ORIGINAL ARTICLE

Immunogenicity of *Phleum pratense* Depigmented Allergoid Vaccines: Experimental Study in Rabbits

V Iraola,[1] MT Gallego,[1] MA López-Matas,[1] M Morales,[1] I Bel,[2] N García,[2] J Carnés[1]

[1]R&D Department, Laboratorios LETI, Tres Cantos, Spain
[2]Quality Control, Laboratorios LETI, Tres Cantos, Spain

Abstract

Background: Immunogenicity studies are based on accurate preclinical and clinical assessment of pharmaceutical products. The immunogenicity of modified allergen vaccines has not been fully elucidated, and the mechanisms involved are not well understood. Animal and human models have recently shown that depigmented allergoids induce specific immunoglobulin (Ig) G against individual allergens, thus supporting the clinical efficacy of these vaccines.
Objective: The aim of this study was to investigate the production of specific IgG against individual antigens and their isoforms in rabbits injected with depigmented allergoid extracts of *Phleum pratense* pollen.
Methods: Two New Zealand rabbits were immunized with depigmented-polymerized extracts adsorbed onto aluminum hydroxide (Depigoid) of *P pratense*. Rabbits were injected 3 times (35 µg Phl p 5). Specific IgG titers against native, depigmented, and depigmented-polymerized extracts and individual allergens (rPhl p 1 and rPhl p 5a) were analyzed by direct enzyme-linked immunosorbent assay. The capacity of these synthesized antibodies to recognize individual native and depigmented allergens and different isoforms was evaluated by immunoblot and 2-D analysis.
Results: All rabbits produced high titers of specific IgG against the 3 extracts. Rabbits injected with depigmented allergoids produced similar specific antibody titers against native, depigmented, and depigmented-polymerized extracts. Serum samples recognized individual allergens and their isoforms in the nonmodified extracts.
Conclusion: Vaccines containing depigmented allergoid extracts of *P pratense* induce immunogenicity in vivo. The antibodies produced after injection of these extracts clearly recognized allergens and different isoforms in their native configuration.

Key words: Allergen extracts. Allergoids. *Phleum pratense*. Allergen immunotherapy. Immunogenicity. 2-D immunoblots. Specific IgG. Phl p 1. Phl p 5. Isoforms.

Resumen

Introducción: Los estudios de inmunogenicidad se centran principalmente en investigaciones preclínicas y en la evaluación clínica de productos farmacéuticos. Sin embargo, la inmunogenicidad de los extractos alergénicos modificados químicamente usados en inmunoterapia, no ha sido totalmente dilucidada y los mecanismos implicados no son del todo bien conocidos. Recientemente se ha demostrado, en modelos animales y en humanos, que extractos alergénicos despigmentados y polimerizados (alergoides) inducen antígenos específicos contra los alérgenos individuales del extracto, apoyando la eficacia clínica demostrada de estas vacunas.
Objetivo: El objetivo de este estudio fue investigar la producción de IgG específica contra alérgenos individuales, proteínas y sus isoformas, en conejos inmunizados con alergoides despigmentados de polen de *Phleum pratense*.
Metodos: Dos conejos New Zealand fueron inmunizados con extractos depigmentado-polimerizados adsorbidos en hidróxido de aluminio (Depigoid®) de *P. pratense*. Los conejos fueron inmunizados con tres inyecciones (35 µg Phl p 5). Los títulos de IgG específica contra el extracto natural, despigmentado y despigmentado-polimerizado, así como contra alérgenos individuales (rPhl p 1 and rPhl p 5a) fueron analizados mediante ELISA directo. La capacidad de estos anticuerpos para reconocer alérgenos individuales naturales y despigmentados, así como sus diferentes isoformas fue determinado mediante immunoblot (1-D y 2-D).
Resultados: Los conejos, tras su inmunización con alergoides despigmentados, produjeron títulos elevados y similares de IgG específica contra el extracto natural, despigmentado y despigmentado-polimerizado, así como contra los alérgenos individuales. Así mismo, reconocieron los alérgenos individuales y sus isoformas del extracto natural.
Conclusión: Las vacunas que contienen alergoides despigmentados de *P. pratense* inducen inmunogenicidad in vivo. Los anticuerpos producidos tras la inyección de estos extractos reconocen claramente alérgenos individuales y sus diferentes isoformas en su configuración natural.

