Depigmented and Polymerised House Dust Mite Allergoid: Allergen Content, Induction of IgG4 and Clinical Response

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Key Words

Allergic asthma · Mites · Allergens · Depigmented and polymerised extracts · Sequencing allergens · IgG4

Abstract

Background: Polymerised allergenic extracts (allergoids) are commonly used in allergen immunotherapy. Clinical efficacy and safety of these extracts have been demonstrated. Recently, allergen sequences have been identified by mass spectrometry in depigmented and polymerised (Dpg-Pol) extracts. The objectives of this study were to investigate the presence of allergens in Dpg-Pol extracts of house dust mite and to analyze the immunological changes induced by these extracts in asthmatic patients enrolled in a double-blind, placebo-controlled study. **Methods:** Dpg-Pol extracts were manufactured and vaccines with a composition of 50% *Dermatophagoides pteronyssinus* and 50% *D. farinae* (100 HEPL/ml) were prepared. Allergen composition was analyzed by mass spectrometry. Patients with asthma and rhinoconjunctivitis were treated in a 1-year, double-blind, placebo-controlled, parallel-group study with 6 up-dosing and monthly maintenance injections. Specific IgE and IgG4 titres to *D. pteronyssinus*, Der p 1 and Der p 2 were measured in patients' sera using the CAP system and direct ELISA experi-

ments. **Results:** Sequences from the major allergens Der p 1 and Der p 2 and from other allergens were identified in native and Dpg-Pol extracts. There was a statistically significant increase in specific IgG4, a decrease in the ratio of IgE/IgG4 to *D. pteronyssinus* and a significant increase in specific IgG4 to Der p 1 and Der p 2 in the patients allotted to active treatment. **Conclusions:** The detection of allergen sequences suggests preservation of major and minor allergens in Dpg-Pol allergoids from house dust mites. Efficacy in asthma treatment and the increase in specific IgG4 seem to be associated with the presence of major allergens in Dpg-Pol allergen extracts.

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Introduction

Allergen-specific immunotherapy is recognised by the World Health Organisation as the only disease-modifying treatment for allergic diseases, and it may also prevent the development of new allergic sensitisation or progression from rhinitis to asthma [1]. Although the efficacy of this approach for the treatment of rhinoconjunctivitis and asthma has been confirmed in many controlled studies [2, 3], conventional subcutaneous immunotherapy in-

volves injection of allergen extracts with the potential to cause severe allergic reactions due to cross-linking of allergen-specific IgE. This small risk of anaphylaxis has limited the use of subcutaneous immunotherapy and led to the development of modified allergen extracts, termed allergoids, with reduced IgE-binding activity (allergenicity) [4], thus increasing safety [5]. On the other hand, although not many studies have analyzed T cell reactivity (immunogenicity), some studies have suggested that reactivity of T helper cells with allergoids is dependent on the type of antigen-presenting cells [6] and the individual [7]. Several standardised allergoids are in clinical use, and clinical efficacy has been confirmed in double-blind placebo-controlled studies [9–11].

A further step in allergen modification for immunotherapy vaccines is a mild acid treatment and dialysis step to remove non-allergenic material and to purify the allergenic extracts prior to polymerisation with glutaraldehyde. This step is termed depigmentation. The resulting depigmented and polymerised (Dpg-Pol) extracts have greatly reduced IgE binding and have shown clinical efficacy for asthma and rhinoconjunctivitis in response to a variety of allergens [8–12].

Although allergoids and Dpg-Pol extracts are effective and are used in clinical practice, there has been some debate regarding whether the process of chemical modification does indeed preserve immunogenicity as well as reducing allergenicity. One difficulty with assessing allergoids is that the polymerisation process blocks access to antibodies, making it difficult to use antibody-based assays to measure allergen content in these vaccines. Recently, we described the use of mass spectroscopic techniques to confirm preservation of major allergen content in Dpg-Pol birch pollen extracts [13], which may allow the development of methods for standardisation of allergoids [14].

The objectives of this study were to assess which allergens of *Dermatophagoides pteronyssinus* were present in a Dpg-Pol extract and whether a significant immunological response accompanied the clinical response in a double-blind placebo-controlled trial in asthma patients.

