Comparison of Intact Allergen Extracts and Allergoids For Subcutaneous Immunotherapy: The Effect of Chemical Modification Differs Both Between Species and Between Individual Allergen Molecules

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Abstract

Background: Allergen products for subcutaneous immunotherapy (SCIT) contain intact allergen extracts or chemically modified allergoids. Chemical modification was introduced to reduce allergenicity while retaining immunogenicity and thereby enable safer and more efficient allergy immunotherapy.

Methods: Experimental allergoids were produced from intact allergen extract for birch, grass, and house dust mite (HDM) to evaluate the effects of chemical modification. Preparations were compared with commercial allergoids and analyzed using SDS-PAGE/immunoblotting, IgE-inhibition assays, and crossed immunoelectrophoresis (CIE). Dermatophagoides pteronyssinus (Der p) vaccines were also tested for protease activity and immunizing capacity in a mouse model.

Results: The composition of IgE-binding epitopes in allergoids differed from that of intact allergen vaccines. Birch and grass allergoids produced smears of protein aggregates on SDS-PAGE, whereas intact allergen preparations showed distinct protein bands as expected. Der p allergoid vaccines, however, showed a distinct protein band corresponding to major allergen Der p 1 in both SDS-PAGE and CIE analysis, and commercial Der p allergoid vaccines showed Der p 1–related cysteine protease activity.

Conclusion: Allergoids and intact allergen preparations differ with respect to the composition of IgE-binding epitopes. However, chemical cross-linking does not affect every allergen molecule to the same degree. Der p 1, for example, remains largely unmodified. Furthermore, the investigational HDM allergoid vaccines showed reduced and delayed immune responses when used for immunization of mice.

Key words: Allergoid. Immunotherapy. House dust mite. Allergenicity. Allergy vaccine.
Summary box

- **What do we know about this topic?**
  The chemical modification of a protein/allergen with aldehyde is dependent on the protein sequence and structure. Therefore, some proteins/allergens are less susceptible to modification than others.

- **How does this study impact our current understanding and/or clinical management of this topic?**
  Given the different compositions of allergoids and intact allergen extracts, clinical documentation regarding safety and efficacy of SCIT with intact allergen vaccine is not applicable to chemically modified allergoid vaccines and vice versa.

Introduction

Subcutaneous immunotherapy (SCIT) is used to treat allergic patients by repeated administration of an allergen extract for the purpose of inducing immunological tolerance, thereby addressing the underlying cause of the disease in the immune system [1]. Commercially available SCIT vaccines contain either intact allergen extracts or allergoids as their active ingredient. An allergoid is an allergen extract that has undergone chemical modification with the aim of reducing its allergenicity, while retaining its immunogenic potency [2].

The allergoid concept was developed in the 1970s by Marsh et al [2], who described the process of modifying rye grass group I allergen using formaldehyde. The chemical agent used in allergoid production (eg, formaldehyde or glutaraldehyde) induces intramolecular as well as intermolecular cross-linking. The precise nature of this chemical reaction leading to intramolecular cross-linking is not clearly understood, although it is likely to involve several different simultaneous reactions, owing to the multiple forms in which aldehydes exist [3]. It is believed that aldehydes act on several functional groups in proteins (eg, amino, thiol, phenol, and imidazole groups), with the most reactive moiety being the ε-amino group, such as that found in lysine [3,4]. Kawahara et al [5] suggested that glutaraldehyde, a bifunctional aldehyde, could be converted to polymeric forms by reaction with a protein’s amino groups (via aldol condensation), with this polymer forming multiple Schiff base (imine) linkages with the protein, thus producing a cross-linked protein structure [3-5].