Palabras clave: Extractos alergénicos. Alergoides. *Phleum pratense*. Inmunoterapia. 2-D inmunoblots. IgG específica. Phl p 1. Phl p 5. Isoformas.

Introduction

Current concerns regarding immunogenicity are focused on accurate preclinical and clinical assessment of pharmaceutical products. Several factors contribute to immunogenicity, including adjuvants, aggregation, impurities, route of administration, and T-cell and B-cell epitopes [1]. Nowadays, efficacy of allergenic vaccines is associated with the capacity of these vaccines to induce specific immunoglobulin (Ig) G (mainly IgG4) against the allergens responsible for sensitization. The immunogenicity of individual proteins and immunological mechanisms has been elucidated in different studies [1]. However, the immunogenicity of molecular aggregates or large molecules containing different proteins remains unknown, and the mechanisms of action of these molecules have not been fully discussed. Using birch extracts, allergoids were recently shown to induce an immune response in vivo against individual allergens [2]; however, little is known about the isoforms recognized or the induction of specific antibodies against different isoallergens.

Allergoids are defined as allergenic extracts subjected to chemical treatment in order to reduce allergenicity while maintaining immunogenicity. Prior to polymerization, some allergenic extracts are semipurified using a mild acid treatment, and substances attached to the proteins by non-covalent bonds are removed. This process is known as depigmentation. The resulting product is a semipurified allergen extract containing a significantly higher proportion of proteins and retaining the same protein composition and characteristics as the native extract, with similar IgE and IgG binding capacity and inactivation of enzymatic activity [3]. After depigmentation and polymerization with glutaraldehyde, the depigmented-polymerized (Dpg-Pol) extracts contain large aggregates with a molecular weight of 1-3 MDa. In addition, these allergoids were recently shown to contain all the individual proteins present in the native extract [1,4]. After polymerization, Dpg-Pol extracts are adsorbed onto aluminum hydroxide in order to prepare a vaccine for use in humans. The clinical efficacy of these modified allergen extracts has been demonstrated in allergic sensitized individuals in clinical trials carried out with house dust mites, grasses, weeds, and trees [5-8]. Furthermore, Dpg-Pol extracts retain or even increase immunogenicity by inducing synthesis of specific IgG, IgG1, and IgG4 after immunotherapy [4,9].

The objectives of this study were to determine the production of specific IgG antibodies in rabbits injected with Dpg-Pol extracts of *Phleum pratense* (Depigoid) (Laboratorios LETI, S.L.U., Madrid, Spain) and to analyze the capacity of these antibodies to recognize different isoallergens.

Methods

In Vivo Immunization

After collection of pre-immune sera, 2 New Zealand White rabbits were immunized with Dpg-Pol allergen extract of *P pratense* adsorbed onto aluminum hydroxide (Depigoid; 1000 DPP/mL). The doses administered were similar to those used in humans (0.5 mL). Rabbits were immunized with 3 subcutaneous injections (1 immunization and 2 boosters) containing 70 μg/mL of Phl p 5. Major allergen content was measured in the extract before polymerization using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Indoor Biotechnologies, Virginia, USA).

Twenty-one days after the first immunization, booster 1 was injected. Booster 2 was administered 51 days after the first immunization. Rabbits were bled after 58 days, and their serum was collected. Individual and pooled sera from both rabbits were kept at –20ºC. The study was conducted at the Vivotecnia Research facilities (Madrid, Spain). All the procedures were approved by the Institutional Review Board of Vivotecnia Research and followed national regulations for animal experimentation [10].

Determination of Specific Immunoglobulin G

Specific IgG—induced after immunization of rabbits with Dpg-Pol allergenic extracts against native, depigmented, and Dpg-Pol extracts and recombinant allergens rPhl p 1 and rPhl p 5 a (Indoor Biotechnologies)—was measured using direct ELISA. Microtiter plates (Immulon IV; Dynex Technologies, Chantilly, Virginia, USA) were coated overnight (100 μL/well) with native, depigmented, and Dpg-Pol *P pratense* extracts at a concentration of 10 μg/mL in carbonate/bicarbonate buffer (pH, 9.6). After washing, the plates were incubated with serial dilutions of rabbit sera in 1% bovine serum albumin (BSA) phosphate-buffered saline (PBS)-Tween (Panreac, Barcelona, Spain) for 2 hours at room temperature. Plates were washed and incubated with peroxidase-labelled goat antirabbit IgG (Nordic Immunology, Tilburg, the Netherlands) diluted at 1:30 000 in 1% BSA-PBS-Tween for 1 hour at room temperature. The reaction was developed, and optical densities were measured at 450 nm. Pre-immune sera were used as negative controls.

