Original Article

Skin tests with native, depigmented and glutaraldehyde polymerized allergen extracts

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Abstract. *Introduction:* Dose-response skin prick tests are an important tool to standardise allergen extracts and to evaluate changes in skin test response as a consequence of allergen modifications.
Objectives: To evaluate *in vivo* and in *vitro* characteristics of 3 different types of extracts of *Phleum pratense, Olea europaea, Parietaria judaica* and *Dermatophagoides pteronyssinus.*
Material and methods: Three types of extracts were used: native unmodified extracts (N), depigmented extracts (DP) (extracts subjected to a mild acid treatment under controlled conditions and dialysis), and a depigmented glutaraldehyde polymerised extract (DPP). Adult patients were skin tested in duplicate with the 3 types of extracts. The dose-response relationship between the geometric mean of the wheal areas and the allergen concentrations was calculated for each patient using regression line analysis. The amount of freeze-dried allergen preparation needed to produce the same wheal size as histamine was calculated in each patient (individual 10 HEP) and for each of the 3 types of extracts. *In vitro* analysis consisted of major allergen determinations and specific IgE and IgG inhibitions.
Results: The respective 10 HEP values for N, DP and DPP preparations were 0.20 mg, 0.15 and 2.11 for *D. pteronyssinus*. For *P. pratense*, these values were 0.02 mg, 0.02 and 0.99; for *O. europaea* 0.15, 0.44 and 4.9; and for *P. judaica* 0.01, 0.008 and 1.78 mg.
Conclusions: The polymerised depigmented extracts are significantly less allergenic than the corresponding native and depigmented extracts. This could provide a greater safety margin for the administration of higher doses of immunotherapy in a shorter period of time.

Key words: skin tests, depigmented allergen extracts, glutaraldehyde polymerized allergen extracts, allergoids

Introduction

Polymerization with glutaraldehyde is a modification procedure of allergen extracts that has been extensively studied for the production of high molecular weight allergoids. With this process, IgE-binding epitopes are hidden within a complex macromolecular network, which leaves most of the antigenic determinants accessible for the induction of IgG antibodies. T cell epitopes are left untouched for T cell recognition [1-5]. Therefore, glutaraldehyde-modified therapeutic allergic vaccines are safer than unmodified vaccines, while retaining clinical efficacy [4, 5].

A depigmentation step before the polymerization with glutaraldehyde has been developed. This previous step inactivates the enzymatic activity of mite extracts [6], removes the pigments [7] and enhances the solubility of the final allergoid. The resulting depigmented polymers have been used in several clinical studies [8-13], and showed clinical efficacy.

The aim of this study was to evaluate the *in vivo* (skin prick test) reactions provoked by native, depigmented and

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polymerized extracts of *Phleum pratense*, *Olea europaea*, *Parietaria judaica*, and *Dermatophagoides pteronyssinus*, in order to establish the relative potency of each allergen preparation.

Material and Methods

Allergen extracts

One hundred grams of a full grown whole *D. pteronyssinus* culture and 100 grams of pollen of *P. pratense*, *O. europaea* and *P. judaica* were extracted 1:20 weight/volume in PBS followed by diafiltration in 3 kDa Pellicon membranes (Millipore, Madrid, Spain) to eliminate salts and the bulk of unbound low molecular weight components. Afterwards, the extracts were freeze-dried, thus obtaining the native (N) extracts.

The next depigmentation step followed a patented procedure [14]. Briefly, the N extract was incubated at pH 2 and extensively dialysed overnight in 3.5 kDa cut-off membranes (Spectra/Por, Houston, TX, USA). The pH gradually increased until the starting pH was reached. This procedure further removed pigments and originated the depigmented (DP) extracts. The resultant depigmented (DP) allergen preparations were treated with glutaraldehyde to produce a polymer (DPP), using previously described methods [15, 16]. This step was followed by dialysis to remove components with a molecular weight of less than 100 kDa.

The protein contents of each extract was measured using the method of Bradford [17]. The micro-Kjeldahl method was used to determine the protein nitrogen units (PNU) per mg of each freeze-dried extracts.

