

Allergenicity, immunogenicity and dose-relationship of three intact allergen vaccines and four allergoid vaccines for subcutaneous grass pollen immunotherapy

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Summary

Different vaccines containing intact allergens or chemically modified allergoids as active ingredients are commercially available for specific immunotherapy. Allergoids are claimed to have decreased allergenicity without loss of immunogenicity and this is stated to allow administration of high allergoid doses. We compared the allergenicity and immunogenicity of four commercially available chemically modified grass pollen allergoid products with three commercially available intact grass pollen allergen vaccines. The allergenicity was investigated with immunoglobulin (Ig)E-inhibition and basophil activation assays. Human T cell proliferation and specific IgG-titres following mouse immunizations were used to address immunogenicity. Furthermore, intact allergen vaccines with different contents of active ingredients were selected to study the influence of the allergen dose. In general, a lower allergenicity for allergen vaccines was clearly linked to a reduced immunogenicity. Compared with the vaccine with the highest amount of intact allergen, the allergoids caused reduced basophil activation as well as diminished immunogenicity demonstrated by reduced T cell activation and/or reduced induction of murine grass-specific IgG antibodies. Interestingly, intact allergen vaccines with lower content of active ingredient exhibited similarly reduced allergenicity, while immunogenicity was still higher or equal to that of allergoids. The low allergenicity observed for some allergoids was inherently linked to a significantly lower immunogenic response questioning the rationale behind the chemical modification into allergoids. In addition, the linkage between allergenicity, immunogenicity and dose found for intact allergen vaccines and the immunogen as well as allergenic immune responses observed for allergoids suggest that the modified allergen vaccines do not contain high doses of immunologically active ingredients.

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Introduction

Specific immunotherapy (SIT) is the practice of administering specific allergen(s), intact or chemically modified (allergoids), to an allergic patient in order to induce immunological tolerance and ameliorate symptoms associated with subsequent exposure to the causative allergen [1]. SIT with intact allergens has been documented extensively as an effective treatment for immunoglobulin (Ig)E-mediated allergic disorders in several studies during the past decades [2].

Most allergoid vaccines are allergen extracts which have been modified chemically by, for example, glutaraldehyde or formaldehyde. The chemical process causes irreversible inter- and intramolecular polymerizations of the proteins at the primary amino groups in the amino-terminus or at the amino acid lysine. Theoretically, this results in destruction of some conformational IgE epitopes and should have no impact on sequential T cell epitopes [3]. Thus, the claim of allergoids is that they have decreased allergenicity, thereby allowing faster and higher up-dosing [4] with preserved or even improved immunogenicity because of formation of

higher molecular weight complexes after modification [4–6]. However, it is not possible to measure the potency of allergoids or the amount of major allergens in these complexes after the chemical modification, and to our knowledge no double-blind placebo-controlled dose–response studies have been performed with commercially available allergoids. There is only one study on non-commercial allergoids showing no clear dose-dependence of the allergoid treatment [7].

In order to evaluate how the functional potency of allergoids after the modification compares with intact allergens (in which major allergen and protein content can be measured reliably in the final product), we decided to compare the allergenicity and immunogenicity of four commercially available chemically modified grass allergoid products with three commercially available intact allergen grass vaccines covering a range of active ingredient content. IgE inhibition and basophil activation assays were selected as model systems for allergenicity, and human T cell proliferation and specific IgG-titres following mouse immunizations were used to address immunogenicity.

Material and methods

Allergen preparations and patients

Three commercially available intact allergen vaccines with three different maintenance doses: Alutard SQ grass mix (ALK-Abelló A/S, Hoersholm, Denmark), Pangramin Plus grass mix (ALK-Abelló A/S) and ALK7 grass mix (ALK-Abelló A/S) containing unmodified extracts of grass pollen (Intact-Allergen-1, -2 and -3 respectively); and four commercially available allergoid grass mix products: Allergovit (Allergopharma, Reinbek, Germany), Pollinex Quattro (Allergy Therapeutics, West Sussex, UK), Purethal (HAL Allergy, Haarlem, the Netherlands) and Depigoid (Leti Laboratorios, Barcelona, Spain) (Allergoid-A, -B, -C and -D respectively) for subcutaneous immunotherapy were included.