Rabbit IgG antibodies targeting rPhl p 1and rPhl p 5 were detected by direct ELISA. Microtiter plates (Immulon II; Dynex Technologies) were coated overnight at 4ºC (100 μL/well) with 1 μg/mL of recombinant allergen in sodium carbonate buffer (pH, 9.6). Plates were washed 3 times with PBS-Tween and blocked with 1% BSA-PBS-Tween for 1 hour at room temperature. After washing, the plates were incubated with serial dilutions (100 μL/well) of rabbit serum in 1% BSA-PBS-Tween for 2 hours at room temperature. Plates were washed 5 times and incubated with 100 mL/well of peroxidase-labelled goat antirabbit IgG (Nordic Immunology) diluted at 1:30 000 in 1% BSA-PBS-Tween for 1 hour at room temperature. Color was developed with tetramethyl benzidine after incubation for 30 minutes. The reaction was stopped with sulfuric acid 1 N, and absorbance was read at 450 nm. Pre-immune sera were used as negative controls.

The linear parts of the curve were used to compare the differences between specific IgG titers of absorbance values. The Mann-Whitney rank sum test was applied to identify the differences between pairs. A P value ≤.05 was considered significant. Statistical analysis was carried out using SigmaPlot 10.0 (Port Richmond, California, USA).

2-D Analysis

The *P pratense* extract was semipurified and concentrated with ammonium sulfate in 2 different steps until the saturation percentage (40% and 80%) was reached and then maintained at 4°C overnight. Samples were centrifuged, the pellet was collected, and samples were reconstituted in ultrapurified water. Concentrated extract was washed using the ReadyPrep 2-D Cleanup Kit (BioRad, Hercules, California, USA), following the manufacturer's instructions. The sample was separated according to its isoelectric point in ReadyStrip IPG Strips (BioRad) (pH, 3-10), using Protean IEF Cell (BioRad). The strips were equilibrated with the ReadyPrep 2-D Kit buffers (BioRad) and separated in the second dimension according to their molecular weight. Gel was stained with SyproRuby (BioRad) following the manufacturer's instructions.

Immunoblots

Fifty micrograms of native freeze-dried *P pratense* or 2 μg of rPhl p 1 and rPhl p 5a (Indoor Biotechnologies), was run in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and electro-transferred onto an Immobilon-P membrane (Millipore, Bedford, Massachusetts, USA). After transfer, membranes were blocked for 2 hours and incubated overnight with the pool of pre-immune sera and with the pool of immunized rabbit sera diluted 1:10 000. Specific IgG binding was detected using peroxidase-labelled goat antirabbit IgG (Nordic) diluted at 1:30 000. Bands were visualized by chemiluminescence (Chemidoc XRS, BioRad). The pre-immune sera were used as a control.

2-D immunoblots were prepared following a similar procedure. First, 150 μg of *P pratense* native extract was run and transferred to a polyvinylidene fluoride membrane. Afterwards, 2 different experiments were carried out, as follows: *a)* In order to detect Phl p 5 isoforms, membranes were incubated after transfer with a biotinylated anti-Phl p 5 monoclonal antibody (Indoor Biotechnologies) diluted 1/16 000; and *b)* Membranes were incubated with pre-immune serum or immunized rabbit serum (diluted 1:4000) before being developed using chemiluminescence.

Results

Allergen Vaccines

The standardized Dpg-Pol allergen vaccines used in the experiments met all the criteria for human use. Physicochemical, immunological, and microbiological tests were performed before administration in animals following the European Pharmacopoeia [11] and European Medicines Agency guidelines on allergenic products [12].