Methods

Extract Manufacturing

A native extract from *D. pteronyssinus* (Laboratorios LETI, Madrid, Spain) and its corresponding Dpg-Pol extract were manufactured following previously described methods that are detailed in depth in the European patent of the depigmentation process [15]. One hundred grams of *D. pteronyssinus* (Laboratorios

LETI) were extracted in phosphate-buffered saline buffer (PBS), 0.01 M, pH 7.4, at 4°C. After centrifugation at 16,000 g for 30 min, the supernatant was collected, sterile filtered (pore size 0.22 µm under sterile conditions) and dialysed overnight against highly purified water (Ph. Eur. specification). Finally, the extract was again sterile filtered, frozen (–50°C) and lyophilised.

Lyophilised native extract was reconstituted in highly purified water (1% w/v) at room temperature until the product was completely dissolved. The pH of the solution was reduced to 2 by the drop-wise addition of 6 M HCl at room temperature in order to remove all the low-molecular-weight substances and components attached to the proteins/allergens. Afterwards, the extract was redialysed overnight at room temperature against highly purified water in dialysis membranes with a cut-off of 3.5 kDa (Cellu Sep Membrane; Membrane Filtration Products, Seguin, Tex., USA) and under constant agitation. The pH was again adjusted to physiological conditions (pH 7.4) by the drop-wise addition of 1 N NaOH at room temperature, and the extract was sterile filtered, frozen (–50°C) and freeze-dried. The depigmented extract was reconstituted in PBS, 0.01 M (1% w/v), polymerised with glutaraldehyde (50%) and maintained overnight at room temperature under constant agitation. After polymerisation, the resulting material was then dialysed overnight at room temperature in 100-kDa dialysis membranes (Millipore, Bedford, Mass., USA) against highly purified water, sterile filtered, frozen (–50°C) and freeze-dried.

The protein content of native, depigmented and Dpg-Pol extracts was measured by the Lowry-Biuret method (Bio Rad Laboratories, Hercules, Calif., USA). Major allergen content (Der p 1 and Der p 2) was measured (Indoor Biotechnologies, Charlottesville, Va., USA) in native and depigmented extracts, and the biological potency was calculated by ELISA competition assays [16] and adjusted to a final concentration of 100 HEPL/ml. The extracts were adsorbed to alum hydroxide (Brenntag, Mülheim, Germany) for clinical use.

Extract Characterisation: Mass Spectrometry-Based Sequencing

Dpg-Pol *D. pteronyssinus* freeze-dried extract was dissolved in water at a concentration of 1 mg/ml and digested with trypsin (Proteoextract trypsin digestion kit; Calbiochem, San Diego, Calif., USA) or pepsin (Sigma, St. Louis, Mo., USA) in 0.1 M HCl or 5% formic acid. Resulting peptides were separated by reversed-phase capillary HPLC (Nanoease Symmetry 300™ trap column and Nanoease Atlantis dC18™ separating column; Waters, Milford, Mass., USA) directly coupled to an electrospray ionisation quadrupole time-of-flight mass spectrometer (Q-ToF Ultima Global; Waters) in data-dependent analysis mode. To avoid sample cross-contamination, the chromatographic system was flushed with an empty purge run after every sample run.

Spectra were analysed using the ProteinLynx Global Server software (version 2.2.5, Waters) with both automatic and manual data validation. Positive identification of peptides by collision-induced dissociation was based on at least 4 consecutive unequivocally identified γ -ions in MS/MS mode. For MS/MS-based sequencing, an in-house database consisting of all International Union of Immunological Societies-known *D. pteronyssinus* allergens was used. Proteins were considered identified if at least 2 peptides were positively assigned.

Fig. 1. Flow chart of the prospective, double-blind, placebo-controlled, randomised clinical trial. Patients were treated with a Dpg-Pol allergenic extract containing 50% *D. pteronyssinus* and 50% *D. farinae*.

Searches against the UniProtKB/Swiss-Prot and TrEMBL release 54.6 of December 4, 2007 did not identify other allergens present in this batch [17].