Blood samples were obtained from 20 grass allergic patients tested positive for *Phleum pratense*-specific IgE (> IgE class 2) and shown to release histamine towards purified major grass allergens Phl p 1 and/or Phl p 5 (> 10 nM histamine at 5 µg/ml major allergen). Twelve patients responded to tree pollen, 10 to mites, seven to animal dander, six to weeds and four patients responded to moulds in addition to grass pollen. None of the patients had detectable IgE to all allergen sources tested. All patients had a clinical history describing grass pollen as the major cause of allergy symptoms and none of the patients received SIT before the study.

Vaccines were analysed in concentrations normalized according to the recommended maximal therapy dose. Blood donors were recruited and informed according to the project description approved by the local ethics committee.

Allergenicity

Immunoglobulin E-inhibition

The IgE-inhibition experiments were performed on an ADVIA Centaur system (Bayer Diagnostics, Tarrytown, NY, USA), as described previously [8,9]. A serum pool obtained from mixing equal amounts of serum from 15 grass allergic patients (IgE classes 3 and 4) was used for the inhibition experiments. Serial dilutions of the grass pollen extracts, either Intact-Allergen-1, -2 and -3 or Allergoid-A, -B, -C and -D were used as inhibitors. Assay read-out values in relative light units were determined in triplicate.

Basophil activation test

Activated human basophils express the degranulation/activation marker CD63 on their membrane [10]. CD63 was determined by flow cytometric analysis as a marker of basophil activation [11,12].

The BD-FastImmune CD63FITC/CD123PE/Anti-HLA-DR (BD Bioscience, San José, CA, USA) was used according to the manufacturer's recommendations. Basophils in diluted whole blood (1 : 10) were activated in duplicate by incubation with vaccines for 1 h at 37°C in 96-deep-well plates (Eppendorf, Hamburg, Germany). The vaccines were diluted in RPMI-1640 (BioWhittaker, Cambrex, Verviers, Belgium), 0.1% human serum albumin (Sigma-Aldrich, St Louis, MO, USA) in 10-fold dilutions ranging from 1 : 200 to 1 : 2×10^7 . The reaction was stopped by adding 10 µl 0.1 M ethylenediamine tetraacetic acid/well (Sigma-Aldrich) and supernatant was removed by 10 min centrifugation at 800 g. The cells were stained with BD-FastImmune antibody mix and treated according to the manufacturer's recommendations, followed by analysis on flow cytometer: fluorescence activated cell sorter ARIA (BD Bioscience).

CD63 expression was measured on > 1000 basophils. Activation was calculated as percentage of basophils moving to a positive gate. For statistical comparison, median basophil activation values were calculated for each patient at 1 : 20 000 dilution of each product as this dilution was in the dose–response range of the curves in the majority of the products and patients tested. Statistical analysis of other dilutions provided similar results.

Immunogenicity

Establishment of Phl p 1- or Phl p 5-specific T cell lines

T cell lines specific to Phl p 1 or Phl p 5 were established from peripheral blood mononuclear cells (PBMCs) of 18 grass allergic patients as described previously [8]. Briefly, freshly isolated PBMCs (2×10^6 /ml), CD8 depleted by magnetic antibody cell sorting magnetic depletion according to the

manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany), were stimulated with purified Phl p 1 or Phl p 5 (1 µg/ml) for 14 days. Recombinant interleukin (IL)-2 (Chiron, Emeryville, CA, USA) was added at days 5, 6 and 7. After 14 days T cells were isolated, washed and restimulated with autologous PBMCs; Phl p 1 or Phl p 5 (1 µg/ml); and phytohaemagglutinin-P (PHA-P) (Difco, Detroit, MI, USA). Recombinant IL-2 was added at days 3, 4 and 5. The lines were kept in culture with restimulation every 10 days with allogeneic Epstein-Barr virus B cells, allogeneic PBMCs and PHA-P and addition of recombinant IL-2 at days 3–5. The cell line cultures were terminated after a period of 8 weeks.