Major allergen content

Major allergen content was measured in each preparation. Der p 1, Der p 2 were measured by ELISA using monoclonal antibodies (Indoor Biotechnologies Ltd., Charlottesville, VA, USA). Phl p 1, Phl p 5, Par j 1 and Ole e 1 were measured by scanning densitometry [18]. The results were expressed as the quantity, in micrograms, of major allergen per mg of freeze-dried extract.

Patients and skin testing

The patient population consisted of 25 adult individuals (14 male and 11 female) sensitive patients to *D. pteronyssinus*; 30 to *P. pratense* (18 male and 12 female), 32 to *O. europaea* (12 male and 20 female), and 34 to *P. judaica* (9 male and 25 female). All these patients were skin prick tested according to the rules of the Nordic Council on Medicines [19] with slight modifications. Skin prick tests were conducted in duplicate in the back using 3 serial 10-fold dilutions of each extract. Histamine HCl 10 mg/ml and glycerinated saline solution were used as positive and negative controls, respectively. Skin tests were done between 9:00 A.M. and 11 A.M. None of the patients was pretreated with drugs which could affect the performance of the test. Reactions were recorded after 15 minutes of application [20]. Wheal areas were outlined with a fine tip marker and transferred, with transparent tape, to the corresponding sheet of a case report form. The area of each wheal was measured by planimetry using a Wacom pallette (Wacom Tehnology Co., USA) connected to the MacDraft software (Microspot Inc., USA).

A serum sample from each patient was collected, and a pool of sera from the sensitive patients tested for each allergen was prepared and stored in 0.5 ml aliquots at -70º C until use for specific IgE and IgG binding inhibition assays.

Specific IgE and IgG Inhibition assays

The inhibition of specific IgE binding was evaluated by a reverse phase specific IgE-binding competition ELISA [21] and the IgG binding activity was measured by means of an ELISA specific IgG inhibition assay [22]. In both cases, the in-house reference preparation of each native allergen extract was used as standard and the results were expressed as the quantity (micrograms) of freeze-dried material added to the well of the microplate to achieve the 50% inhibition.

Statistical analysis

For each patient, the bioassay of dose-response relationship between the allergen concentration and the skin reaction was estimated by regression line analysis $\log(A) = a + b \times \log(C)$, in which A is the geometric mean of the 2 wheal areas provoked by each allergen concentration and C is this allergen concentration [23]. Regressions were carried out with the statistics package SPSS (SPSS, Inc., Chicago, IL, USA). Linearity was tested by F test (Analysis of Variance). For testing the regression lines for parallelism (i.e., equality of slopes) the *t* test was used [24].

For each patient, the individual bioequivalent dose of allergen extract to achieve a wheal of the same size as positive control (individual 10 HEP) was calculated [19]. General descriptive statistics of these values were calculated, including the median which is the value of 10 HEP for the population sensitive to each allergen [19]. The non-parametric test of Friedman was used to compare the individual 10 HEP of each preparation from each allergen extract. The Wilcoxon test was used to compare these results between pairs.

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Table I. Values (µg of each allergen preparation) of the 50% binding inhibition to specific IgE and IgG, major allergen contents (µg per mg of freeze-dried extract), protein contents (µg per mg of freeze-dried extract) and PNU (mg of freeze-dried extract). (*ND: Not Detectable).

	IgE			IgG			Major allergens			Protein contents			PNU		
	N	DP	DPP	N	DP	DPP	N	DP	DPP	N	DP	DPP	N	DP	DPP
D. pteronyssinus	0.43	33.33	27.78	0.02	0.01	0.01	Der p 1: 15.6 Der p 2: 3.1	Der p 1: 0.4 Der p 2: 3	Der p 1: 0.3 Der p 2: ND*	473	432	556	9013	9891	6995
P. pratense	0.09	0.45	16.13	0.18	0.02	0.82	Phl p 1: 31.4 Phl p 5: 43.8	Phl p 1: 27.7 Phl p 5: 49.5	Phl p 1: ND* Phl p 5: ND*	382	526	786	9310	10310	10290
O. europaea	0.12	2.91	20.83	0.11	0.16	0.31	Ole e 1: 226.6	Ole e 1: 288.8	Ole e 1: ND*	207	247	343	9674	10520	10066
P. judaica	0.03	0.01	2.29	0.34	0.14	0.15	Par j 1: 583.4	Par j 1: 819.7	Par j 1: ND*	147	260	455	9687	10635	8771

Table II. Parameters of the regression analysis of the dose-response bioassay (slope, intercept, correlation coefficient, F test for linearity and its P value) of each allergen extract.

