The proportion of free amino groups after GA polymerization and diafiltration decreases by more than 91%.

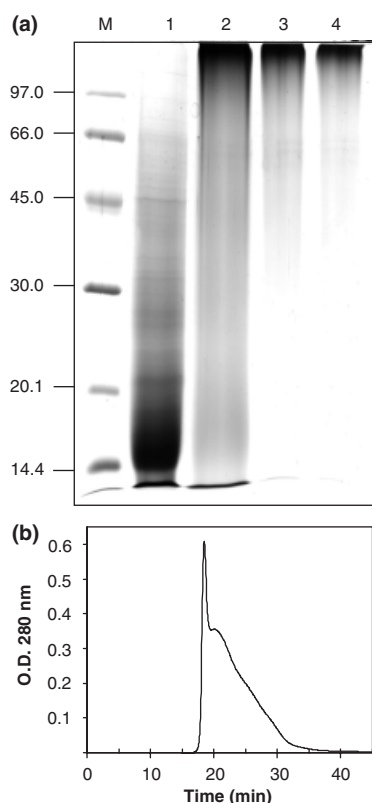


Fig. 1. (a) Coomassie-stained SDS-PAGE of pollen native extract (lane 1), glutaraldehyde-treated *Parietaria judaica* pollen extract before and after diafiltration (lanes 2 and 3, respectively), final product pollen polymerized extract (PPE) (lane 4). (b) Gel permeation chromatography of PPE.

Comparative allergenicity

The allergenic activity of both native and polymerized extracts was determined by EAST inhibition (Fig. 2a). The dose-response curves were linear ($r^2 = 0.94$ – 0.97) and parallel ($m = 16.5$ – 16.4), therefore the relative potencies measured at 50% inhibition could be compared. Eight hundred and fifty times more allergoid was needed to achieve the same degree of inhibition as that found in the non-modified allergen, indicating a 99.9% decrease in allergenicity. Skin test results showed that a 670-fold reduction in allergenicity was found in PPE, which it is also a 99.9% decrease (Fig. 2b).

The biological activity of native or polymerized *P. judaica* was analysed by FAST and HRT. Blood from 10 *P. judaica* allergic patients with positive SPT responses to *P. judaica* extract (Bial Aristegui; median 45 mm^2), and five controls was used. Table 1 summarizes the results obtained with the different allergen concentrations, which produced basophil activation or mediator release in blood samples. Ten out of 10 patients showed positive FAST both with native and polymerized *P. judaica* and HRT was considered positive in nine and eight patients for PNE and PPE, respectively. None of the controls were positive in any tests at any of the concentrations assayed. PNE was tested at 7, 0.14, and $0.07 \mu\text{g/mL}$ and PPE was assayed at 10-fold of the concentration used for the native extract (70, 1.4 and $0.7 \mu\text{g/mL}$), as preliminary assays showed a very low response