Specific IgG Determination

Both rabbits immunized with Dpg-Pol extract of *P pratense* synthesized high titers of specific IgG antibodies against modified extracts and native and depigmented extracts (Figure 1A). Statistically significant differences in IgG production were obtained between Dpg-Pol and native or depigmented extract (*P*=.029). Specific IgG values were negative against pre-immune sera.

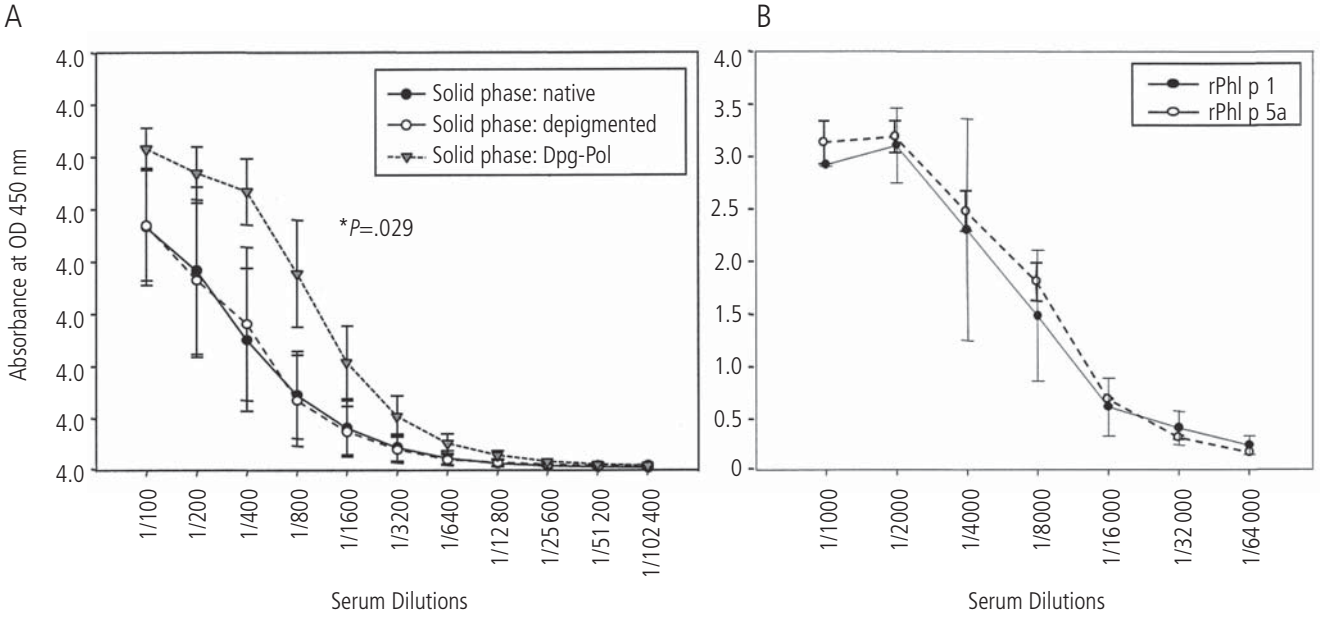

Figure 1. Serum titers of specific immunoglobulin G of immunized rabbits with depigmented allergoids of *Phleum pratense*. A, Native, depigmented, and depigmented-polymerized extracts of *P pratense*. B, recombinant allergens (rPhl p 1 and rPhl p 5). Dpg-Pol indicates depigmented polymerized extract; OD, optical density.

Detection of Major Allergens Phl p 1 and Phl p 5

High titers of specific IgG against rPhl p 1 and rPhl p 5a were obtained with direct ELISA (dilution 1/1000) (Figure 1B) when animals were immunized with whole Dpg-Pol *P pratense* extract, indicating that the immune response in immunized animals is directed against individual allergens. No signal was detected in pre-immune sera.

1-D and 2-D Immunoblots

The protein profile can be seen in Figure 2. The pool of sera from rabbits immunized with the Dpg-Pol vaccine recognized allergens present in native extract at a molecular weight range of between 10 kDa and 100 kDa, approximately. Bands at 30 kDa were clearly recognized (Figure 3A). When Phl p 1 and Phl p 5a were used in the solid phase, they were clearly recognized by the pooled sera after immunization (Figure 3B and 3C).