Commercially available allergoids differ considerably with respect to composition and, hence, allergenicity and immunogenicity [6]. In the present study, allergoid vaccine A was produced using formaldehyde whereas allergoid vaccines B and C were produced using glutaraldehyde. Formaldehyde has 1 aldehyde group and promotes inactivation of IgE binding epitopes primarily by reaction with primary amino groups, but also through cross-linking, whereas glutaraldehyde promotes cross-linking by reaction with 2 primary amino groups. The concentration of allergen extract determines the degree of intra- and intermolecular cross-linking. Furthermore, in the original description of allergoid production by Marsh et al [2], only a single incubation in a low aldehyde concentration was prescribed. However, in a later publication, Marsh et al [7] introduced a second incubation with a higher concentration of aldehyde. Whereas the first method only introduces mild modification, the second method represents a more thorough approach, inducing modifications with a profound effect on the structure and function of proteins in the allergen extract. As the details of the production processes of the commercial allergoids included in this study are not known, it is not possible to fully address these matters.

The resulting allergoids, however, show variable reduction in the capacity to trigger histamine release from leukocytes [2] (ie, reduced allergenicity) and variable immunogenicity (ie, capacity to induce IgG-based immune responses). Indeed, early studies comparing the immunogenicity of modified and intact allergen extracts appeared to confirm that the modification process did not affect the immunogenicity of the allergoid [2].

Reduced allergenicity and retained immunogenicity in theory offer the potential for improved safety over intact allergen vaccines (while retaining comparable efficacy) and have led to the claim that high-dose administration and short updosing may be suitable for allergoid-based SCIT products [8]. However, results published by the German vaccine authority, the Paul-Ehrlich-Institute [9], reported no superiority in the clinical safety profile of allergoid vaccines over intact allergen vaccines. Furthermore, evidence of the clinical efficacy of allergoids is limited, and several recent double-blind, placebo-controlled studies did not meet their primary endpoint. A systematic literature review identified only 6 double-blind, placebo-controlled studies with positive evidence of efficacy according to the World Allergy Organization criteria [10]. A recent PubMed search identified a further 10 randomized double-blind, placebo-controlled studies with positive evidence of efficacy published after 2010.

Previous investigations compared the allergenicity and immunogenicity of birch allergoid vaccines [6,11] and grass allergoid vaccines [12] with intact allergen vaccines. The results indicated that allergoid vaccines are not always associated with reduced allergenicity, whereas all allergoid vaccines tested showed reduced immunogenicity (T-cell stimulation and IgG-mediated responses in mice) compared with the intact allergen vaccine [6,12], thus indicating that commercial allergoids do not fulfill the allergoid concept as originally described by Marsh et al [2].

These comparisons, however, depend on the composition and the concentration of the allergen extracts that constitute the drug substance of the different products. As the composition of allergen products differs between manufacturers and the concentration of allergoids is difficult to assess, 2 conditions of
Intact Allergen Extracts Versus Allergoids

Methods

Allergen Extracts and Products

Allergen extracts were produced by aqueous extraction of allergenic source materials and freeze-dried as described elsewhere [14]. Experimental allergoids were produced by incubating reconstituted freeze-dried extracts with glutaraldehyde (investigational allergoid extracts). In brief, allergen extracts were incubated with glutaraldehyde for 4 hours before the concentration of glutaraldehyde was increased, and incubation at room temperature was continued for another 18 hours. Reactions with glutaraldehyde were stopped by addition of glycine in excess, and the preparations were subsequently purified by size exclusion chromatography and concentrated as described previously [11]. Aqueous allergen extracts and allergoids were coupled to aluminum hydroxide (investigational vaccines) as described previously [14].

Allergen products were obtained from commercial suppliers as follows: Alutard SQ (ALK-Abelló) intact allergen vaccine 1, comprising intact allergen products derived from birch pollen (Betula verrucosa, Bet v), grass pollen (6-grass mix + secale; and Phleum pratense, Phl p), and house dust mite (HDM) (Dermatophagoides pteronyssinus, Der p); Pangramin Plus (ALK-Abelló) intact allergen vaccine 2, comprising intact allergen products derived from grass pollen (5-grass mix) and HDM (Der p); Allergovit and Acaroid (Allergopharma) allergoid vaccine A, comprising allergoids derived from birch pollen (Bet v), grass pollen (6-grass mix), and HDM (Der p); Purethal (HAL Allergy) allergoid vaccine B, comprising allergoids derived from birch pollen (Bet v), grass pollen (10-grass mix), and HDM (Der p); Depigoid (Leti) allergoid vaccine C; comprising allergoids derived from birch pollen (Betula alba, Bet a), grass pollen (5-grass mix), and HDM (Der p).