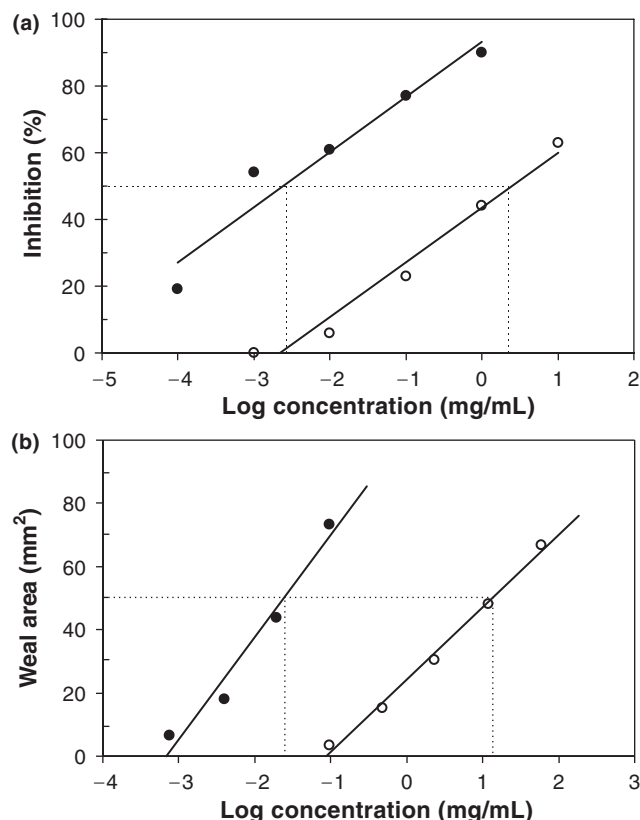


Fig. 2. (a) Enzyme allergosorbent test inhibition of pollen native extract (PNE) (●) and pollen polymerized extract (PPE) (○). The results are expressed as percentage inhibition of binding to solid-phase PNE by different amounts of PNE and PPE. (b) Results of the skin prick test testing of PNE (●) and PPE (○) performed on 10 *Parietaria judaica*-allergic subjects.

when PPE was used at a similar concentration as PNE. We observed lesser responses in FAST and HRT with 10 times higher polymer concentrations than with the monomer ones, so that PPE provoked a basophil activation and histamine release significantly inferior to the monomer, in spite of using a concentration 10 times higher. In FAST, we observed a basophil activation significantly inferior to the polymerized extract than the monomer extract when the highest and lowest concentrations of both allergenic extracts were compared (monomer $7 \mu\text{g/mL}$ vs. polymerized $70 \mu\text{g/mL}$, $P = 0.007$; monomer $0.07 \mu\text{g/mL}$ vs. polymerized $0.7 \mu\text{g/mL}$, $P = 0.047$). In HRT, a greater histamine release was observed when the monomer $0.14 \mu\text{g/mL}$ (median 41.5) was compared with the polymer $1.4 \mu\text{g/mL}$ (median 5.4) ($P = 0.021$).

Comparative immunogenicity

In order to verify the ability of PPE to induce blocking IgG antibodies against the native allergen, rabbits were immunized with either native or polymerized allergens. PPE-immunized rabbits produced similar IgG responses as those immunized with PNE as determined by ELISA (Fig. 3a). Inhibition studies were also carried out to demonstrate whether new antigenic determinants were introduced by GA treatment (Fig. 3b). Inhibition curves were similar, thus

Table 1. FAST and HRT responses induced by monomeric and polymeric extract of *Parietaria judaica* (PNE and PPE, respectively) in blood from patients allergic to *P. judaica*

Patient	FAST (% basophil activation/stimulation index)						HRT (% histamine release)			
	PNE ($\mu\text{g/mL}$)			PPE ($\mu\text{g/mL}$)			PNE ($\mu\text{g/mL}$)		PPE ($\mu\text{g/mL}$)	
	7	0.14	0.07	70	1.4	0.7	7	0.14	70	1.4
1	87.8/43	85.6/38	89.4/40	51.4/23	89.2/40	74.5/33	75.0	38.9	87.5	0.0
2	71.3/28	73.7/29	71.9/28	59.9/23	75.6/30	67.8/27	26.7	28.2	54.2	0.0
3	86.5/36	58.7/24	30.5/12	54.8/22	25.7/10	22.1/9	15.4	0.6	1.3	0.6
4	81.2/36	80.4/36	66.6/30	16.7/7	2.9/1	0.65/0.2	6.7	0.0	0.0	2.2
5	77.6/26	60.2/20	48.2/16	63.6/21	13.2/4	4.4/1	65.4	57.7	83.3	64.1
6	76.8/13	81.5/14	85.6/14	79.4/15	87.0/13	85.2/15	51.7	51.7	65.5	16.1
7	69.3/2	68.9/2	59.1/2	27.0/0.8	73.7/2	76.1/2	50.9	44.1	10.2	8.5
8	66.8/7	74.1/5	66.9/4	13.1/0.8	57.5/3	54.7/3	31.8	34.9	51.5	4.6
9	57.3/143	29.1/72	30.1/75	13.2/33	26.2/65	28.5/71	52.3	54.3	38.5	6.2
10	89.8/74	64.6/53	70.8/59	19.2/16	52.1/43	67.4/56	85.2	74.2	60.5	15.2
Median										
	$P = 0.007$						$P = 0.021$			
Patients	77.2	71.3	66.8	39.2	54.8	61.1	51.3	41.5	52.9	5.4
Controls ($n = 5$)	2.2*	0.5*	2.4*	1.4*	0.8*	2.7*	2.1*	2.2*	2.3*	3.2*