T cell stimulation assay

The capacity of the different vaccines to stimulate allergen-specific T cells was analysed in a standard 72-h T cell stimulation assay, as described previously [8], with autologous PBMC as antigen-presenting cells (10^5 /well, irradiated 3000 Rad). Threefold dilutions of the recommended maintenance dose of each product (from 1 : 100 to 1 : 2700) were used for T cell stimulation. Purified Phl p 1 or Phl p 5 and alum (aluminium hydroxide) alone were used as positive and negative controls respectively. Cells cultured in medium alone were used to determine background proliferation. All assays were repeated twice as three or four replica cultures and results are expressed as stimulation index [SI = counts per minute (cpm) stimulated cultures/cpm medium control]. Only experiments with SI > 3 for Phl p 1 or 5 were accepted for further analysis.

Cytokine measurements were performed on supernatants from eight T cell lines harvested after 48 h of stimulation using cytometric bead assay (CBA assay) (BD Bioscience Pharmingen) measuring IL-2, IL-4, IL-5, IL-10, tumour necrosis factor- α and interferon (IFN)- γ according to the manufacturer's instructions.

All vaccines were tested for toxicity in activated non-Phl p 1- or 5-specific T cell lines without significant T cell toxicity or mitogenic effect.

Immunization of mice

BALB c/A mice, eight per group, were immunized a total of six times on days 0, 14, 28, 42, 56 and 70 with each product. All products were administered subcutaneously in a volume per injection corresponding to 1/10 of the recommended human maintenance dose. Blood was drawn from the mice approximately 1 week after immunization on days 0, 35, 63 and 82.

Antibody analyses

Analyses of *P. pratense*-specific IgG (sIgG) antibodies from 56 mice were performed by direct enzyme-linked immun-

osorbent assay according to the method described in [8] using microtitre plates coated with *P. pratense* extract.

Statistical analysis

All data were analysed using the statistical software program GraphPad Prism version 4.03. Comparison of basophil activation data and T cell responses was performed using the non-parametric Dunn's multiple comparison test because of inhomogeneous variance. Comparison of IgG-data between the intact allergen vaccines and each allergoid product after five immunizations was performed by one-way analysis of variance using a planned Bonferroni multiple comparison *post hoc* test. Equal variances were demonstrated by Bartlett's test.

Results

Allergenicity

The IgE-inhibition curves (data not shown) of the intact allergen vaccines were parallel to the inhibition curve of an aqueous grass pollen extract, indicating that the epitope composition of the alum absorbed intact allergen vaccines did not differ from that of the aqueous grass pollen extract. The allergoids caused very low activity in the inhibition assay; therefore, a direct comparison of the ability of the allergoids and intact allergen vaccines to inhibit IgE-binding was not possible, as none of the allergoids tested revealed inhibition curves that could be fitted for statistical analysis.

For basophil activation, blood from 20 grass pollen allergic patients was analysed; four of these are shown in Fig. 1 as examples of the different patterns of reactivity obtained for individual patients. Two patients were omitted from the statistical analysis because of missing data points for Intact-Allergen-3. The results revealed a fairly consistent ranking of the different products with respect to basophil activation, even though a high degree of variation was seen between individual patients (Fig. 1). Median values of the percentage of CD63 activation at one dilution (1 : 20 000 of the maintenance dose) are shown (Fig. 2). Compared with Intact-Allergen-1 (the vaccine with the highest amount of intact allergen), the basophil activation was significantly lower for all four allergoids. In comparison with Intact-Allergen-3 (the vaccine with the lowest amount of intact allergen), Allergoid-B, -C and -D activated basophils to a similar extent, and Allergoid-A showed significantly higher basophil activation than Intact Allergen 3. Allergoid-A and -B activated basophils to the same extent as Intact-Allergen-2. Thus, the data show that the allergoids investigated exhibit a wide range of basophil activation comparable to some of the intact allergen vaccines and that the activation levels observed for intact allergens clearly reflected the concentration of active ingredients in the vaccine preparation.