Patient Population and Sera

Serum samples from a previous published trial [10] were used. Briefly, a prospective, double-blind, placebo-controlled, randomised study was performed (fig. 1). The mean age of patients allotted to active treatment was 23.5 years (SD 9.3), and the mean age of patients receiving placebo was 23.8 years (SD 7.7). Adolescents and adults were allocated to receive an alum-adsorbed mixture of modified allergen extracts of 50% *D. pteronyssinus*/50% *D. farinae* or a placebo containing all components of the active vaccine except the allergen extract for 54 weeks. The allergen extract of *D. farinae* was prepared and processed as described for *D. pteronyssinus*. Patients were eligible if they had a history of allergic asthma to house dust mites, positive skin tests to house dust mites, negative skin tests to other mite species and common aero-allergens, detectable specific IgE (CAP ≥ 2) and a positive bronchial challenge test using a standardised extract of *D. pteronyssinus* (all performed as previously described [10]). Twenty-nine patients in each group were taking inhaled corticosteroids for their asthma. Serum samples were collected from each patient at the first visit (baseline) and at the end of the study and stored at -20°C until use. These studies were approved by the local Ethical Review Board, and all patients gave written informed consent.

D. pteronyssinus-Specific IgE and IgG4

Specific IgE and IgG4 to *D. pteronyssinus* were determined in individual serum samples. Briefly, commercially available *D. pteronyssinus* ImmunoCaps (Pharmacia, Uppsala, Sweden) were used in the solid phase. After the addition of serum samples from each patient, specific IgE or IgG4 to *D. pteronyssinus* was detected using the UniCAP[®] 100 (Phadia), following the manufacturer's instructions.

Results were obtained from a standard curve and expressed as kilounits per litre in the case of IgE and as milligrams of antigen per litre for IgG4. Five samples from the placebo group and 4 from the active group were excluded because of insufficient serum for analysis.

Specific Der p 1 and Der p 2 IgE and IgG4

Specific IgE and IgG4 to Der p 1 and Der p 2 were measured in serum samples before and after treatment by direct ELISA according to the method of Mastrandrea et al. [18], with modifications. Briefly, purified natural Der p 1 and Der p 2 allergens (Indoor Biotechnologies) were dissolved in carbonate/bicarbonate buffer, pH 9.6, at a concentration of 1 $\mu\text{g}/\text{ml}$. Then, 100 μl were coated onto plastic high-binding microtitre plates (Immulon II; Thermo Scientific, Milford, Mass., USA). Each serum sample was diluted 1:2 in PBS, 0.01 M, and incubated (100 μl) in the wells for 2 h. Microplates were washed 3 times and incubated for 2 h with anti-human IgE (Ingenasa, Madrid, Spain) conjugated with per-

Table 1. Clinical data at baseline and after 1 year of treatment

Variable	Baseline	One year	p value
Actively treated group (n = 27)			
Total symptom score	13.8 (7.5–23.8)	8.2 (3.4–12.9)	0.001
Bronchial symptom score	3.3 (1.3–7.3)	1.7 (0.0–3.7)	0.006
Nasal symptom score	8.6 (4.8–12.1)	4.7 (1.2–7.2)	<0.001
Ocular symptom score	0.7 (0.4–3.1)	0.6 (0.0–2.5)	0.03
Total medication score	15.6 (8.9–20.5)	7.1 (0.0–15.8)	0.013
Placebo group (n = 27)			
Total symptom score	17.8 (5.5–33.3)	29.5 (11.6–43.2)	0.01 ¹
Bronchial symptom score	3.5 (2.0–8.0)	5.0 (2.0–13.0)	0.17 ¹
Nasal symptom score	9.9 (4.1–16.4)	27.0 (24.0–32.6)	0.05 ¹
Ocular symptom score	2.17 (0.0–3.6)	2.17 (0.33–10.0)	0.06
Total medication score	13.7 (4.0–27.0)	21.0 (7.3–31.6)	0.08

Values are medians with interquartile ranges.

¹ Trend for worsening of symptoms.

oxidase (Roche Diagnostics, Basel, Switzerland) or with peroxidase-labelled anti-human IgG4 (Menarini Diagnostic, Florence, Italy). After 5 washes, the reaction was developed for 30 min, stopped with 1 N sulphuric acid and read at 405 nm.

Statistical Analysis

Descriptive statistics were expressed as absolute and relative frequencies for categorical outcomes and as means, medians, quartiles and range for quantitative outcomes. A normal distribution test was conducted in all cases. A t test or the Mann-Whitney rank sum test was used to compare the active and placebo groups. In order to detect differences in the active or placebo group from the baseline to the end of the study period, Student's t test for paired data or the Wilcoxon signed rank test were used. Analyses were performed using SPSS, version 17 (SPSS Inc., Chicago, Ill., USA).