* $P < 0.01$ between controls and patients. FAST, flow-cytometric cellular allergen stimulation test; HRT, histamine release test; PNE, pollen native extract; PPE, pollen polymerized extract.

providing no indication that polymerization produced new antigenic determinants.

Homogeneity in the T cell reactivity of PBMC from *P. judaica* allergic subjects to PNE as compared with PPE was observed. No significant differences ($P < 0.05$; Wilcoxon Test) were found between both extracts at the three concentrations tested (Fig. 4). In all except one, PBMC proliferated to a similar extent with both allergenic extracts at all the tested concentrations (12, 6, 3 $\mu\text{g/mL}$). Both extracts were able to produce a proliferation between 25 and 175 % over the control cells. None of the extracts induced proliferation when PBMC from no allergic subjects were used.

Discussion

The clinical benefits of immunotherapy can be achieved more rapidly, safely and with fewer injections by the use of polymerized pollen allergens instead of the conventional native extracts [8–12]. The high molecular weight of the polymerized extract components could have more difficulty in crossing the mucosa and, therefore, these extracts have mainly been administered by subcutaneous injection in clinical trials up to now. These modified allergens must have a greatly reduced allergenic reactivity compared with the native allergen while retaining immunogenicity, thus maintaining the capacity of inducing tolerance. Modified allergens, lacking B cell epitopes, utilize phagocytic or pinocytic antigen-uptake mechanisms through monocytes, macrophages, or dendritic cells, by-passing IgE-cross-linking and IgE-mediated antigen presentation in mast cells, basophils, Langerhans cells, eosinophils, and epidermal cells. This effect induces a balanced Th0 or Th1-like cytokine pattern by T cells, resulting in a lower IgE and higher IgG production by memory B cells [7, 22]. Because of the low IgE-mediated side-