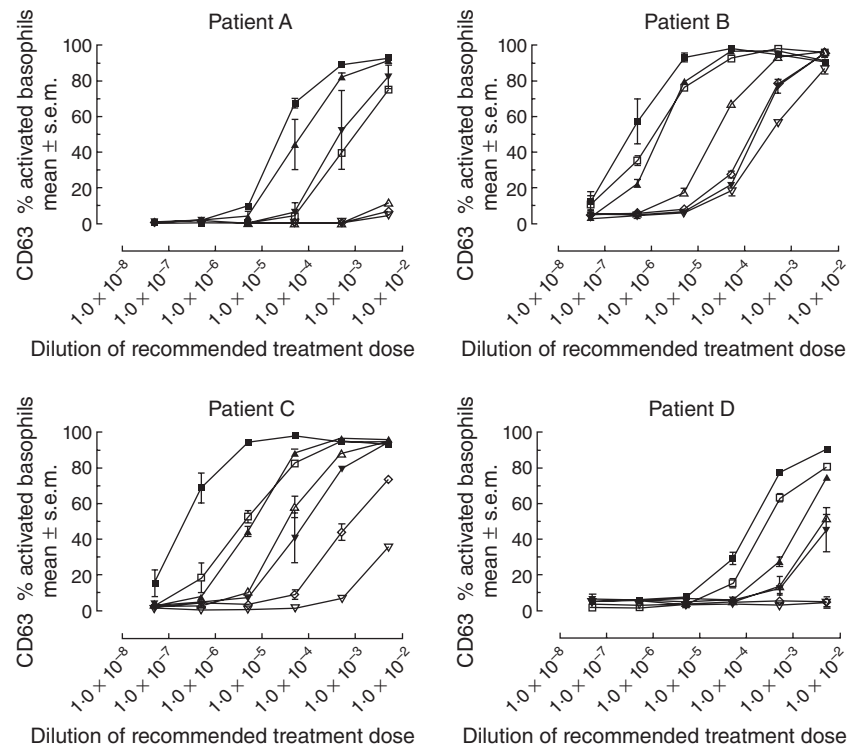


Fig. 1. Basophil activation test with four allergoid products and three intact allergen vaccines in blood from four grass pollen allergic patients. Intact-Allergen-1 (■), Intact-Allergen-2 (▲), Intact-Allergen-3 (▼), Allergoid A (□), Allergoid B (△), Allergoid C (▽) and Allergoid D (◇).

Immunogenicity

A total of 29 Phl p 1- and Phl p 5-specific T cell lines from 18 grass pollen allergic patients were established, and exhibited strong proliferation when stimulated with the intact allergen vaccines (Fig. 3a). Comparable results were obtained with several dilutions; only data for 1 : 300 dilution are shown as SI values (the average background value was $2482.9 \text{ cpm} \pm 2740.2$). In general, the allergoids induced significantly lower or completely abrogated T cell activation when compared with all Intact-Allergen vaccines. Only the T cell activation of Allergoid-A was not significantly lower than Intact-Allergen-2 and -3 (Fig. 3b). The lowest T cell stimulation was observed with Allergoid-D, which also showed the lowest basophil activation. The cytokine levels produced in response to the individual vaccines directly reflected the T cell proliferation and comparable T helper type 2 (Th2) cytokine profiles dominated by IL-5 with very low IFN- γ and IL-10 were observed after T cell activation with all vaccines investigated (data not shown).