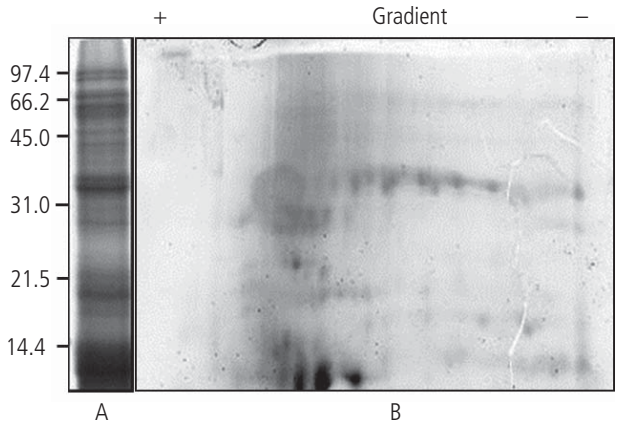

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *Phleum pratense* extract. A, 1-Dimensional. B, 2-Dimensional.

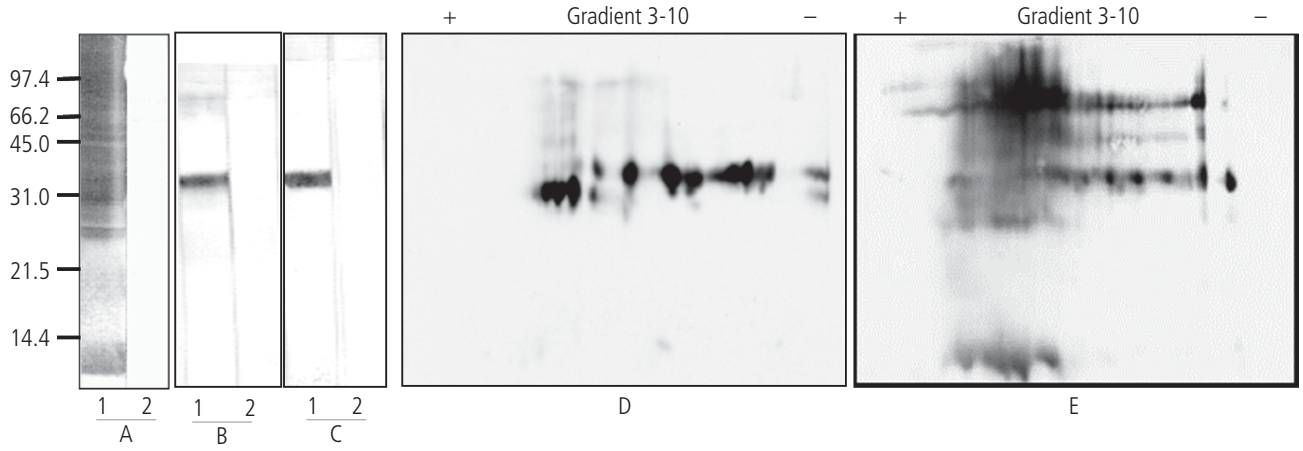

Figure 3. Immunoblot. A, *Phleum pratense* extract 1-dimensional. B, rPhl p 1. C, rPhl p 5 (lane 1, immunized rabbit sera; lane 2, pre-immune sera). D, 2-dimensional *P pratense* extract using anti-Phl p 5 monoclonal antibody to recognize Phl p 5 isoforms. E, *P pratense* 2-dimensional immunoblot (immunized rabbit sera).

2-D immunoblot analysis demonstrated the existence of at least 12 different Phl p 5 isoforms in native extract (Figure 3D) and the IgG binding capacity of the pooled sera to recognize them (Figure 3E).

Pre-immune sera did not recognize bands in any immunoblot experiment.