All commercial products were formulated in aluminum hydroxide and stored under the recommended storage conditions and used prior to the day of expiry. Proteins from commercial products were eluted from aluminum hydroxide using 50 mM phosphate buffer pH 7.2 as described elsewhere [15] and analyzed.

Antibody Reagents

Polyclonal, monospecific antiserum against major allergen was raised by immunizing rabbits with the purified major allergen. The antibody reagent was prepared from rabbit blood samples as described [16]. Polyclonal, polyspecific rabbit antiserum against allergen extract was prepared in a similar way, although only allergen extract was used for immunization instead of purified major allergen.

SDS-PAGE and Immunoblotting

Samples eluted from aluminum hydroxide complexes were analyzed using SDS-PAGE and immunoblotting using standard methodology. Analyzed samples were upconcentrated 7.5 times in the desorption process, and the maximum volume (20 µL) was applied to gels.

Crossed Immunoelectrophoresis

Crossed immunoelectrophoresis (CIE) was performed as described elsewhere [17]. Briefly, antigens were precipitated in a crossed immunoelectrophoretic gel system using polyspecific antibodies raised against Bet v, Phl p, or Der p extracts in rabbits.

IgE Inhibition Analysis

IgE inhibition assays were performed on the Centaur platform (Bayer Diagnostics). Pools of sera from at least 10 sensitized individuals with specific IgE levels to the relevant...
assay in the absence of DTT enabled the evaluation of the proteolytic activity of serine proteases exclusively. In the serine protease assay, the cysteine protease inhibitor E64 was substituted with the serine-specific protease inhibitor aprotinin.

Mouse Immunization and Antibody Analyses

SJL mice (8 per group) were immunized by subcutaneous injection in a volume corresponding to 1/10 of the recommended human maintenance dose of ‘intact allergen product 1’. Analysis of Der p 1– and Der p 2–specific IgG (sIgG) antibodies in individual mouse sera was performed by direct ELISA as described [6]. All animal work was performed in accordance with EU regulations and ISO 10993-2: ‘Animal Welfare Requirements’ and was approved by the Danish Ethics Committee under the Ministry of Justice. All necessary legal, regulatory, and ethical permissions were obtained. Animals were handled by trained personnel under veterinary supervision, and records and decisions concerning animal welfare were made daily.

Results

Investigational Allergoids

In order to enable direct comparison of intact allergen extract with allergoids, investigational allergoid vaccines and intact allergen vaccines were derived from the same drug substance batches. Investigational birch, grass, and HDM allergoids were produced using a simple glutaraldehyde modification procedure. SDS-PAGE and immunoblot analyses showed comparable band patterns when comparing investigational

![Figure 2](image_url)
and commercial allergoid vaccines, suggesting that they were similar in composition, except for allergoid C, where insufficient protein was eluted to perform gel staining.

Comparison of Allergen Content in Intact Allergen Extracts and in Allergoids

The SDS-PAGE band patterns of the 3 investigational allergoids compared with the 3 investigational intact allergen extracts are shown in lanes 2 and 3 in Figure 2. The birch and grass intact allergen extracts produced distinct band patterns, whereas the corresponding allergoids produced a “smear” with no distinct bands (Figure 2), indicating that the reaction with glutaraldehyde had produced larger protein aggregates. These data are consistent with the findings from size exclusion chromatography performed during the allergoid production process, where an increase in molecular size was observed after the chemical reaction with glutaraldehyde.

CIE analyses showed that the chemical modification process had altered the charge of the proteins present in the investigational birch and grass allergoids, leading to different precipitate patterns, as compared with the investigational intact allergen extracts (grass data not shown, birch, Figure 3).