The kinetics of sIgG responses following immunization of mice is shown in Fig. 4a. An increase in sIgG was already observed after three immunizations with Intact-Allergen-1 and -2 and Allergoid-A. Mice immunized with the intact allergen vaccines reached a maximum level after five immunizations, whereas except for Allergoid-A, mice immunized with allergoids had not reached comparable sIgG levels after six immunizations. The sIgG responses induced by five immunizations using Intact-Allergen-1 and -2 were

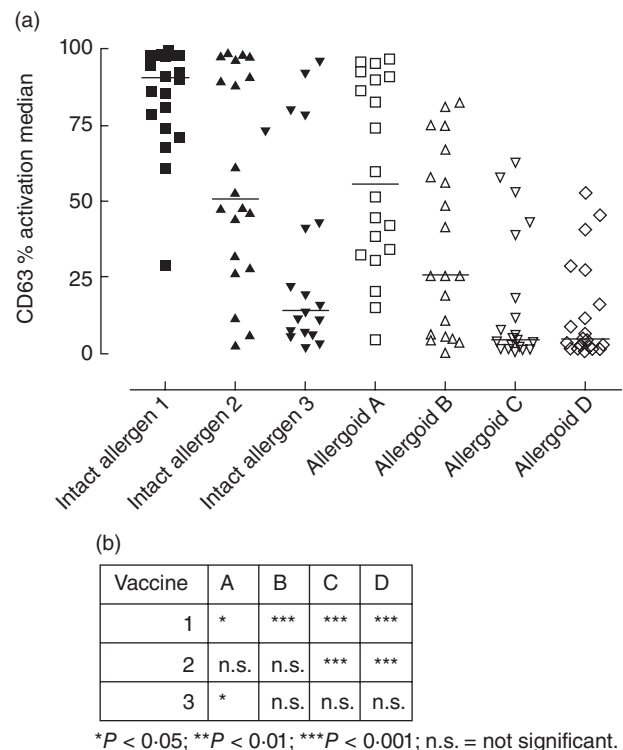


Fig. 2. (a) Median values of percentage of CD63 activation at one dilution (1 : 20 000 of the maintenance dose). (b) Summary table of statistical comparison between intact allergen vaccines and allergoid products. Intact-Allergen-1 (■), Intact-Allergen-2 (▲), Intact-Allergen-3 (▼), Allergoid A (□), Allergoid B (△), Allergoid C (▽) and Allergoid D (◇).

significantly higher than sIgG responses induced by Allergoid-A, -B, -C and -D (Fig. 4b,c).

Discussion

The objective of the present study was to investigate the allergenicity and immunogenicity of commercially available vaccines based on intact grass pollen allergens or chemically modified grass pollen allergoids. In addition, our study was designed to enable a comparison of biological potency of four commercially available allergoids with three intact allergen vaccines containing different amounts of active ingredient (protein and adjuvants). Because of the fact that it is not possible to measure the allergen content of allergoids after the chemical modification, all vaccines were normalized with respect to the manufacturer's recommended maintenance dose.

The tested commercially available allergoids for subcutaneous grass pollen immunotherapy were seen to constitute a diverse group with respect to both allergenicity and immunogenicity – low allergenicity of the allergoids was linked inherently to reduced immunogenicity.