	D. pteronyssinus			*P. pratense*			*O. europaea*			*P. judaica*		
	N	DP	DPP	N	DP	DPP	N	DP	DPP	N	DP	DPP
slope (b)	0.384	0.395	0.373	0.444	0.424	0.451	0.339	0.394	0.389	0.384	0.395	0.373
standard error or the slope	0.001	0.011	0.021	0.039	0.046	0.036	0.019	0.040	0.051	0.001	0.011	0.021
intercept (a)	1.807	1.813	1.403	2.415	2.419	2.036	1.924	1.819	1.109	1.892	1.901	1.486
r^2	1.00	1.00	1.00	0.99	0.99	0.99	1.00	0.99	0.98	0.98	1.00	1.00
F test	100248	1397.69	323.92	224.56	194.13	261.79	325.15	525.14	5779.53	100248	1397.69	323.92
P	0.002	0.017	0.035	0.042	0.046	0.039	0.035	0.028	0.008	0.002	0.017	0.035

Table III. Descriptive statistics of the values of the skin prick test, and relative potency of each preparation (N, DP, and DPP) of the extracts.

	D. pteronyssinus			*P. pratense*			*O. europaea*			*P. judaica*		
	N	**DP**	**DPP**	**N**	**DP**	**DPP**	**N**	**DP**	**DPP**	**N**	**DP**	**DPP**
Mean	0.48	1.26	190.42	0.03	0.03	3.05	0.25	0.90	636.42	0.02	0.04	3.74
95% upper limit	0.49	1.30	200.67	0.03	0.03	3.11	0.25	0.91	652.51	0.02	0.04	3.83
95% lower limit	0.47	1.21	180.17	0.03	0.03	2.99	0.24	0.88	620.33	0.02	0.04	3.64
St. dev.	0.73	3.38	783.88	0.02	0.04	4.79	0.25	1.26	1118.69	0.03	0.12	8.54
Median (10 HEP)	0.20	0.15	2.11	0.02	0.02	0.99	0.15	0.44	4.19	0.01	0.00	1.78
95% upper limit	0.410	0.410	5.520	0.044	0.030	3.243	0.340	0.770	751.300	0.017	0.008	3.682
95% lower limit	0.080	0.080	1.180	0.008	0.006	0.234	0.070	0.150	1.000	0.003	0.001	1.217
Relative potency	1.000	1.383	0.096	1.000	1.339	0.025	1.000	0.347	0.037	1.000	2.648	0.004

The relative potency of each allergen preparation was calculated comparing the value of 10 HEP of the DP and DPP with N.

Results

Extracts

The protein contents, the protein nitrogen units, the quantity of each preparation to produce a 50% inhibition in specific IgE and IgG binding and the contents of major allergen are shown in Table I.

Skin tests

All the preparations of each allergen produced measurable skin response, except the DPP preparation of *O. europaea*, which gave negative skin test responses in most sensitive patients. Table II shows the parameters of the regression analysis of the dose-response bioassay (slope, intercept, correlation coefficient, F test for linearity and its P value) for each allergen extract. The F test of the analysis of variance had a P value of < 0.05 in all cases indicating that the dose-response is linear. The analysis of parallelism (t test) showed that, in each allergen, the differences in the value of the slopes are not significant ($p > 0.05$), thus the lines could be considered parallel and the results used for comparative purposes.

Figure 1 shows the regression lines of each preparation of the allergen extracts studied. Table III shows the descriptive statistics of the individual 10 HEP of each allergen preparation, the relative potency and the quantity of extract needed to obtain 10 HEP in the studied population.