Discussion

Although some authors claim that allergoids have reduced immunogenicity [13,14] due to the structure of the molecules formed after polymerization, others conclude that allergoids have a high allergen-specific T-cell–stimulating capacity in vitro [15] and induce the synthesis of specific IgG in vivo after immunotherapy [4,16,17]. In recent years, the immunogenic capacity of these vaccines and their significantly reduced IgE binding capacity has resulted in a considerable increase in the use of allergoids for treatment of allergic diseases. In addition, the clinical efficacy of Dpg-Pol extract has been extensively demonstrated in double-blind placebo-controlled clinical trials [5-8], and this likely depends on the preserved immunogenicity of Depigoid. The immunogenicity of these vaccines has been documented in animals, with significant production of specific IgG after injection of Dpg-Pol extracts of *Betula alba* [2]. A significant increase in specific IgG and IgG1 titers has been observed in humans after only 2 weeks of treatment (11 injections) with Dpg-Pol extract of *P pratense* [9] and in specific IgG4 after 1 year of immunotherapy with Dpg-Pol extract of *Dermatophagoides* [4]. The studies mentioned above confirmed that Dpg-Pol vaccines retain their immunogenic capacity. Based on similar effects, Mutschlechner et al [18] used genetic engineering to obtain allergenic extracts or allergens with significantly reduced IgE binding capacity but maintained capacity to induce specific IgG against individual allergens.

In the present study, the production of specific IgG was evaluated by direct ELISA in rabbits after immunization with

Dpg-Pol extracts from grass pollen (*P pratense*) adsorbed onto aluminum hydroxide. The results showed that injection of Depigoid, a Dpg-Pol allergenic extract, induced significant production of specific IgG against grass allergens. The immunogenicity of Dpg-Pol molecules was compared using native, depigmented, and Dpg-Pol extracts in the solid phase. Serum samples contained significantly higher titers of specific IgG to Dpg-Pol extracts than to the 2 other nonpolymerized extracts, suggesting that polymerization could induce new epitopes in the polymerized molecules. Regardless of stimulation of the immune response, the clinical implications of these specific antibodies to epitopes not present in native extracts should be studied. Additionally, as discussed by Kahlert et al [15], quantitative differences in the stimulating capacity of native allergens and allergoids could be due to the chemical modification that affects binding of the allergoid peptide to the T-cell receptor, thus modifying T-cell responses.

Another probable explanation could be the molecular size of the new molecules. A study performed by our group (in press) has demonstrated that polymerized *P pratense* allergens have a molecular weight of between 1 and 2 MDa (data not published). This huge molecular size could be acting as an adjuvant itself, stimulating the immune response. Ongoing studies are attempting to establish the immunological mechanisms involved.

In addition to our immunoblotting experiments, we verified that the rabbits immunized with the Dpg-Pol extracts recognized not only individual allergens, but also different isoforms present in the extracts, as evidenced in the 2-D immunoblots. These findings confirm that the immune response is induced after administration of the high-molecular-weight allergen components present in the polymerized extracts. Polymerized molecules are processed by different immunological mechanisms (not yet fully elucidated), and the synthesized antibodies can react with the epitopes present in native allergens, thus confirming that the allergen composition of Dpg-Pol extracts contains numerous individual allergens and their isoforms and that the specific IgGs synthesized may protect against the common inhaled allergens present in pollen grains. This conclusion was confirmed when the pool of sera was incubated with the recombinant allergens rPhl p 1 and rPhl p 5a. Both allergens were clearly recognized after rabbit immunization, and no signal was detected in the pre-immune sera. These results are consistent with those of Casanovas et al [9] in humans, where, after treatment with the same product used in our study (Dpg-Pol extract of *P pratense*), a significant increase was observed in specific IgG and IgG1 levels. This increase was significantly higher than that observed with nonpolymerized extract.

In conclusion, our results propose an explanation for the effect of depigmented allergoids, suggesting that, although patients are treated with a modified molecule containing the individual allergens in complex chains, the immune response could target those allergens and isoforms that patients are exposed to. Future studies comparing immunization induced by native and modified allergens should be performed to confirm this finding.

Acknowledgments

All the authors are currently employed by Laboratorios LETI. The study was financially supported by Laboratorios LETI.