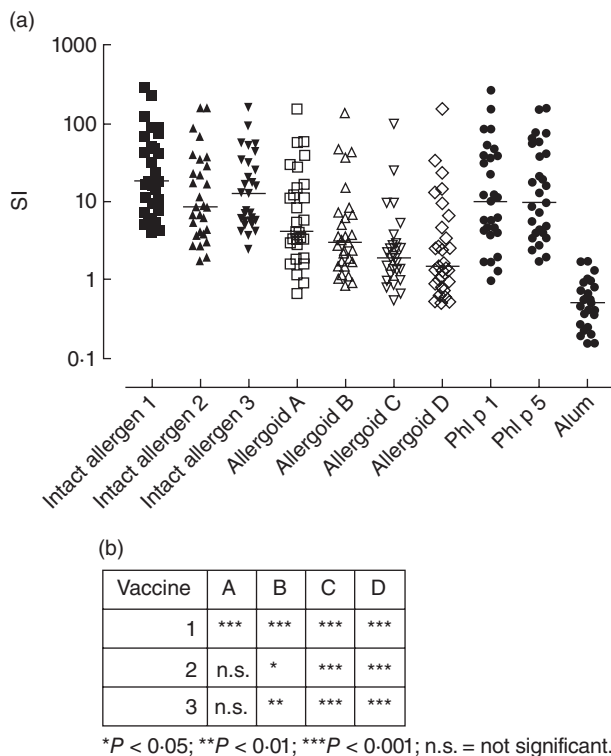
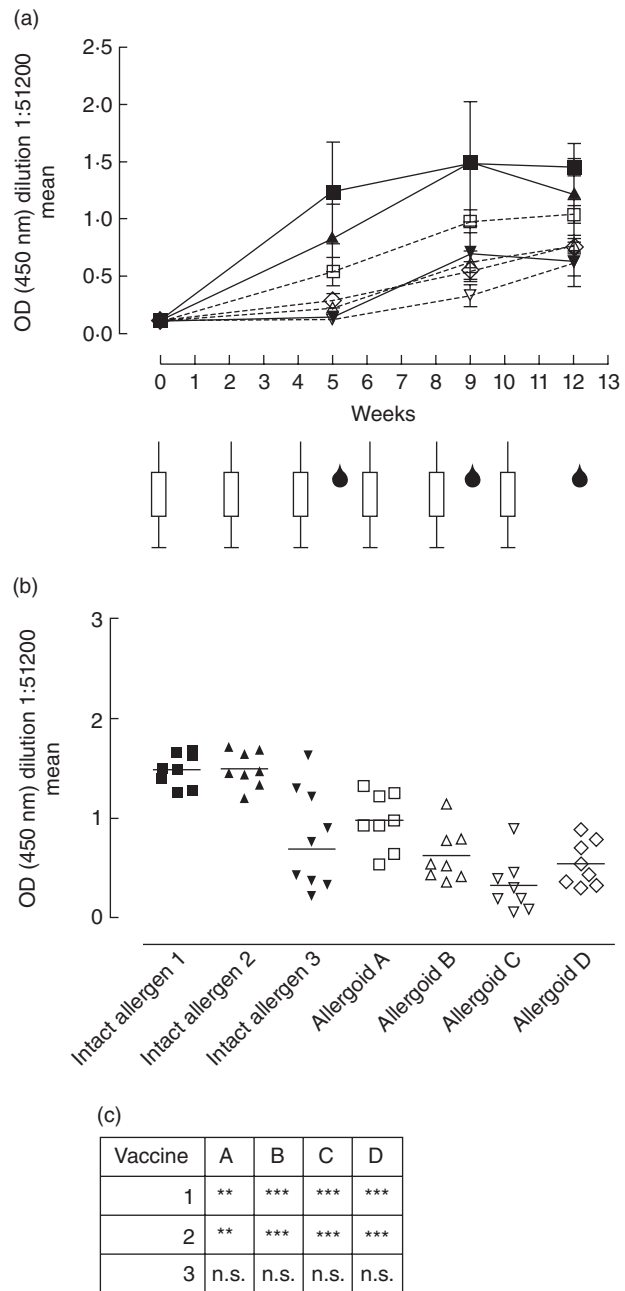


Fig. 3. (a) Median values (logarithmic scale) of stimulation index (SI) in T cell stimulation assay. T cell lines from 18 allergic patients were tested with peripheral blood mononuclear cells (PBMCs) as antigen-presenting cells (APC). (b) Summary table of statistical comparison between intact allergen vaccines and allergoid products. Intact-Allergen-1 (■), Intact-Allergen-2 (▲), Intact-Allergen-3 (▼), Allergoid A (□), Allergoid B (△), Allergoid C (▽), Allergoid D (◇), and controls (●) Phl p 1, Phl p 5, and alum.



* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s. = not significant.

Fig. 4. (a) The kinetics of Phl p-specific immunoglobulin (Ig)G antibody responses following immunization of the mice. Immunization points are indicated by the syringe symbol. Blood samples were analysed after three, five and six immunizations (corresponding to weeks 5, 9 and 12) indicated by ●. (b) Mean values of Phl p-specific IgG in mice after five immunizations. (c) Summary table of statistical comparison. Intact-Allergen-1 (■), Intact-Allergen-2 (▲), Intact-Allergen-3 (▼), Allergoid A (□), Allergoid B (△), Allergoid C (▽) and Allergoid D (◇).