The Friedman test showed that the differences obtained with the 3 preparations of each extract are significant in all cases ($P < 0.0001$). The Wilcoxon test demonstrated that the significant differences occur between N and DPP, and DP and DPP, whereas the comparisons between N and DP were not significant, except in the case of *O. europaea*, in which the wheal sizes induced by the DP preparation were smaller than those obtained with N. Table IV summarises these statistical comparisons.

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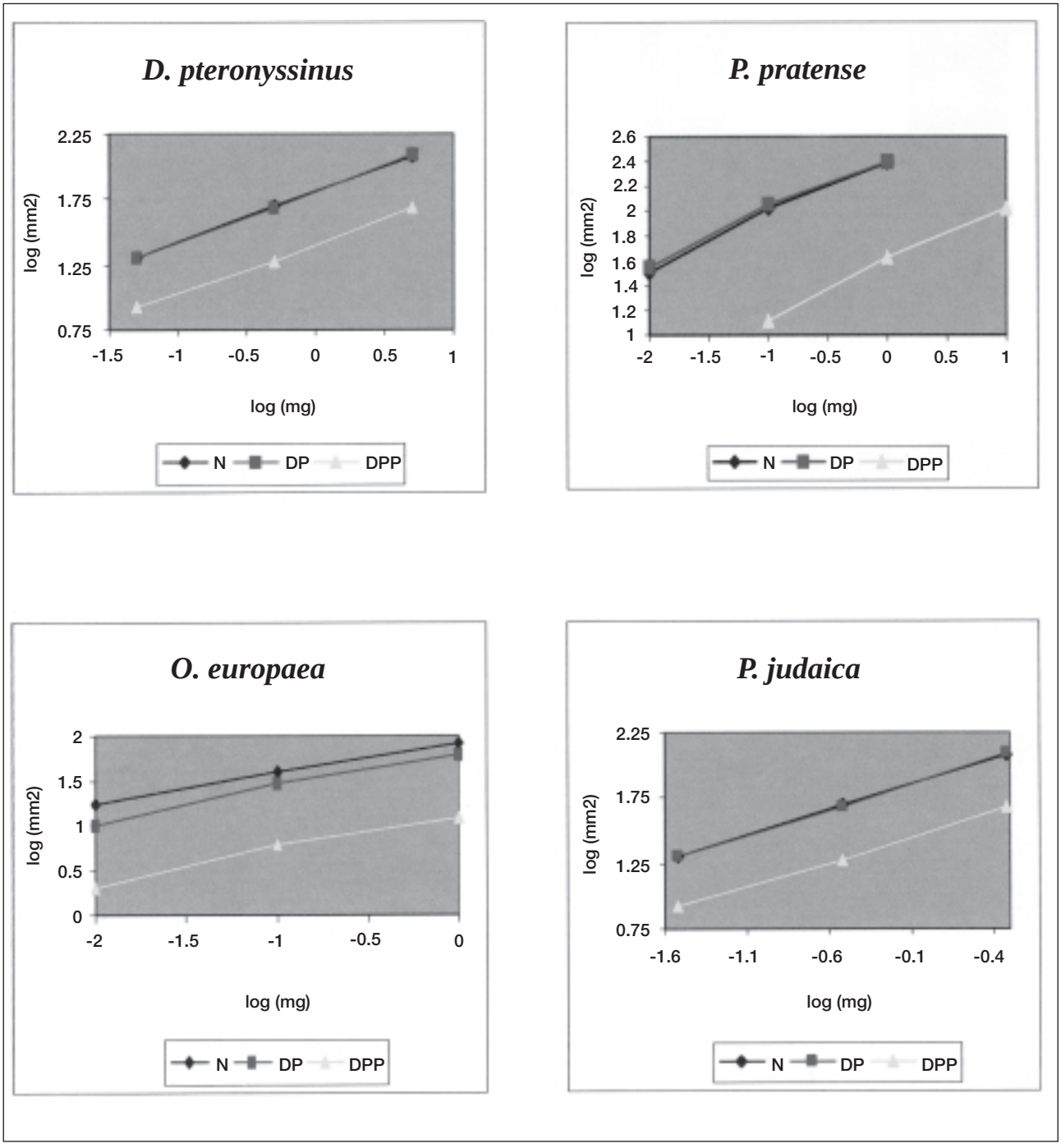

Figure 1. Regression lines (log-log) of the studied extracts. In all cases, the wheals induced by DPP preparations are significantly lower than those induced by N and DP.