Results

Patient Population

Fifty-four subjects completed the clinical study. Clinical data from the whole study period have been presented previously [10].

Subjects treated with alum-adsorbed Dpg-Pol extract of *D. pteronyssinus*/*D. farinae* (Depigoid®) achieved a significant improvement in total, bronchial, nasal and ocular symptom scores and rescue medication use after treatment compared to baseline (table 1), whereas subjects treated with placebo had no significant changes or increases in symptom scores.

Comparison of Depigoid and placebo with regard to the difference between post-treatment and baseline measurements of total, bronchial, nasal and ocular symptoms

and rescue medication use showed a significant improvement with Depigoid compared to placebo ($p \leq 0.02$ for all comparisons). There were 4 local reactions (2 in each group, all within 15 min of injection and all less than 5 cm in diameter). There were no systemic adverse reactions.

Allergen Content of Dpg-Pol Extracts

The allergen content in the native and Dpg-Pol extracts as determined by LC-MS/MS-based sequencing was similar. Different isoforms of the major allergens Der p 1 and Der p 2, as well as the minor allergens Der p 3, Der p 4, Der p 8, Der p 10 and Der p 20, were detected in both extracts. Der p 11 was only identified in Dpg-Pol extract. Native and polymerised extracts from the same source were always analysed in parallel. It is therefore likely that Der p 11 was also present in the native extract but not detected by mass spectrometry. Der p 9 and Der p 14 were only identified in native extracts. Results of the mass spectrometric analyses are summarised in table 2. Shown are the probability of occurrence as calculated by ProteinLynx Global Server, a mass spectrometry analysis software based on a probability-driven algorithm, and the percentage of sequence coverage. Figure 2 shows the sequences of Der p 1 and Der p 2 isoforms identified by MS/MS in native and Dpg-Pol extracts.

Serum Antibody Responses

Specific IgE and IgG4 to *D. pteronyssinus*, Der p 1 and Der p 2 at baseline and after 1 year of treatment were measured in the placebo and active groups.

Der p 1

Isoform	
0101	MKIVLAIASL LALSAVYARP SSIKTFEEYK KAFNKSYATF EDEEAARKNF LESVKYVQSN GGAINHLSL SLDEFKNRFL MSAEAFEHLK
0113	-----RP SSIKPFEEYK KAFNKSYATF EDEEAARKNF LESVKYVQSN GGAINHLSL SLDEFKNRFL MSAEAFEHLK
0101	TQFDLNAE TN ACSINGNAPA EIDLR QMRTV TPIRMQGGCG SCWAFSGVAA TESAYLAYRN QSLDLAEQEL VDCASQHGCH GDTIPR
0113	TQFDLNAE TN ACSINGNAPA EIDLR QMRTV TPIRMQGGCG SCWAFSGVAA TESAYLAYRN QSLDLAEQEL VDCASQHGCH GDTIPR
0101	GIEY IQHNGVVQES YYR YVAREQS CRRPNAQRFG ISNYCQIYPP NVNKIREALA QTHSAIAVII GIKDLL FRH YDGR IIQRD NGYQP
0113	GIEY IQHNGVVQES YYR YVAREQS CRRPNAQRFG ISNYCQIYPP NANKIREALA QTHSAIAVII GIKDLL FRH YDGR IIQRD NGYQP
0101	NYHAV NIVGYSNAQG VDY WIVRNSW DTNWGDNGYG YFAANIDLMM IEEYPYVVIL
0113	NYHAV NIVGYSNAQG VDY WIVRNSW DTNWGDNGYG YFAANIDLMM IEEYPYVVIL

Der p 2

Isoform	
0101	MMYKILCLSL LVAAVARDQV DVKDCANHEI KK VLVPGCHG SEPCIIHRGK PFQLEAVFEA NQNTKTAKIE IKASIDGL EV DVP GIDPNAC
0109	-----DQV DVKDCANHEI KK VLVPGCHG SEPCIIHRGK PFQLEAVFEA NQNSKT AKIE IKASIDGL EV DVP GIDPNAC
0114	MMYKILCLSL LVAAVAADQV DVKDCANHEI KK VLVPGCHG SEPCIIHRGK PFQLEALFEA NQNTKNKIE IKASIDGL EV DVP GIDPNAC
0101	HYMKCPLVKG QQ YDIKYTN VPKIAPKSEN VVVTVK VMGD DGVLCAIAT HAKIRD
0109	HYMKCPLVKG QQ YDIKYTN VPKIAPKSEN VVVTVK VLGD NGVLCAIAT HAKIRD
0114	HYVKCPLVKG QQ YDIKYTN VPKIAPKSEN VVVTVK VLGD NGVLCAIAT HAKIRD