For the HDM preparations, a band corresponding to Der p 1 (25 kDa) was identified on SDS-PAGE gels and immunoblot of the intact allergen extract, investigational allergoid vaccine, and commercial vaccines, except for allergoid vaccine C (Figures 2 and 4).

A single band corresponding to another HDM major allergen, Der p 2, was observed in the intact allergen extracts, but not in the investigational allergoids (Figure 2) or commercial allergoid vaccines (eluted from alum). Commercial intact allergen vaccine (+alum, eluted) also failed to show a distinct Der p 2 band. The lack of a distinct Der p 2 band in alum-adsorbed intact allergen vaccines is probably due to very tight binding to alum and, consequently, poor elution of the allergen.

Overall, the SDS-PAGE/immunoblot data were consistent with the CIE analyses. Some antigen precipitates present in the CIE pattern of the intact allergen extract were not visible in the CIE patterns representing the investigational allergoid, whereas other precipitates appeared more diffuse, indicating a modified epitope structure, except for the precipitate representing Der p 1. The morphology of the Der p 1 precipitate did not change after the modification procedure (Figure 3). In the birch system, no distinct precipitate was visible after modification, indicating that the different antigens were fully incorporated into the allergoid complex. For all preparations, it was observed that after modification, precipitates displayed altered mobility in the first dimension, indicating a modified electric charge. Furthermore, for the Der p 2 allergen, the precipitate was modified to such a degree that the antibodies could not form complexes, as indicated by the lack of precipitates (Figure 3, CIE B3).

Figure 3. CIE of Der p and Bet v intact allergen extracts (-alum) and investigational allergoid (-alum). A1-3, Der p intact allergen extract (-alum); A4-5, Bet v intact allergen extract (-alum). B1-3, Der p investigational allergoid (-alum); B4-5, Bet v investigational allergoid (-alum). Antigens were visualized using polyclonal rabbit antibodies raised towards either Der p extract (áDer p), purified Der p 1 (áDer p 1), purified Der p 2 (áDer p 2), Bet v extract (áBet v), and purified Bet v 1 (áBet v 1).
Assessment of Epitope Modification in Allergoids

The IgE-inhibition curves of the grass, birch, and HDM commercial intact allergen vaccines were parallel to the inhibition curves of the corresponding intact allergen extracts (Figure 5), indicating that the epitope composition of the alum-adsorbed commercial intact allergen vaccines did not differ from those not adsorbed to alum. Indirectly, this is an indication that Der p 2, which is detected by SDS-PAGE/immunoblot in the intact allergen extracts, is actually present in the HDM intact allergen vaccines.

When comparing investigational intact allergen extracts with the corresponding investigational allergoids, nonparallel hill slopes for all 3 species indicating that the composition of IgE epitopes present in the investigational allergoids differed from the composition of IgE epitopes in intact allergen extracts. Similar results were found with the commercial birch allergoid vaccine (data not shown); however, the commercial grass and HDM allergoids did not contain a sufficient amount of protein to perform this analysis.

For all 3 investigated species, the IgE-binding epitopes of the allergoids appeared significantly affected by the aldehyde modification process, which therefore altered the allergenicity/immunogenicity of the vaccines (see Discussion). These considerations are relevant for the allergen extracts, although the data do not enable conclusions to be drawn for individual allergen molecules, such as the Der p 1 and Der p 2 major allergens.

Enzymatic Activity of HDM Vaccines

Several HDM allergens are proteolytic enzymes secreted into the mite intestine and present in fecal pellets. Thus, Der p 1 is a cysteine protease (Table 1) and Der p 3, 6, and 9 display serine protease activity. Der p 2 has no known enzymatic activity.

Analysis of the commercial allergen vaccines showed that cysteine protease activity was present in all the HDM vaccines analyzed (Figure 6), indicating that Der p 1 enzymatic activity was resistant to aldehyde treatment. In contrast, serine protease activity was only present in the intact allergen vaccines (Figure 6), indicating that the chemical modification process had inactivated all serine protease activity (ie, Der p 3, 6, and 9).

