

Modulation of the Humoral Response to *Dermatophagoides pteronyssinus* Allergens in BALB/c Mice by Extract Modification and Adjuvant Use

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Key Words

House dust mite • Allergen-specific immunotherapy • Depigmented allergoid • CpG oligodeoxynucleotide

Abstract

Background: Currently, several strategies are being used in order to improve the safety and efficacy of allergen-specific immunotherapy; these strategies include the use of modified hypoallergenic extracts as well as different adjuvants with immunomodulatory properties in combination with native or modified extracts. The objectives of this study were to investigate the humoral response generated in mice to modified *Dermatophagoides pteronyssinus* extracts in the presence or absence of two different adjuvants. **Methods:** BALB/c mice were inoculated either with native, depigmented or depigmented-polymerised *D. pteronyssinus* without adjuvants or combined with aluminium hydroxide or oligodeoxynucleotides containing CpG motifs. IgE concentration, specific total IgG, IgG1 and IgG2a titres were measured in mice sera and cross-reactivity inhibition experiments were performed. IgG antigenic profiles were obtained by immunoblotting for all formulations. **Results:** Inoculation of depigmented-polymerised extract induced statistically significant lower IgE levels than the native extract even when ad-

sorbed onto aluminium hydroxide. When this extract was inoculated in the presence of oligodeoxynucleotides containing CpG motifs, it elicited high IgG levels, a high IgG2a/IgG1 ratio and low IgE production. Furthermore, the antigenic profiles observed after extract inoculation showed punctual differences between the depigmented-polymerised extract and the native or depigmented extracts. **Conclusions:** Our results suggest that the depigmentation and polymerisation process modifies the native extract's antigenic and immunogenic properties and converts the depigmented-polymerised extract into a better choice for allergen-specific immunotherapy.

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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]. Currently, several strategies are being developed in order to improve the safety and efficacy of traditional allergen-specific immunotherapy (SIT). These alternatives mainly involve the admin-

istration of hypoallergenic vaccines with low IgE-binding properties which can be generated by chemical modification of allergen extracts [2] or by recombinant DNA technology [3]. One of the techniques used for extract modification is polymerisation with glutaraldehyde, a process that modifies IgE epitopes, resulting in a safer vaccine that allows the administration of increased concentrations of allergen per dose. These formulations are known as allergoids and are composed of high molecular weight molecules that include all the individual allergens present in native (N) extracts forming proteic macrocomplexes [4]. A further step in this sense implies the production of allergoids using highly purified extracts. In this process, the allergen extract first undergoes depigmentation, a mild acid treatment and dialysis that removes most non-allergenic materials and purifies the allergenic extract prior to polymerisation with glutaraldehyde. Depigmented-polymerised (DPP) extracts from the house dust mite *Dermatophagoides pteronyssinus* have proven to be hypoallergenic [5], safe at high doses [6–8] and have proven their efficacy in several clinical trials [8–12].

A second approach for SIT improvement includes the use of novel adjuvants that contribute to a Th1-oriented balance of the Th2 response that takes place during an allergic reaction. Currently, aluminium hydroxide (AIOH) is the adjuvant of choice in conventional SIT; however, some studies have suggested that it might not be the ideal immunostimulatory agent due to its association with the generation of Th2 responses [13]. On the contrary, CpG-containing immunostimulatory oligodeoxynucleotides (ODNs) have shown to be able to generate Th1 responses through activation of TLR-9 receptors [14] and may be a better adjuvant choice for SIT. CpG ODNs have proven their efficacy as both a therapeutic alternative and as an adjuvant in mouse models of allergic rhinitis and asthma, acting as inductors of Th1 responses and inhibitors of allergen-induced airway hyperresponsiveness, eosinophilia as well as airway remodelling [15–19].

Mouse models have an established place in the development of novel intervention strategies and they have been successfully used as a model in studies of allergic response modulation and SIT trials [20–24]. The objective of this study is to analyse the immune response in BALB/c mice to extracts from the house dust mite *D. pteronyssinus* when several formulations that included one N extract and two chemically modified extracts (depigmented, DP, and DPP extract) from the house dust mite *D. pteronyssinus* were inoculated in the presence or absence of two adjuvants: AIOH or CpG-containing immunostimulatory ODNs.