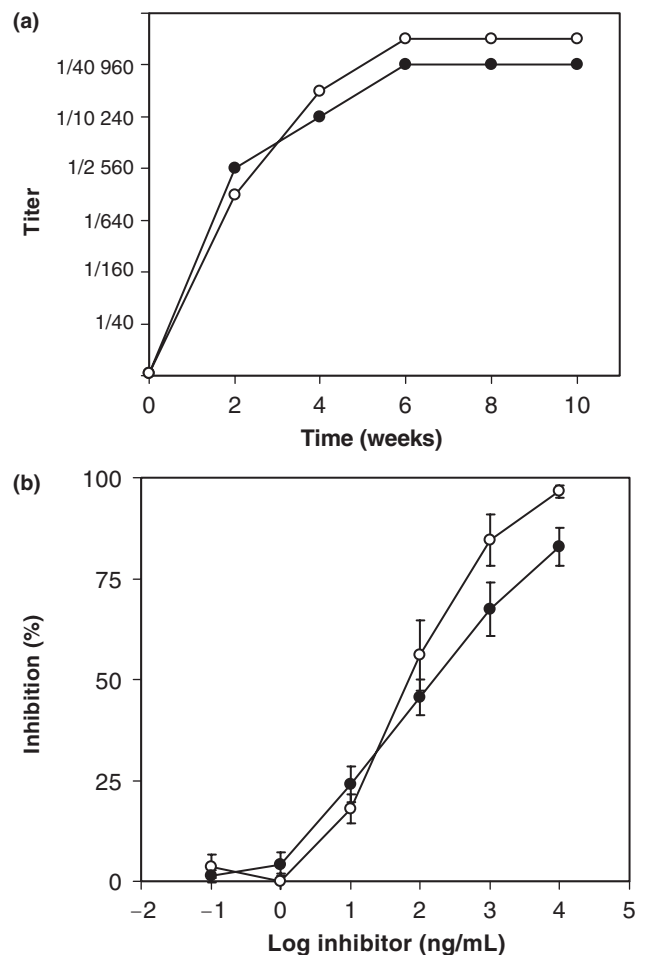


Fig. 3. (a) Rabbit IgG antibody response to immunization with pollen native extract (PNE) (●) and pollen-polymerized extract (PPE) (○). (b) Absence of new antigenic determinants in PPE as illustrated by the inhibition of rabbit IgG against PPE by PNE (●) and PPE (○).

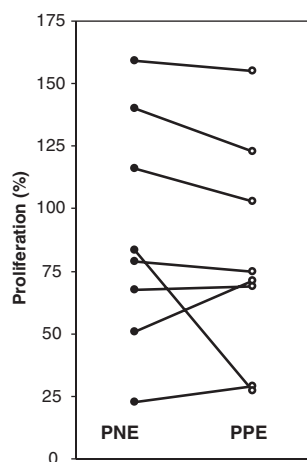


Fig. 4. Comparison of the proliferative response of peripheral blood mononuclear cell from *Parietaria judaica*-allergic subjects to pollen native extract (PNE) (●) and pollen-polymerized extract (PPE) (○). The percentage of proliferation was calculated as follows: $(S - C)/C \times 100$, where *S* and *C* represent the absorbance values for antigen-stimulated and control cells, respectively. A proliferation of 100% indicates that cells are duplicated after stimulation with native (PNE) or polymerized (PPE) extract.

effects of modified allergen extracts, they can be administered in a high-dose schedule with a low risk, but the diminution of allergenicity of these products can be routinely assured with laboratory tests.

Several *in vitro* and *in vivo* methods are available to characterize GA-polymerized allergoids, but not all of them are applicable as routine assays. An accurate assessment of the polymerization process can be performed by electrophoresis and chromatography, checking the absence of proteins with molecular masses lower than 100 kDa and high molecular mass components. Moreover, reduction of free amino groups in PPE (>91%) is consistent with the loss of IgE reactivity conferred by GA modification. The overall reduction of free amino groups (>90%) found in our preparations of pollen and mite-modified extracts are higher than the reported data of only 70% reduction in tree-modified extracts [23]. The diafiltration process is faster and more reliable than the classical gel filtration chromatographic method [24] and furthermore produces the elimination of flavonoids, pigments, and other non-proteinaceous substances found in the native extract, resulting in extracts with a higher protein content than the native counterparts.

Direct skin testing (intradermal and prick testing) of allergic subjects has been the main *in vivo* method used to compare the allergenicity of the modified extracts with the native extracts [4, 25, 26]. PPE induced a significant decrease (higher than 600-fold) in skin test reactivity, which is higher than the 90-fold decrease found with allergoid preparations from timothy pollen extracts [25]. In FAST and HRT, PPE provoked basophil activation and histamine release significantly inferior to the monomer, in spite of using a concentration 10 times higher. EAST-inhibition experiments showed that PPE had an 850-fold less allergenicity than the native counterpart, which is in accordance with skin test data. A 100- to 1000-fold less allergenicity in PPE than PNE was also obtained with the 2-step conventional gel filtration method for GA-modified extracts [6]. Thus, for reasons of practicability and better reproducibility, EAST inhibition was

considered the preferential method for the routine test of residual allergenicity in GA-modified extracts. The slopes of the curves for PPE and PNE, in both EAST inhibition and prick test, were not significantly different, which indicated that there were no changes at IgE epitopes because of the chemical modification.