References

1. Flower DR. Advances in predicting and manipulating the immunogenicity of biotherapeutics and vaccines. BioDrugs. 2009;23(4):231-40.
2. Carnés J, Himly M, Gallego M, Iraola V, Robinson DS, Fernández-Caldas E, Briza P. Detection of allergen composition and in vivo immunogenicity of depigmented allergoids of *Betula alba*. Clin Exp Allergy. 2009;39(3):426-34.
3. Fernández-Caldas E, Gallego M, Carnés J, Iraola V. Enzymatic activity of *Dermatophagoides pteronyssinus* extracts after acidic treatment. Int Arch Allergy Immunol. 2008;145:298-304.
4. Gallego MT, Iraola V, Himly M, Robinson DS, Badiola C, García-Robaina JC, Briza P, Carnés J. Depigmented-polymerised house dust mite allergoid: allergen content, induction of IgG4 and clinical response. Int Arch Allergy Immunol. 2010;153:61-9.
5. García-Robaina JC, Sánchez I, de la Torre F, Fernández-Caldas E, Casanovas M. Successful management of mite-allergic asthma with modified extracts of *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* in a double-blind, placebo-controlled study. J Allergy Clin Immunol. 2006;118:1026-32.
6. Colás C, Monzón S, Venturini M, Lezaun A. Double-blind, placebo-controlled study with a modified therapeutic vaccine of *Salsola kali* (Russian thistle) administered through use of a cluster schedule. J Allergy Clin Immunol. 2006;117:810-6.
7. Alvarez-Cuesta E, Aragoneses-Gilsanz E, Martín-Garcia C, Berges-Gimeno P, Gonzalez-Mancebo E, Cuesta-Herranz J. Immunotherapy with depigmented glutaraldehyde-polymerised extracts: changes in quality of life. Clin Exp Allergy. 2005;35:572-8.
8. Guerra F, Daza JC, Almeda E. Immunotherapy with a depigmented, polymerised vaccine of *Olea europaea* pollen allergens. Significantly reduces specific bronchial and skin test reactivity in sensitized patients after one year of treatment. J Investig Allergol Clin Immunol. 2003;13:108-17.
9. Casanovas M, Sastre J, Fernández-Nieto M, Lluch M, Carnés J, Fernández-Caldas E. Double-blind study of tolerability and antibody production of unmodified and chemically modified allergen vaccines of Phleum pratense. Clin Exp Allergy. 2005;35:1377-83.
10. On the protection of animals used for experimentation and other scientific purposes. BOE, 2005. Royal Decree 1201/2005, October 10th. Boletín Oficial del Estado 252, 21/10/2005. pp. 34367-34391. [In Spanish].
11. Monograph on Allergen Products, European Pharmacopoeia 01/2010:1063.
12. European Medicines Agency. Committee for Medicinal Products for Human Use (CHMP) and Biologics Working Party (BWP) Guideline on Allergen Products: Production and Quality Issues. 2008. EMEA/CHMP/BWP/304831/2007.
13. Lund L, Henmar H, Würtzen PA, Lund G, Hjortskov N, Larsen JN. Comparison of allergenicity and immunogenicity of an intact allergen vaccine and commercially available allergoid products

for birch pollen immunotherapy. Clin Exp Allergy. 2007;37:564-71. 14. Henmar H, Lund G, Lund L, Petersen A, Würtzen PA. Allergenicity, immunogenicity and dose-relationship of three intact allergen vaccines and four allergoid vaccines for subcutaneous grass pollen immunotherapy. Clin Exp Immunol. 2008;153:316-23
15. Kahlert H, Stüwe H, Cromwell O, Fiebig H. Reactivity of T cells with grass pollen allergen extract and allergoid. Int Arch Allergy Immunol. 1999;120:146-57.
16. Cirković TD, Bukilica MN, Gavrović MD, Vujcić ZM, Petrović S, Jankov RM. Physicochemical and immunologic characterization of low-molecular-weight allergoids of *Dactylis glomerata* pollen proteins. Allergy. 1999;54:128-34.
17. Ibarrola I, Sanz ML, Gamboa PM, Mir A, Benahmed D, Ferrer A, Arilla MC, Martínez A, Asturias JA. Biological characterization of glutaraldehyde-modified *Parietaria judaica* pollen extracts. Clin Exp Allergy. 2004;34:303-9.
18. Mutschlechner S, Deifl S, Bohle B. Genetic allergen modification in the development of novel approaches to specific immunotherapy. Clin Exp Allergy. 2009;39:1635-42.

Manuscript received April 12, 2011; accepted for publication July 4, 2011.

Jerónimo Carnés, PhD

Research & Development
Laboratorios LETI, S.L.
Calle del Sol nº 5
28760 Tres Cantos
Madrid, Spain
E-mail: jcarnes@leti.com