Materials and Methods

Allergen Extracts

N, DP and DPP *D. pteronyssinus* extracts were supplied by the allergen manufacturer (Laboratorios LETI, Madrid, Spain). Extracts were prepared and standardised as previously described [12]. Briefly, N extract was prepared starting from 100 g of *D. pteronyssinus* mite culture (Laboratorios LETI) that were extracted in 0.01 M phosphate-buffered saline, sterile filtered, frozen and lyophilised. Lyophilised N extract was then reconstituted in highly purified water and the pH of the solution was reduced using a mild acid treatment with HCl in order to remove all low molecular weight substances and components attached to the proteins/allergens. Afterwards the extract was dialysed overnight in dialysis membranes with a cut-off of 3.5 kDa (Cellu Sep Membrane, Seguin, Tex., USA). The pH was again adjusted to physiological conditions and the extract was sterile filtered, frozen and lyophilised. This D extract was reconstituted in PBS 0.01 M (pH 7.4), polymerised with glutaraldehyde and maintained overnight at room temperature under magnetic stirring. The resulting material was then dialysed in 100-kDa dialysis membranes (Millipore, Bedford, Me., USA), filtered and freeze-dried. Total protein content in each extract was quantified by a Bradford colorimetric assay (BioRad, Madrid, Spain).

Mice

Female BALB/c mice (6–8 weeks old) were obtained from Harlan Ibérica (Barcelona, Spain). Mice were housed under specific pathogen-free conditions and a 12:12-hour light-dark cycle. Cages, food and bedding were autoclaved, and all mice were handled in a laminar flow hood by gloved, gowned and masked personnel. All procedures were approved by the Institutional Review Board and followed the local ethical rules for animal experimentation [25].

Adjuvants

AIOH was supplied by Laboratorios LETI. An ODN with several consensus CpG sequences [26] including the optimal motif for activating human cells [27] (5'-TCGTCGTTAACGTTGTC-GCCTT-3') was designed. Synthesis with a nuclease-resistant PS backbone was carried out by ISOGEN Bioscience BV (De Meern, The Netherlands).

Immunisation Protocol

Animals were immunised subcutaneously in the footpad on days 0, 15 and 30 with 25 µg total protein from each extract in the absence of adjuvants, adsorbed onto AIOH (0.3% final concentration) or in combination with 20 µg ODN-CpG (n = 5–7). On day 37, sera were collected and the animals were euthanised.

Specific IgG Determination

Standard ELISA plates (Nunc-Immunoplate Maxisorp) were coated overnight at 4°C with 100 µl of 20 µg/ml N extract, blocked with 5% non-fat dried milk powder in PBS-0.5% Tween 20. Sera diluted in blocking solution (1:1,000 or 1:500) were added to the wells and incubated for 2 h at room temperature. Specific total IgG and IgG subclasses 1 and 2a were detected with horseradish peroxidase-conjugated goat anti-mouse antibodies (Nordic Immunology) added to each well and the plate was incubated for 1 h at room temperature. Plates were washed and developed with or-