Limited IgE-binding was observed with the allergoids demonstrated by IgE-inhibition assay. This could be due to disruption of the IgE epitopes during the chemical modification process to such a large extent that the allergoids were

unable to inhibit IgE-binding of the aqueous grass pollen extract. However, all allergoids induced basophil activation, suggesting that IgE-binding and cross-linking on the cell surface was induced by some intact IgE epitopes. Other studies with allergoids have demonstrated reduced allergenicity by various *in vitro* methods [8,13,14], as well as skin prick testing [15,16]. Ibarrola *et al.* [15] investigated allergenicity and immunogenicity and several immunochemical aspects of a *Parietaria* allergoid preparation and concluded that allergoids may be close to non-allergenic with fully retained immunogenicity. However, the above study and other studies demonstrating strongly reduced allergenicity of allergoids have been based mainly on IgE-binding assays, which seem to overestimate the effect of the chemical modifications.

In the present study, the allergoids as a group did not show reduced basophil activation as a measure of allergenicity. The two allergoids with the lowest allergenicity (Allergoid-C and -D) also exhibited significantly lower immunogenicity than all intact allergens. Our results demonstrated that the reduction in allergenicity observed for some allergoids was accompanied by a clearly reduced immunogenicity. The mouse immunization experiments showed a clear reduction in the capacity to induce sIgG by all allergoids compared with the Intact-Allergen-1 and -2. The low immunogenicity was demonstrated further by a generally reduced or even completely abrogated capacity of the allergoids to activate human Phl p 1- and Phl p 5-specific T cell lines compared with the intact allergens. This is in accordance with previous studies [8,17–19]. Dormann *et al.* [18] demonstrated that the majority of PMBCs and T cell lines showed a reduced response to a birch pollen allergoid, possibly as a result of loss of T cell epitopes, as several Bet v 1-specific T cell clones failed to recognize the allergoid [17,18].

The major allergens, including Phl p 5, are used to standardize the allergen content of the investigated intact vaccines. In the vaccines investigated in the present study, the content of Phl p 5 is comparable in Intact-Allergen-2 and -3, being 10 times lower than in Intact-Allergen-1, and this may explain the reduced responses observed towards the two latter vaccines. The major allergen content of allergoids cannot be measured because of the chemical modification and attempts to compare total protein contents or major allergen contents before modification is questionable. As a result of these differences between the vaccines investigated all comparisons were performed based upon the manufacturer's recommended maximal dose or maintenance dose. However, when compared with Intact-Allergen-3, all allergoids revealed similar allergenicity and lower levels of T cell activation, suggesting that the effective allergen dose of these allergoids is low.

Whether products based on grass mix and single species can be compared depends upon the cross-reactivity between the entailed species. To address this, an intact allergen vaccine based on a single grass (Phl p) was included in the

present investigation and responses obtained in all assay systems were almost identical to the response to intact allergen 1, which is composed of a mixture of extracts from seven temperate grasses (results not shown). Thus, even though the measurements of immunogenicity are confined to responses towards one grass species (Phl p-specific mouse IgG) or even individual allergens from a single species (Phl p 1- or 5-specific T cell lines), the composition of the individual vaccines did not influence the evaluation of the allergenicity and immunogenicity because of extensive cross-reactivity between the grass species. This is in line with the high degree of cross-reactivity for both antibody [20] and T cell responses demonstrated for various temperate grasses [21–24].