One of the claims for modified allergens is to retain immunogenicity. Studies performed in rabbits demonstrated that PPE was able to induce blocking IgG antibodies against native allergen in a similar way as native allergen. Moreover, there was no indication that GA treatment introduced new antigenic determinants (Fig. 3b), as demonstrated in wasp venom [27]. PBMC proliferation assay (Fig. 4) showed that both native and polymerized extracts were able to provoke lymphoproliferation to the same extent. In contrast, Dormann et al. found a lower proliferation capacity in PBMC induced with formaldehyde-modified birch extract than those induced with the native counterpart. Structural changes within the allergen molecule could produce differences in the availability of T cell epitopes between formal allergoid and unmodified allergens [28]. In our case, it has been proven that the modified extract was able to produce similar T cell activation, thus suggesting that T cell determinants were intact. However, the degree of T cell stimulation by the allergoids seems to be dependent on the type of antigen-presenting cell [29]; using dendritic cells and macrophages, allergoids exhibited superior T cell stimulating capacity as that found with PBMC [30].

The initial process of antigen standardization should include both *in vivo* and *in vitro* assay procedures, but subsequent extract batches could be screened for potency with a simple *in vitro* assay. Quality control tests for modified allergens should include the control of polymerization by the measurement of free amino group diminution (>90%), lack of protein bands of less than 100 kDa by SDS-PAGE, and reduction of allergenicity greater than 500-fold, determined by EAST inhibition.

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References

- 1 The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. *Lancet* 1998; 351:1225–32.
- 2 Durham SR, Walker SM, Varga EM et al. Long-term clinical efficacy of grass-pollen immunotherapy. *N Eng J Med* 1999; 341:468–75.