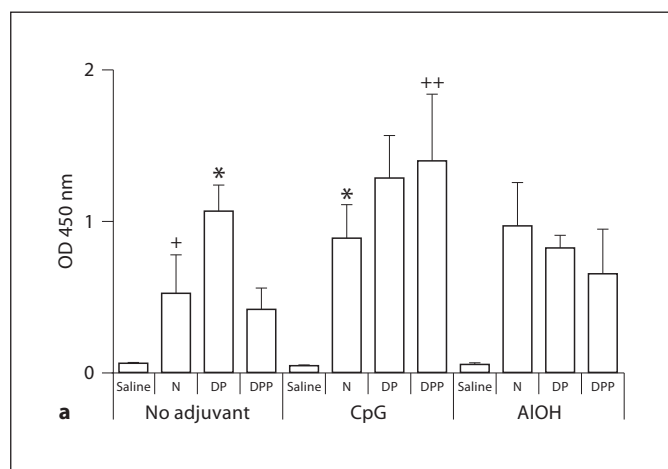
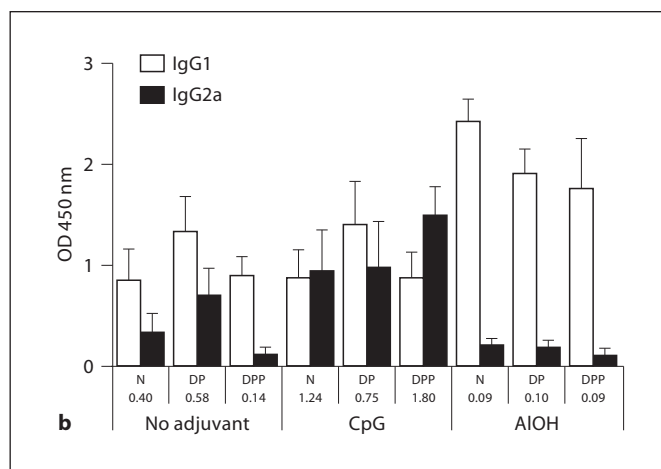


Fig. 1. IgG reactivity against N extract as determined by ELISA in sera from animals inoculated with N, DP and DPP extracts in combination with different adjuvants. Five mice were inoculated with each formulation. Results are expressed as the mean $\pm$ SD. **a** Total IgG levels. Results were analysed by two-way ANOVA followed by post hoc Fisher LSD tests. * Significant differences between extracts when the same adjuvant was used ($p < 0.05$). + Significant



nificant ($p < 0.05$) and ++ highly significant ($p < 0.001$) differences between adjuvants when the same extract was used. **b** IgG1 and IgG2a levels. Kruskal-Wallis one-way analysis of variance on ranks was performed, followed by post hoc analysis using the Mann-Whitney U test with Bonferroni correction. The value represented under the name of each extract corresponds to the IgG2a/IgG1 ratio.

tho-phenylenediamine (DAKO) as substrate and reactions were stopped after 30 min by addition of 1 N H_2SO_4 . Optical density was measured at 450 nm.

Total IgE Levels

IgE levels were measured in the sera of individual animals at a 1:200 dilution using BD OptEIA™ Mouse IgE ELISA Set (BD Biosciences) according to the manufacturer's instructions.

SDS-PAGE and Immunoblotting

N extract was separated by SDS-PAGE in a 4–20% gradient gel and transferred onto a PVDF transfer membrane (Millipore) using a tank transfer system (Biorad). Pooled sera from each formulation were diluted 1:400 in a buffer containing PBS, 5% non-fat dried milk powder, 0.05% Tween 20. Detection was performed with goat anti-mouse IgG-AP (Invitrogen) diluted 1:2,000 and developed with BCIP/NBT substrate kit (Invitrogen).

ELISA Cross-Reactivity Inhibition Experiments

Sera from each of the groups inoculated without adjuvants were pooled and pre-incubated for 2 h with each extract at room temperature. After pre-incubation, reactivity against each of the extracts was measured by ELISA.

The extract concentration needed for complete inhibition was determined after pre-incubation of each serum at increasing concentrations of inhibitor. Reactivity was considered inhibited when it dropped below 10% of the uninhibited reactivity.

Statistical Analysis

Normality (Shapiro-Wilk) and equal variance (Levene) tests were applied to all ELISA results. In the case that these tests were passed, total IgG results were analysed by two-way ANOVA fol-

lowed by post hoc Fisher LSD tests. When non-parametric tests were required for analysis of IgG1 and IgG2a data, Kruskal-Wallis one way analysis of variance on ranks was performed, followed by post hoc analysis using the Mann-Whitney U test with Bonferroni correction.

Results

Specific IgG Levels

Sera from pre-immune mice showed no reactivity against the extracts used in this study (data not shown).