Allergoids-A, -C and -D and all the intact allergens contain alum as adjuvant, whereas Allergoid-B contains L-tyrosine and monophosphoryl lipid A (MPL) as adjuvant. Intact-Allergen-1 and -2, and Allergoid-A and -C have a similar content of alum, which is 10 times higher than the alum content in Intact-Allergen-3. Despite the lower allergen and alum content of Intact-Allergen-3, this vaccine induces T cell activation at a higher level than that observed with three of the four allergoid products. The low level of sIgG that was observed in mice immunized with Intact-Allergen-3 may be explained by the low content of alum, because alum is known to influence this parameter strongly in mice [25,26]. Thus, among the three intact allergen vaccines a clear dose–response relationship was seen when taking both the major allergen amount and alum content into consideration.

If the observed reduction in allergenicity and immunogenicity of the allergoids are due to obstruction of both IgE and T cell epitopes, the chemical modification can be regarded as a simple 'dilution' of epitopes comparable to a reduction of dose. This is also in accordance with our recent findings of linked allergenicity and immunogenicity in birch pollen allergoids [8].

Moreover, the increased T cell immunogenicity of Intact-Allergen-3 with the lowest allergen content, when compared with Allergoid-B, -C and -D, could suggest that besides destruction of IgE epitopes T cell epitopes are also particularly influenced by the modification process of allergoids, thereby explaining the loss of immunogenicity. This could, in practice, further compromise the efficacy of allergoids.

Allergoid-A was the allergoid with the highest allergenicity and immunogenicity. This vaccine was modified using formaldehyde, whereas the other allergoids were modified using glutaraldehyde. Both chemicals produce similar protein modifications by reaction with primary amino groups, and therefore do not affect epitopes at random, as the degree of modification depends upon the number of amino groups available for chemical reaction as well as on reaction conditions such as time, concentration, etc. Whether the difference in allergenicity and immunogenicity between Allergoid-A and the other allergoids can be

explained by different effects of the modification process remains to be determined. However, the variation between the allergoids emphasizes that each product must claim its justification *per se*; i.e. clinical results obtained with one allergoid product may not apply to allergoids in general. Moreover, these results demonstrate clearly that the extensive documentation for the effect of intact allergen vaccines cannot be used to verify the effect of vaccines based on modified allergens.

The assay parameters selected in this study represent human *in vitro* and murine *in vivo* model systems that cannot be extrapolated directly to human clinical aspects. However, our findings showing lack of reduction in allergenicity for some allergoids are in accordance with clinical data from a risk–benefit assessment performed by the Paul Ehrlich Institute in Germany. This risk–benefit assessment based on estimated incidences of serious, non-fatal adverse drug reactions clearly did not report a better safety profile of allergoids compared with intact allergens [27], and the risk of inducing systemic adverse reactions has not been eliminated with allergoids [28]. Furthermore, the recent Cochrane meta-analysis by Calderon *et al.* [2] demonstrates clearly that the conclusive clinical documentation for the beneficial effect of allergen immunotherapy on symptom, medication and quality-of-life scores is based primarily on data from patients treated with intact allergen vaccines.

Theoretically, vaccines based on a reduced spectrum of epitopes may be capable of inducing qualitatively similar immunological changes compared with intact allergens; however, quantitatively, our results suggest that reduced allergenicity is linked inherently to reduced immunogenicity. Moreover, our results indicate that despite equal or higher protein content in all the allergoids before modification, their functional immunogenicity was either equal to or lower than the lowest-performing intact allergen (Intact-Allergen-3) in all assays. This result indicates that after modification, the allergoids are not inducing immunological responses in accordance with the content of active ingredient measured before the modification, thereby questioning further the rationale behind the allergoid concept.

The link demonstrated between allergenicity, immunogenicity and dose questions the concept behind allergoids as well as the perception that allergoids contain high doses of low allergenic active ingredients, which are claimed to justify shorter up-dosing regimens. Furthermore, the results show significant differences in allergenicity and immunogenicity, which calls for specific documentation of clinical safety and efficacy for each individual vaccine – both intact allergen and allergoid vaccines. Taken together currently no sufficient clinical studies exist comparing the efficacy of intact allergen and allergoids vaccinations and the number of papers documenting the clinical efficacy of intact allergens vaccines by far exceeds the published documentation on allergoid efficacy.

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