IgG Antibody Responses to HDM Vaccines

Mice immunized with the commercial HDM intact allergen vaccine 1 and investigational HDM allergoid vaccine

### Table 1. Biochemical Properties and Lysine Content of House Dust Mite Allergens.

| Allergen      | Primary accession number | Biochemical name                      | Molecular weight, kDa | No. of lysine residues | % lysine |
|---------------|--------------------------|---------------------------------------|-----------------------|-----------------------|---------|
| Der p 1<sup>a</sup> (mature) | P08176                   | Cysteine protease                     | 24                    | 2                     | 0.9     |
| Der p 2<sup>b</sup> | P49278                   | NPC2 family                           | 15                    | 14                    | 10.9    |
| Der p 3       | P39675                   | Trypsin-like (serine protease)        | 31                    | 19                    | 8.2     |
| Der f 6<sup>c</sup> | P49276                   | Chymotrypsin-like DP5 (serine protease) | 25                    | 16                    | 7.0     |
| Der p 9       | Q7Z163                   | Trypsin-like (serine protease)        | 24                    | 8                     | 3.6     |
| Der p 23<sup>d</sup> | L7N6F8                   | Peritrophin-like protein domain       | 8                     | 4                     | 5.8     |

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<sup>a</sup> The exact number of lysine residues may vary for some isoallergens.

<sup>b</sup> Major allergens

<sup>c</sup> Considered equivalent to Der p 6, for which only a sequence fragment has been published.
showed similar IgG responses to Der p 1 (Figure 7), whereas the response to Der p 2 decreased and was delayed in mice immunized with allergoid vaccine.

**Discussion**

The original theory behind allergoids was that they would offer improved safety over intact vaccines because chemical cross-linking would eliminate some of the IgE epitopes and, at the same time, immunogenicity would be enhanced because chemical cross-linking would produce larger molecules with better capacity to stimulate immune responses [2]. However, more recent studies have shown that commercial allergoids may be equally potent in IgE binding as native allergen vaccines and that the immunogenicity of commercial allergoids is not superior to that of native allergen products [6,11-12]. These results were obtained for allergoids made from birch allergen extracts [6,11] and grass allergen extracts [12]. Furthermore, statistics from the Paul-Ehrlich-Institute do not indicate improved safety of allergoids over native allergen products in real-life immunotherapy [9].

Previous studies indicated that commercial allergoids differ widely in allergenicity and immunogenicity [6,12].
The therapy ordinance from the German authority, the Paul-Ehrlich-Institute, now requires all nonregistered allergen products on the German market for the frequent allergen groups (e.g., grass and birch pollen, HDM, and insect venoms) to be documented by clinical dose finding trials and properly designed confirmatory phase 3 trials. In contrast to many SLIT tablet–based studies with intact allergens, several recent state-of-the-art double blind, placebo controlled clinical trials with allergoids did not meet their primary endpoints, even if many trials were performed with doses significantly higher than those currently marketed (Table 2).

Analyses comparing investigational and commercial birch and grass allergoid extract and vaccines with intact allergen extracts and vaccines have generally demonstrated evidence of chemical modification in the form of aggregated proteins (SDS-PAGE), alterations in protein charge (CIE analysis), and IgE epitope modification (IgE inhibition assays).

Table 2. Outcome of Double-Blind Placebo-Controlled Phase 3 SCIT Studies With Allergoid Products and a Primary Endpoint of Symptom and/or Medication Scorea.