The three extracts were immunogenic in the absence of adjuvants, when administered adsorbed to AIOH and in the presence of CpG (fig. 1a). Two-way ANOVA showed that the differences in immunogenicity observed between each formulation depended on the effect of the interaction between adjuvant and extract ($p < 0.001$) as well as the independent effect of the adjuvant ($p < 0.001$) or the extract used ($p < 0.05$). The differences in immunogenicity of each formulation depended on the extract ($p < 0.05$) and the adjuvant ($p < 0.001$) used as well as the interaction between both ($p < 0.001$). In the absence of adjuvants, inoculation of DP extract resulted in higher IgG reactivity than N or DPP extract ($p < 0.05$ and $p < 0.001$, respectively; table 1). When CpG was used, both DP and DPP extracts were significantly more immuno-

Table 1. Summary of the Fisher LSD post hoc analysis of the IgG reactivity levels

		No adjuvant			CpG			AlOH		
		N	DP	DPP	N	DP	DPP	N	DP	DPP
No adjuvant	N									
	DP	*								
	DPP	–	**							
CpG	N	*	–	*						
	DP	**	–	**	*					
	DPP	**	–	**	*	–				
AlOH	N	*	–	*	–	–	*			
	DP	–	–	*	–	*	*	–		
	DPP	–	*	–	–	**	**	–	–	

– = No significant differences. * $p < 0.05$; ** $p < 0.001$.

genic than the formulation that included N extract ($p < 0.05$). Finally, no statistically significant differences in IgG reactivity were observed between the three formulations that included AlOH as an adjuvant. The highest IgG reactivity was observed when DPP extract was inoculated in combination with CpG as an adjuvant, with significant or highly significant differences when compared with all formulations with the exception of DP and DP + CpG. Table 1 shows the complete Fisher LSD post hoc analysis of the data shown in figure 1a.

The adjuvant effect was also visible when the IgG2a/IgG1 ratio was studied (fig. 1b). In the absence of adjuvants, inoculation of the three extracts resulted in an IgG1-predominant response (N extract IgG2a/IgG1 = 0.4; DP extract IgG2a/IgG1 = 0.58 and DPP extract IgG2a/IgG1 = 0.14). When AlOH was used as an adjuvant, the IgG1 response was exacerbated ($p < 0.001$ compared to formulations without adjuvant), resulting in lower IgG2a/IgG1 ratios (N + AlOH IgG2a/IgG1 = 0.09; DP + AlOH IgG2a/IgG1 = 0.10 and DPP + AlOH IgG2a/IgG1 = 0.06). The use of CpG as an adjuvant had no significant effect on IgG1 levels, but increases in IgG2a levels were observed ($p < 0.05$ when inoculated in combination with N or DPP extract, compared to inoculation in the absence of adjuvants). These increases resulted in a mixed response in the case of N + CpG (IgG2a/IgG1 = 1.24) and DP + CpG (IgG2a/IgG1 = 0.75) and an IgG2a-biased response when DPP extract was formulated in combination with CpG (IgG2a/IgG1 = 1.80).

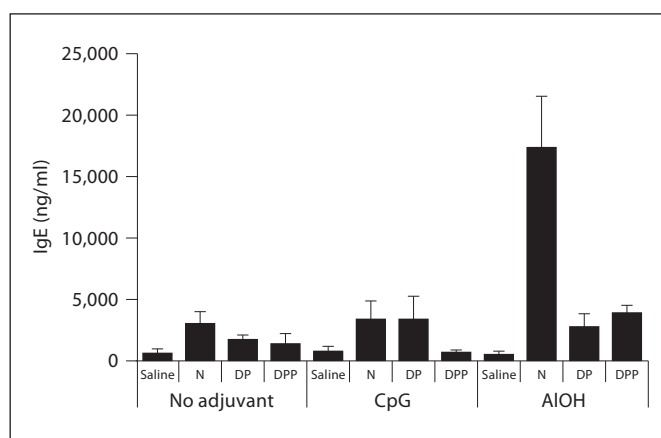


Fig. 2. Total IgE levels. Concentration in sera as determined by BD OptEIA™ Mouse IgE ELISA set. Results are shown as the mean $\pm$ SD from 5 animals.