Fig. 2. Allergen sequences identified by nano-LC-MS/MS-based peptide mapping. Results from analyses obtained from tryptic and peptic samples were combined. Sequences identified in the native extract X-121LN are indicated in red; sequences identified in the Dpg-Pol extract 137LP are highlighted in blue.

Table 2. Probabilities of occurrence and sequence coverage of *D. pteronyssinus* allergens in 3 different native extracts (X-95LN, X-42LN, X-121LN) and their corresponding Dpg-Pol extracts (X-104LP, X-56LP, 137LP) as determined by LC-MS/MS

Allergen	Probability and sequence coverage								
	native extracts						Dpg-Pol extracts		
	X-95LN	X-42LN	X-121LN	X-104LP	X-56LP	137LP			
Der p 1.0113	high (6.6%)	high (7.8%)	high (10%)	high (14.7%)	high (14.7%)	high (13.1%)			
Der p 1.0102	high (6.9%)	high (8.3%)	high (10.6%)	high (15.6%)	high (15.5%)	high (13.9%)			
Der p 2.0101	low (21.9%)	low (32.9%)	low (21.9%)	high (16.4%)	high (16.4%)	high (22.6%)			
Der p 2.0109	high (34.9%)	high (53.5%)	high (34.9%)	high (18.6%)	high (18.6%)	high (25.6%)			
Der p 2.0114	low (21.9%)	low (32.9%)	low (21.9%)	high (16.4%)	high (16.4%)	high (22.6%)			
Der p 3.0101	high (7.6%)	high (4.6%)	high (4.2%)	high (10.3%)	high (3%)	high (10.3%)			
Der p 4.0101	low (0%)	high (3.6%)	low (0%)	high (4.4%)	high (1.8%)	high (2.6%)			
Der p 5.0101	low (0%)	low (0%)	low (0%)	low (0%)	low (0%)	low (0%)			
Der p 6.0101	low (0%)	low (0%)	low (0%)	low (0%)	low (0%)	low (0%)			
Der p 7.0101	low (0%)	low (0%)	low (0%)	low (0%)	low (0%)	low (0%)			
Der p 8.0101	high (9.1%)	high (14.1%)	high (9.1%)	high (5%)	high (10.9%)	high (5%)			
Der p 9.0101	low (0%)	high (4%)	high (4%)	low (0%)	low (0%)	low (0%)			
Der p 9.0102	low (0%)	high (4.2%)	high (4.2%)	low (0%)	low (0%)	low (0%)			
Der p 10.0101	high (10.5%)	high (13.4%)	low (0%)	high (22.2%)	high (19.7%)	high (12%)			
Der p 11.0101	low (0%)	low (0%)	low (0%)	high (2.5%)	high (3.4%)	high (5%)			
Der p 14.0101	high (5.5%)	high (1.4%)	high (7.8%)	low (0%)	low (0%)	low (0%)			
Der p 20.0101	high (3%)	high (3.1%)	high (3.1%)	high (4.5%)	low (0%)	high (4.5%)			
Der p 21.0101	low (0%)	low (0%)	low (0%)	low (0%)	low (0%)	low (0%)			

Probability values were determined by ProteinLynx Global Server and are based on sequence coverage, signal intensities and the quality of the MS/MS data as described in the text (high: 60–100% probability; medium: 5–60% probability; low <5% probability). The percentage of the allergen sequence identified by MS/MS is given in parentheses.

Fig. 3. Comparison of individual specific IgG4 levels in the active group before and after treatment. Coloured lines indicate median values.

Specific IgG4

At baseline, no significant differences in specific IgG4 were observed between the active and placebo groups.