| Did not meet primary endpoint | No study results available | Met primary endpoint |
|------------------------------|---------------------------|----------------------|
| NCT00263627/2005-000025-35  | NCT00540631/2006-000934-11| NCT00263640/2004-003892-35|
| 2016-002781-31              | NCT01012531               | NCT00263601          |
| 2006-005269-20              | NCT00423787               | NCT00414141          |
| 2012-000414-11              | NCT00831025               | 2015-000984-15       |
| 2006-003066-34              | NCT00537342               | 2006-003067-31       |
| 2015-000188-15              | NCT00916422               | 2004-001538-18       |
| NCT01012531                |                           | 2016-000051-27       |
| 2008-002264-34b             |                           |                      |
| 2007-004255-10b             |                           |                      |
| 2006-005868-10              |                           |                      |
| 2006-000602-23              |                           |                      |
| 2014-004431-38              |                           |                      |
| 2012-004916-79              |                           |                      |
| 2013-002129-43              |                           |                      |

aRegistered in the clinical trial databases clinicaltrials.gov or clinicaltrialsregister.eu. as of 07-12-2021.

bWrong study reported.
It should be noted, however, that the chemical reaction with aldehyde used in the production of the allergoid does not introduce random inactivation of surface-exposed amino acid residues but is targeted to primary amino groups, thereby introducing a bias in the inactivation of IgE-binding epitopes on the molecular surface. Thus, while Der p 2 is modified thoroughly in the chemical process, Der p 1 showed a much lesser modification effect (SDS-PAGE, immunoblot, CIE, protease activity, and mouse immunization data). As outlined earlier, this difference can be explained by the number of solvent-exposed primary amino groups, e.g., lysine side chains and terminal amino groups, which react rapidly with aldehydes [2]. Der p 1 contains only 3 such amino groups, whereas Der p 2 contains 15 modifiable groups, meaning that Der p 2 is more extensively modified by aldehyde treatment than Der p 1. In addition, it is clear from the crystal structures shown in Figure 1 that the 3 Der p 1 primary amino groups (ie, amino terminal plus 2 lysine residues) are not dispersed evenly over the surface, leaving large areas unaffected. The lack of modification explains the detection of native Der p 1 in the Der p allergoid by immunoblot and CIE.

Der p 23 is also an important HDM allergen. As it was discovered not so long ago, specific reagents to analyze Der p 23 are not available. However, as the molecule, which is very small (approximately 8 kDa), contains 5.8% lysine, we would expect it to readily react with aldehyde and form part of the allergoid complex.

Many HDM allergens have proteolytic activity. As shown here, the activity of the cysteine protease Der p 1 was largely retained during the chemical reaction with aldehyde, whereas the enzymatic activity of the serine proteases Der p 3, 6, and 9 was effectively inactivated after chemical modification.

A recent study based on mass spectrometry showed that peptides of group 1 allergens were found in a modified extract, although none of the peptides contained the amino acid lysine, indicating that the group 1 allergen can indeed be modified [18]. Furthermore, Der p 3 was identified in the modified extract, indicating that the missing enzymatic activity is not due to lack of the protein, but that the chemical modification most likely destroyed the enzymatic activity of Der p 3.

Previous studies showed a prevalence of reactivity to Der p 1 and Der p 2 of up to 96% in HDM-allergic populations [19-22]. Therefore, the presence of both major allergens is considered fundamental to the manufacture of an effective vaccine. Major allergen content can be standardized as part of batch release for intact allergen vaccines, whereas this not possible for allergoids.

Furthermore, the current study demonstrates that the investigational HDM allergoid vaccine showed slower kinetics of induction of Der p 2–specific IgG responses in mice than the corresponding commercial intact allergen vaccine, indicating that Der p 2 immunogenicity was not fully retained after the chemical modification, consistent with published studies on birch and grass allergen extract vaccines, which showed reduced immunogenicity following chemical modification [6,11-12].

In conclusion, the chemical composition of allergoids differs markedly from that of intact allergen extracts, and it is clear that clinical documentation regarding the safety and efficacy of SCIT with intact allergen vaccine is not applicable to chemically modified allergoid vaccines and vice versa. Furthermore, it is an open question whether commercial allergoids fulfill the theoretical concept of reduced allergenicity and enhanced immunogenicity laid out by Marsh et al [2] in 1970.

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**Conflicts of Interest**

All authors were/are employees and/or stockholders of ALK A/S.

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