Total IgE Levels

The effects of extract modification and adjuvant use on the levels of total IgE were analysed (fig. 2). When comparisons between extracts are performed, statistically significant differences are observed: In the absence of adjuvants, inoculation of N extract resulted in higher IgE levels than the inoculation of DP or DPP extract ($p < 0.05$). When the extracts were adsorbed onto AlOH, the highest levels of IgE were observed after inoculation of N extract ($p < 0.05$). If CpG was used as an adjuvant, the formulation that included DPP extract as an adjuvant induced lower IgE levels ($p < 0.05$) than N or DP extract.

Statistically significant differences are also observed when comparison of the adjuvants is performed. AlOH significantly increased the IgE levels induced by N extract inoculation. The differences are significant ($p < 0.05$) when compared to the groups inoculated with N extract only or N extract in combination with CpG. When the adjuvant effect was analysed for groups inoculated with DP extract, significant differences were only observed between the group inoculated in the absence of adjuvants and the group inoculated with DP extract adsorbed onto AlOH ($p < 0.05$). The IgE levels observed when the different formulations that included DPP extract were highest when this extract is inoculated adsorbed onto AlOH ($p < 0.05$).

The highest levels of IgE were induced by the N + AlOH formulation ($p < 0.05$ when compared to all formulations) and the lowest levels were observed after in-

oculation of DPP extract either in the absence of adjuvants or in combination with CpG ($p < 0.05$).

Immunoblot Analysis

The IgG immunoblot experiments were used to analyse the specificity of the antibody response induced by each formulation against N extract (fig. 3). All formulations presented a common recognition pattern in the 116- to 166-kDa range. Two bands of approximately 21 and 31 kDa were observed with varying reactivity intensities when all extracts were inoculated in the absence of adjuvants or in combination with CpG. When ALOH was used as an adjuvant in combination with the three extracts, each of these two bands was visible with high intensity (fig. 3, lanes 10–12). A high molecular weight reactivity band (approx. 160 kDa) was observed when N and DP extracts were inoculated in the absence of adjuvants or in the presence of CpG or ALOH. This band could not be detected in response to the formulations that included DPP extract. Sera from animals inoculated with DPP extract recognised an intense low molecular weight band (approx. 714 kDa). This band is faintly detected by sera from animals inoculated with N and DP extracts without adjuvant or in combination with CpG, and its intensity is higher when these extracts are adsorbed onto ALOH.

Cross-Reactivity Inhibition

In order to study the effect of depigmentation and subsequent polymerisation on the antibody repertoire generated after extract inoculation, the differences in total IgG reactivity after pre-incubation of each serum with each extract were analysed. Figure 4a shows the decrease in reactivity of pools of sera from animals inoculated with N, DP and DPP extracts after pre-incubation with increasing concentrations of their respective extract. In the three cases, concentrations over 35 $\mu\text{g/ml}$ of each inhibitor were enough to reduce serum reactivity over 90% when compared to uninhibited sera. For the following experiments, each inhibitor was used at a two-fold excess concentration (70 $\mu\text{g/ml}$). Analysis of the inhibition capacity of each extract when sera were tested against N extract showed that reactivity was reduced more than 90% when each antiserum was pre-incubated with its own extract (fig. 4b). When the inhibition capacities of DP and DPP extracts were tested, N antiserum retained 14 and 48% of its reactivity, respectively (fig. 4b). The reactivity inhibition assays for DP extract (fig. 4c) showed that while N and DP extracts were able to inhibit the reactivity of their own antisera against DP extract, DPP antiserum retained over 20% of its reactivity against DP extract when pre-incubated