In the active group, significant increases from the baseline level to the end of the year of treatment were found in specific IgG4 to *D. pteronyssinus*, Der p 1 and Der p 2 (fig. 3). The fold changes were 1.4 for *D. pteronyssinus*, 1.8 for Der p 1 and 2.8 for Der p 2.

In the placebo group, a statistically non-significant difference was observed in IgG4 to *D. pteronyssinus* (before treatment: mean $\pm$ SD 0.55 ± 0.47 mg/l, median 0.46 mg/l; after 1 year of immunotherapy: 0.50 ± 0.36 , 0.49 mg/l; $p > 0.05$), IgG4 to Der p 1 [before treatment: 0.68 ± 0.59 optical density (OD), 0.41 OD; after 1 year of immunotherapy: 0.74 ± 0.70 , 0.45 OD; $p > 0.05$] and IgG4 to Der p 2 (before treatment: 2.05 ± 1.26 , 2.11 OD; after 1 year of immunotherapy: 2.00 ± 1.29 , 2.26 OD; $p > 0.05$).

Specific IgE

At baseline, no significant differences in specific IgE were observed between the active and placebo groups.

In the placebo group, a statistically significant decrease was observed in specific IgE to *D. pteronyssinus* (baseline: mean $\pm$ SD 49.6 ± 35.1 kU/l, median 43.4 kU/l; after 1 year: 43.0 ± 35.0 , 28.8 kU/l; $p = 0.008$), but not to Der p 1 and Der p 2 (data not shown).

In the active group, there was also a trend for a decrease in specific IgE to *D. pteronyssinus* (baseline: 44.8 ± 33.5 , 36.8 kU/l; after 1 year: 39.5 ± 31.4 , 30.5 kU/l; $p = 0.06$).

Ratio of Specific IgE/Specific IgG4

A significant decrease in the ratio of specific IgE/IgG4 to *D. pteronyssinus* was observed in the active group (baseline: mean $\pm$ SD 94.8 ± 89.9 , median 64.2; after 1 year: 65.1 ± 54.3 , 52.6; $p = 0.02$).

No changes were observed for placebo-treated patients (baseline: 103.3 ± 83.0 , 80.8; after 1 year: 133.0 ± 204.6 , 51.1).

Discussion

We have examined a Dpg-Pol immunotherapy extract of *D. pteronyssinus* and *D. farinae* used in a previously reported double-blind placebo-controlled clinical trial in asthma patients. We confirm conservation of sequences for the major *D. pteronyssinus* allergens in the Dpg-Pol extract and in vivo immunogenicity with induction of specific IgG4 to *D. pteronyssinus* and to the major allergens Der p 1 and Der p 2, together with clinical efficacy with regard to symptom and medication scores.

A potential drawback of allergoids may be the modification of lysine side chains involved in allergen epitopes during the chemical treatment used in manufacture. As expected, lysine residues were not detected by mass spectrometry in the digested Dpg-Pol extract because they are modified to establish covalent bridges between allergens. Several in vitro studies have suggested partial loss of T cell epitopes for various allergoid preparations [7, 19, 20]. If major allergens were lost or under-represented in allergoid immunotherapy preparations, this could potentially reduce their clinical and immunological efficacy in some patients. The end product of allergoid manufacture is a large polymer with reduced accessibility to antibody binding; this is the basis of the reduced allergenicity. However, this property also reduces binding of antibodies used in assays to detect allergen proteins and has hampered assessment of the allergen content of allergoids. Recently, we described the application of mass spectroscopy-based peptide mapping to determine allergen sequences in Dpg-Pol extracts of *Betula alba* [13]. Using these techniques in the present study, allergen sequences of the major allergens Der p 1 and Der p 2 and minor allergens (Der p 3, 4, 8, 10, 11 and 20) were detected in proteolytic digests of Dpg-Pol extracts of *D. pteronyssinus*. The detection of sequences for Der p 11 in the Dpg-Pol extract but not native extracts may be due to a relative increase in the allergen content of the extract during the depigmentation process. These data suggest a high degree of preservation of the major and minor allergens within the polymerised allergoid product, at least at the level of amino acid sequences.

The immunogenicity of the Dpg-Pol extracts in vivo was confirmed by the demonstration of induction of allergen-specific IgG4 responses in patients treated with these extracts for immunotherapy. Accordingly, an immune response to the major allergens Der p 1 and Der p 2 was confirmed. These data confirm that the Dpg-Pol extract is processed and recognised by the immune system. It can induce a B cell response similar to that de-

scribed for immunotherapy with unmodified allergen extracts [21]. These findings extend previous results showing the induction of IgG in patients treated with allergoid immunotherapy [22].