- 3 Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. *Allergy* 1998; 53:1–42.
- 4 Grammer LC, Shaughnessy MA, Patterson R. Standardization of modified allergens using polymerized ragweed as a model system. *Clin Rev Allergy* 1987; 5:107–16.
- 5 Gieni RS, Yang X, Kelso A, Hayglass KT. Limiting dilution analysis of CD T-cell cytokine production in mice administered native vs. polymerized ovalbumin: directed induction of T-helper type 1-like activation. *Immunology* 1996; 87:119–26.
- 6 Patterson R, Suszko IM, Pruzansky JJ, Zeiss CR, Metzger WJ, Roberts M. Polymerization of mixtures of grass allergens. *J Allergy Clin Immunol* 1977; 59:314–9.
- 7 Gieni RS, Yang X, Kelso A, HayGlass KT. Limiting dilution analysis of CD4 T-cell cytokine production in mice administered native vs. polymerized ovalbumin: directed induction of T-helper type-1-like activation. *Immunology* 1996; 87:119–26.
- 8 Ariano R, Kroon AM, Augeri G, Canonica GW, Passalacqua G. Long-term treatment with allergoid immunotherapy with *Parietaria*. Clinical and immunologic effects in a randomized, controlled trial. *Allergy* 1999; 54:313–9.
- 9 Tari MG, Mancino M, Ghezzi E, Frank E, Cromwell O. Immunotherapy with an alum-adsorbed *Parietaria*-pollen allergoid: a 2-year, double-blind, placebo-controlled study. *Allergy* 1997; 52:65–74.
- 10 Bousquet J, Hejjiaoui A, Soussana M, Michel FB. Double-blind, placebo-controlled immunotherapy with mixed grass-pollen allergoids. IV. Comparison of the safety and efficacy of two dosages of a high-molecular-weight allergoid. *J Allergy Clin Immunol* 1990; 85:490–7.
- 11 Pastorello EA, Pravettoni V, Incorvaia C et al. Clinical and immunological effects of immunotherapy with alum-adsorbed grass allergoid in grass-pollen-induced hay fever. *Allergy* 1992; 47:281–90.
- 12 Hendrix SG, Patterson R, Zeiss CR et al. A multi-institutional trial of polymerized whole ragweed for immunotherapy of ragweed allergy. *J Allergy Clin Immunol* 1980; 66:486–94.
- 13 Colombo P, Duro G, Costa MA et al. An update on allergens. *Parietaria* pollen allergens. *Allergy* 1998; 53:917–21.
- 14 García Ortega P, Martínez J, Martínez A, Palacios R, Belmonté J, Richart C. *Mercurialis annua* pollen: a new source of allergic sensitization and respiratory disease. *J Allergy Clin Immunol* 1992; 89:987–93.
- 15 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 1976; 72:248–54.
- 16 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature* 1970; 227:680–5.
- 17 Willis TW, Tu AT. Purification and biochemical characterization of atroxase, a nonhemorrhagic fibrinolytic protease from western diamondback rattlesnake venom. *Biochemistry* 1988; 27:4769–77.
- 18 Ceska M, Eriksson R, Varga J. Radioimmunosorbent assay of allergens. *J Allergy Clin Immunol* 1972; 49:1–9.
- 19 Sanz ML, Sánchez G, Gamboa Vila L, Uasuf C, Chazot M. Allergen-induced basophil activation: CD63 cell expression detected by flow cytometry in patients allergic to *Dermatophagoides pteronyssinus* and *Lolium perenne*. *Clin Exp Allergy* 2001; 31:1007–13.
- 20 Siraganian RP. Histamine secretion from mast cells and basophils. *Trends Pharmacol Sci* 1983; 4:432–7.
- 21 Resano A, Prieto I, Sanz ML, Oehling A. Reliability of histamine release test in dust mite allergy: influence of the sensitisation degree. *J Invest Allergol Clin Immunol* 1995; 5:289–93.
- 22 Akdis CA, Blaser K. Regulation of specific immune responses by chemical and structural modifications of allergens. *Int Arch Allergy Immunol* 2000; 121:261–9.
- 23 Hopkins M, Lees BG, Richardson DG, Woroniecki SR, Wheeler AW. Standardisation of glutaraldehyde-modified tyrosine-adsorbed tree pollen vaccines containing the Th1-including adjuvant, monophosphoryl lipid A (MPL). *Allergol Immunopathol* 2001; 29:245–54.
- 24 Patterson R, Suszko IM, Grammer LC, Shaughnessy MA. Polymerization of individual species of grass pollen allergens. *J Allergy Clin Immunol* 1983; 72:129–33.
- 25 Wihl JA, Pilstrom L, Maasch HJ. Studies on allergen and allergoid preparations from purified timothy (*Phleum pratense*) pollen extracts. III. Comparative investigations by skin prick tests and nasal provocation tests. *Int Arch Allergy Appl Immunol* 1985; 76:162–7.
- 26 Cirkovic T, Jankulovic MG, Prisc S et al. The influence of a residual group in low-molecular-weight allergoids of *Artemisia vulgaris* pollen on their allergenicity, IgE- and IgG-binding properties. *Allergy* 2002; 57:1013–20.
- 27 Gewurz A, Grammer LC, Shaughnessy MA, Patterson R. Soluble copolymer of wasp venom with human albumin for venom immunotherapy. *J Allergy Clin Immunol* 1986; 77:520–3.
- 28 Dormann D, Ebner C, Jarman ER, Montermann E, Kraft D, Reske-Kunz AB. Responses of human birch pollen allergen-reactive T cells to chemically modified allergens (allergoids). *Clin Exp Allergy* 1998; 28:1374–83.
- 29 Kahlert H, Stüwe H-Th, Cromwell O, Fiebig H. Reactivity of T cells with grass pollen allergen extract and allergoid. *Int Arch Allergy Immunol* 1999; 120:146–57.
- 30 Kahlert H, Griebenow EG, Stüwe H-Th, Cromwell O, Fiebig H. T cell reactivity with allergoid: influence of the type of APC. *J Immunol* 2000; 165:1807–15.