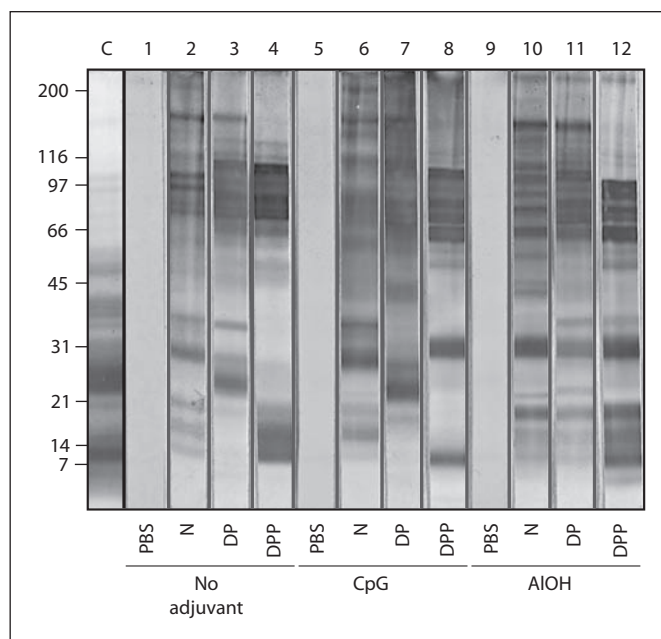


Fig. 3. Immunoblot analysis. Lanes 1–12 represent analysis of the response against N extract. C = Coomassie-stained gel.

with DPP extract. When DP antiserum was pre-incubated with N and DPP extracts, inhibition was also incomplete, the antiserum retained 15 and 48% of its reactivity, respectively (fig. 4c). When reactivity against DPP extract was analysed (fig. 4d), neither N extract nor DP extract were capable of completely inhibiting the reactivity of DPP antiserum which dropped to 21 and 19%, respectively, after pre-incubation with each extract.

Discussion

Future directions in SIT will include the development of better standardised vaccines which should improve the safety profile of SIT, as well as the use of new immunomodulatory agents capable of modifying a naturally occurring Th2 response against an allergen. In this work we have studied the modulation of the response induced after inoculation of DP allergoids in combination with two different adjuvants (CpG and ALOH).

In the absence of adjuvants, the DP extract proved to be the most immunogenic. The differences between this extract and the N extract were greater than initially expected, as the protein content in both extracts has proven to be very similar [28]. The increase in immunogenicity