In the current study, patients were treated with a combination of Dpg-Pol to *D. pteronyssinus* and *D. farinae*, and the dose of *D. pteronyssinus* given was thus 50% of that contained in an extract containing only *D. pteronyssinus*. Whether this affected the levels of specific IgG4 induced will require further study, although there is a high degree of cross-reactivity between these two mite extracts. In a study of patients with atopic dermatitis treated with a Dpg-Pol extract of *D. pteronyssinus* for 6 months, there was an increase in specific IgG4 against Der p 2 and significant decreases in allergen-specific IgE in serum [23]. In the present study, we observed a small reduction in specific IgE in both the actively treated and placebo groups, which we hypothesise could relate to changes in environmental exposure to allergen. However, the ratio of IgE to IgG4 specific for *D. pteronyssinus* decreased only in the active group, which may relate to the clinical benefit observed. The changes in IgG4 concentrations observed here in a clinically effective treatment regimen were statistically significant but modest compared to those reported in some immunotherapy studies (1.4- to 2.8-fold). This most likely relates to the allergen dose used, as a dose-response effect has been reported, and the changes we detected were similar to those seen for other immunotherapy regimens such as for *Alternaria* [24], cat [25] or house dust mite [26]. Notably, not all patients showed an increase in allergen-specific IgG4 after immunotherapy with Dpg-Pol extract at this dose. However, as has been observed in previous studies, the degree of increase in IgG4 did not correlate with the clinical response to treatment (data not shown). In a time course study of native extract grass pollen immunotherapy, a 4-fold increase in allergen-specific IgG4 was detected after 12 weeks of treatment and a cumulative dose of 68 µg of Phl p 5, but significant inhibition of basophil histamine release and binding of IgE-allergen complexes to B cells was observed as early as 6 weeks after treatment (cumulative dose 20 µg) [27]. Thus, functionally relevant changes in IgG4 antibodies may precede changes in the total concentration in serum. Reported increases in IgG4 to house dust mite range from 1.9- to 7.2-fold after immunotherapy [28], somewhat lower than those increases reported for pollen allergens. This may reflect the presence of IgG4 at baseline due to perennial exposure to house dust mite allergens. Analysis of functional blocking antibody responses will be of interest.

In the present study, we have shown preservation of allergen molecules within Dpg-Pol extract of house dust mites and an in vivo immune response to this extract used in a double-blind placebo-controlled study of immunotherapy for asthma. The treatment was effective in reducing symptom scores by 54% compared with placebo [10]. This is in line with numerous studies confirming clinical efficacy of allergoid immunotherapy preparations [9, 11, 12]. The treatment regimen was very well tolerated, with no systemic reactions, no anaphylactic reactions, no asthma exacerbations or requirement of oral corticosteroids, and no hospitalisations or emergency room visits, as previously reported [10]. Indeed, data from a large series of patients reported no grade 3 or 4 systemic reactions in over 2,000 patients treated with Dpg-Pol extracts [29]. This compares with a rate of grade 3 reactions of 4.4% in a study of immunotherapy using unmodified extract of *Phleum pratense* [30], rates of systemic reactions of 2.1 and 2.9% in two large US series and 33 immunotherapy-related fatalities in the USA between 1985 and 2001 [3]. Other approaches to reduce IgE binding of allergen extracts used in immunotherapy include

the use of modified recombinant allergens or allergen peptides [31, 32]. Both of these approaches have been associated with side effects, including delayed asthmatic reactions, and remain experimental. Allergoids are widely used for immunotherapy in Europe, and further studies are planned to determine whether the dose of Dpg-Pol extracts can be increased to improve efficacy whilst maintaining safety.

In summary, we report the detection of peptide sequences in a Dpg-Pol extract of house dust mites, suggesting preservation of major allergens, together with in vivo immunogenicity and efficacy of the extract in the treatment of asthma.

Acknowledgements

We thank Dr. Miguel Casanovas and Dr. Enrique Fernández-Caldas for their contribution to the study and design of the clinical trial.

This study was sponsored by Laboratorios LETI S.L.U.

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