Discussion

We describe the results of skin testing allergic individuals with unmodified, depigmented and depigmented and polymerized extract of *D. pteronyssinus*, *P. pratense*, *P. judaica* and *O. europaea*. The results confirm a significant reduction in skin test reactivity when the patients were skin tested with the allergoid.

Dose-response skin testing provides the possibility to estimate differences in the relative potency of different extracts. This method is well documented and the methodological errors have been carefully analysed and accepted [25, 26]. We have used this bioassay to compare

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Table IV. Statistical analysis of comparing the results of prick tests between the 3 preparations of each allergen extract (Friedman test), and between pairs (Wilcoxon signed rank test) of preparations. (* Chi square corrected for ties).

	Friedman		Wilcoxon					
	N-DP-DPP		N-DP		N-DPP		DP-DPP	
	Chi Sq.*	P	Z	P	Z	P	Z	P
D. pteronyssinus	29.43	< 0.0001	- 0.83	0.41	- 4.17	< 0.0001	- 4.14	< 0.0001
P. pratense	30.64	< 0.0001	- 0.60	0.55	- 4.11	< 0.0001	- 4.07	< 0.0001
O. europaea	30.63	< 0.0001	- 3.90	< 0.0001	- 3.82	< 0.0001	- 3.82	< 0.0001
P. judaica	44.13	< 0.0001	- 1.37	0.17	- 4.86	< 0.0001	- 4.84	< 0.0001

the strengths of native and modified allergen extracts.

In order to compare the results and to calculate the relative potencies of the different extracts, the regression lines must be linear and parallel [27]. The F test demonstrated that there is no evidence of lack of linearity, and the t test indicated that the lines were parallel, suggesting that the analysed extracts retain their specificity in the allergic response and that the allergic patients only recognise quantitative differences in these extracts. The relative potency was calculated using the median of the values necessary to produce a wheal size comparable with 10 HEP, instead of the mean, because the latter is not influenced by extreme results [28]. The use of median values is also recommended by the Nordic Guidelines for allergen standardisation [19]. All DPP demonstrated a significant reduction in skin test reactivity, the relative potency ranging from 0.096 for *D. pteronyssinus* to 0.004 for *P. judaica*. The *in vivo* results are in agreement with the *in vitro* results, which suggest an important reduction in the capacity to produce an allergic response.

Patients were also skin tested with the depigmented preparations. With the exception of *O. europaea*, the relative potency of the DP preparation is > 1, meaning that the potency of the depigmented preparation is greater than that of the native extract. In *D. pteronyssinus* there is a discrepancy between the reduction in specific IgE binding *in vitro* and the result obtained *in vivo*. It seems that the depigmentation process affects IgE binding *in vitro*, whereas *in vivo* the allergenic activity remains without modification.

An important finding is that the Der p 1 content in the depigmented extract used in this trial is lower than in the native extract, whereas Der p 2 is not affected by the modification. Based on these results, it seems that the tertiary structure of Der p 1 may be affected by the modification process, and the molecule is no longer recognised by the monoclonal antibody used. These results are in agreement with those of Van der Zee et al. [29] in which Der p 1 depletion of body extracts did not induce a significant reduction in skin test reactivity.

On the other hand, the same could be applied to the extract of *O. europaea*, in which the quantification of Ole e 1 in DP extract is higher than in N extract, whereas the size of the wheals is lower using DP extracts.

The results obtained showed that the depigmented-glutaraldehyde polymerized allergen extracts have an important reduction of the *in vivo* capacity to induce a specific skin response without modification of the antibody

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binding affinity, as shown by the test of parallelism of the slopes. This reduction in allergenicity provides a safety margin that permits the administration of higher doses of immunotherapy in a shorter period of time. At the same time, the test for parallelism demonstrated that the slopes were parallel, which suggests that all three preparations retain the specific antibody binding affinity.

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