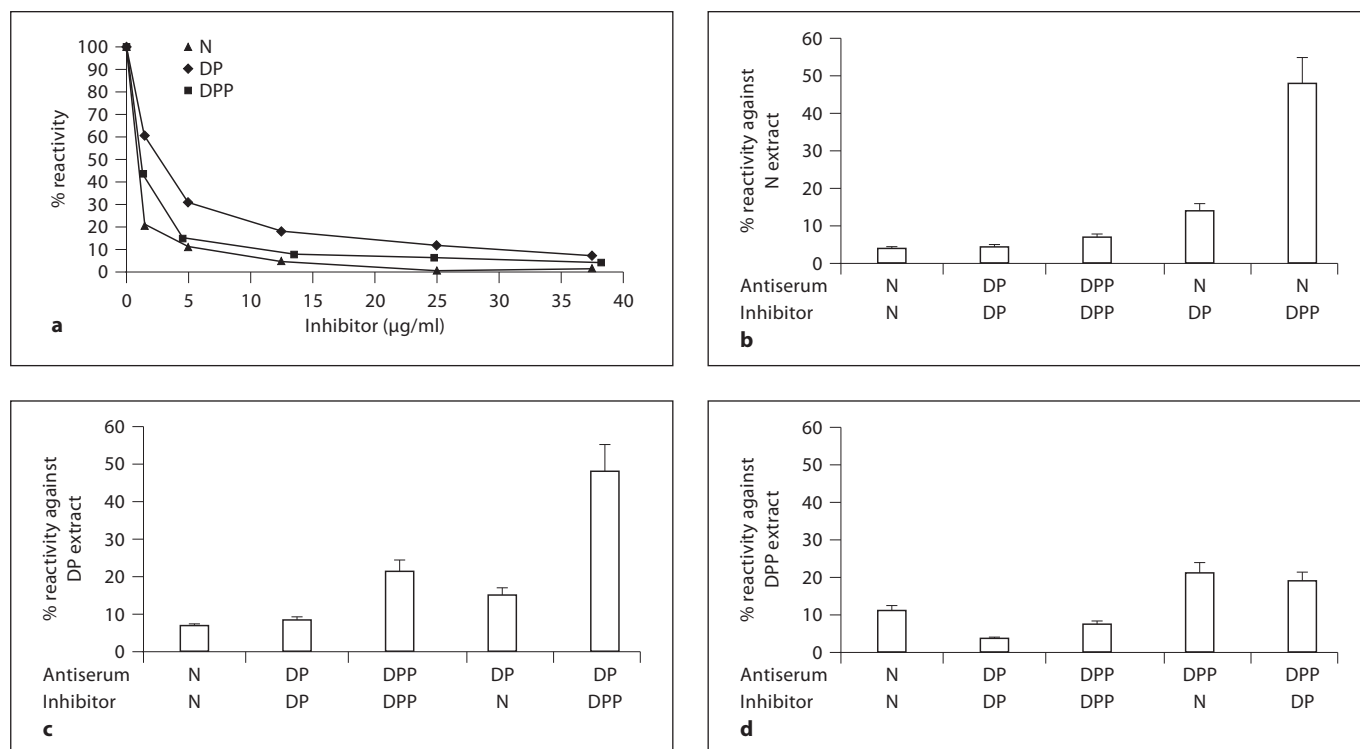


Fig. 4. Cross-reactivity inhibition assays. **a** Saturation curves for N, DP and DPP extracts. Pooled antiserum from each extract was pre-incubated with increasing concentrations of its respective extract and reactivity against the inhibitor was measured by ELISA. Results

are shown as percent reactivity in relation to uninhibited antiserum. **b–d** Changes in reactivity from several pools of sera against N (**b**), DP (**c**) or DPP (**d**) extract in the presence of different inhibitors. Results are shown as the mean $\pm$ SD of 3 independent experiments.

may be a consequence of the depigmentation process, which removes low molecular weight and immunologically irrelevant substances from the N extract, resulting in a purer extract, in terms of protein content.

The increases in IgG reactivity observed when CpG was used as an adjuvant were as expected, with an especially high increase (three-fold) in DPP extract immunogenicity. CpG ODNs have proven to be facilitators of pinocytotic antigen uptake [29], which may improve presentation of large molecules such as those present in the DPP extract, thus increasing its immunogenicity.

When ALOH was used as an adjuvant, it only boosted the N extract's immunogenicity, reducing the differences that had been observed between extracts after inoculation in the absence of adjuvants. A similar effect was observed in the antigen recognition patterns after administration of the extracts with ALOH.

IgG2a/IgG1 ratios are associated with Th1-Th2 polarisation: an IgG2a-predominant humoral response (that is IgG2a/IgG1 > 1) is related to IFN- γ production and a Th1 response [30], whilst a Th2 response driven by IL-4 induc-

es production of IgG1 [31]. Inoculation of the extracts without adjuvants resulted in an IgG1-biased response which was exacerbated when they were formulated adsorbed onto ALOH. This polarisation was due both to increases in IgG1 and decreases in IgG2a levels, suggesting that the naturally occurring Th2 response to the extracts is further enhanced by the adjuvant. These data are consistent with the possible role of aluminium salts as promoters of Th2-type responses as well as Th1 inhibitors [13, 32]. CpG ODNs modulated the polarised response towards a mixed response when formulated with N and DP extracts, but only in the case of DPP extract, a complete reversal was observed. In this case, the IgG2a/IgG1 profile generated in response to DPP + CpG was characteristic of a Th1 response. The combined effect of a modified hypoallergenic extract with the use of CpG as an adjuvant may favour non-IgE-mediated antigen uptake [29, 33], resulting in the production of Th1 cytokines that would induce the production of IgG2a.

These data agree with the IgE levels observed after inoculation of DPP + CpG which were the lowest of all for-

mulations, along with the group inoculated with DPP extract alone. The polymerisation process reduces the IgE-binding capacity of the extract, thus reducing its allergenicity [5–7, 9, 34, 35]. DPP extract did not induce high levels of IgE even when formulated in combination with ALOH, an adjuvant that boosted IgE levels when adsorbed onto N extract. The high IgE levels observed when the N + ALOH formulation was used, along with the IgG2a/IgG1 profile, suggest that mice inoculated with this formulation may have been sensitised against this extract. The DP extract, even though it contains the same antigenic profile as the N extract [28] was not able to boost the IgE levels, even though it has not been proven to be hypoallergenic when used in skin prick tests [5]. However, the depigmentation process is known to reduce its enzymatic activity [28]. This reduction in enzymatic activity may be the cause of the DP extract's inability to induce high IgE levels in a Th2 context, because sensitisation to *D. pteronyssinus* has been associated with the cysteine protease activity of the major allergen Der p 1, which is known to cleave CD23 from activated B cells [36, 37] and CD25 from T cells [38, 39]. Der p 1, as well as Der p 3, 6 and 9, which are also proteases, have been proven to degrade proteolytically tight junctions in lung epithelium and cause the release of pro-inflammatory cytokines from bronchial epithelial cells, mast cells and basophils [for review, see 40]. As a consequence of the reduction in enzymatic activity, DP and DPP extracts allow the inoculation of higher doses without the secondary effects elicited by N extract.

The reactivity pattern as analysed by immunoblotting was similar for all formulations. However, slight differences were observed between extracts, especially between DPP and the non-polymerised extracts. In spite of these differences, sera from animals inoculated with N extract and DPP extract (an extract formed by aggregates of the allergens from the N extract) recognised common protein bands. Indeed, the main principle of DP allergoid use implies that the patient responds to the individual allergens to which he is naturally exposed. The goal of the ELISA inhibition experiments was to find out if the different processes of allergen modification modify the N extract's antigenicity and if there are differences between the antibody repertoire generated in response to extract inoculation. The polymerised extract was only able to inhibit 50% of the reactivity of N and DP antiserum. These data, along with the immunoblotting results, suggest that the polymerisation process induces structural changes in the proteins that are present in the N or DP extracts, hiding some of the IgG epitopes and, therefore, changing the

extracts' antigenicity and immunogenicity, creating new epitopes. DPP antiserum retained 20% of its reactivity after pre-incubation with either N or DP extract, suggesting that there are antibodies generated in response to DPP extract which are specific for this extract, a fact that would imply that during the polymerisation process, new epitopes are generated. Similar results were observed for the DP extract: it only inhibited 85% of N antiserum's IgG reactivity, while DP antiserum retained 15% of its reactivity after pre-incubation with N extract.

Our results indicate that DPP extract is the better choice due to its hypoallergenic properties and the high levels of specific IgG antibodies generated when used with the traditional adjuvant ALOH. Administration of this low IgE-binding extract in combination with CpG-containing immunostimulatory oligonucleotides could be considered as a promising alternative to improve the efficacy of house dust mite-specific immunotherapy. According to our results with the three options investigated, DPP extract combined with the traditional adjuvant ALOH seems to be the best option for the induction of immunogenicity in animals due to the high levels of specific IgG antibodies generated. Clinical trials have already evidenced the high capacity of this vaccine to stimulate specific IgG4 to mite allergens. However, the combination of DPP extract with CpG-containing immunostimulatory oligonucleotides could be considered as a promising alternative. Studies in patients are needed to demonstrate the efficacy of this last combination, but these preliminary results, combined with the high safety profile of DPP allergenic extracts, suggest that it could be considered as an alternative for the future.

Acknowledgements

The authors would like to thank Jimena Cortés for collaborating in several aspects of this work. N.P. and Y.P. are particularly grateful to Prof. Alberto Martínez-Serrano and Prof. Miguel Ángel Íñiguez for allowing them to use their laboratory facilities.

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