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 analysed by 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 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 a similar degree. Der p 1, for example remains largely unmodified. Furthermore, the investigational HDM allergoid vaccines showed reduced and delayed immune responses when used for immunisation of mice.

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

Resumen

Antecedentes: Los productos de alérgenos para inmunoterapia subcutánea (SCIT) contienen extractos de alérgenos intactos o alergoides modificados químicamente. En este trabajo se ha hecho una modificación química para reducir la alergenicidad a la vez que se conservaba la inmunogenicidad y por lo tanto, permitir una inmunoterapia más segura y eficiente.

Métodos: Se produjeron alergoides experimentales a partir de extracto de alérgeno intacto para abedul, hierba y ácaros del polvo doméstico (HDM) y se evaluaron los efectos de la modificación química realizada. Las preparaciones se compararon con alergoides comerciales y se analizaron mediante SDS-PAGE/inmunotransferencia, ensayos de inhibición de IgE e inmunoelectroforesis cruzada (CIE). Las vacunas de Dermatophagoides pteronyssinus (Der p) también se probaron para determinar la actividad de la proteasa y la capacidad de inmunización en un modelo de ratón.

Resultados: La composición de los epítomos de unión a IgE en los alergoides difería de las vacunas de alérgenos intactas. Los alergoides de hierba y abedul produjeron manchas de agregados de proteínas en el SDS-PAGE, mientras que las preparaciones de alérgenos intactos mostraron distintas bandas de proteínas como se esperaba. Las vacunas alergoides Der p, sin embargo, mostraron una banda de proteína distinta de la correspondiente al alérgeno principal Der p 1 en los análisis SDS-PAGE y CIE. Las vacunas alergoides comerciales Der p mostraron actividad de cisteína proteasa relacionada con Der p 1.

Conclusión: Los alergoides y las preparaciones de alérgenos intactos difieren con respecto a la composición de los epítomos de unión a IgE; sin embargo, el entrecruzamiento químico no afecta a todas las moléculas de alérgenos de un modo similar. Der p 1, por ejemplo, permanece prácticamente sin modificar. Además, las vacunas alergoides de HDM produjeron respuestas inmunitarias reducidas y tardías cuando se usaron para la inmunización de ratones.

Palabras clave: Alergoide. Inmunoterapia. Ácaro del polvo. Alergenicidad. Vacuna contra la alergia.

Introduction

Subcutaneous immunotherapy (SCIT) is a therapeutic technique that treats 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 1970's by Marsh et al., who described the process of modifying rye grass group 1 allergen using formaldehyde [2]. The chemical agent used in allergoid production (e.g., formaldehyde or glutaraldehyde) induces intramolecular as well as intermolecular cross-linking. The precise nature of this chemical reaction leading to intra- and inter-molecular cross-linking is not clearly understood, but it is likely to involve several different simultaneous reactions, due to the multiple forms in which aldehydes exist [3]. It is believed that aldehydes act on several functional groups in proteins, such as 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. 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, producing a cross-linked protein structure [3-5].

Commercial allergoids on the market today differ considerably with respect to

composition and hence allergenicity and immunogenicity [6]. In the present study, 'allergoid vaccine A' is produced using formaldehyde whereas 'allergoid vaccine B and C' are produced using glutaraldehyde. Formaldehyde has one 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 two primary amino groups. The concentration of allergen extract determines the degree of intra- versus inter-molecular cross-linking. Furthermore, in the original description of allergoid production by David Marsh [2] only a single incubation in low aldehyde concentration was prescribed, whereas in a later publication David Marsh introduced a second incubation with a higher concentration of aldehyde [7]. Whereas the first method only introduces mild modification, the second method represents a more thorough approach inducing modifications with profound effect on 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 address these matters in completion.

The resulting allergoids, however, show variable reduction in the capacity to trigger histamine release from leucocytes [2], i.e. reduced allergenicity, and variable immunogenicity, i.e. 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 a claim that high-dose and short up-dosing administration may be suitable for allergoid-based SCIT products [8]. However, statistics from the German vaccine authority, the Paul-Ehrlich-Institute [9], reported no superiority in the clinical safety profile of allergoid vaccines, as compared with intact allergen vaccines [9].

Furthermore, evidence of 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 six double-blind, placebo-controlled studies with positive evidence of efficacy according to the World Allergy Organisation (WAO) criteria [10]. A recent PubMed search identified a further ten randomised double-blind, placebo-controlled studies with positive evidence of efficacy, published after 2010.

Previous investigations examined the allergenicity and immunogenicity of birch [6,11] and grass [12] allergoid vaccines in comparison with intact allergen vaccines. 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 responses in mice) as compared to the intact allergen vaccine [6,12] therefore indicating that commercial allergoids do not fulfil the allergoid concept as originally described by Marsh [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, two conditions of the present study were established. Firstly, IgE-binding potency was compared using the concentration of the commercial product recommended by the manufacturer for maintenance treatment. Secondly, an

investigational allergoid was included enabling comparison with an extract made from the same drug substance and therefore of identical composition.

As described above, chemical modification of a protein by aldehyde mainly affects primary amino groups, such as the terminal amino group and lysine amino acid side chains. It should be noted, however, that not all amino acid residues with primary amino groups on their side chains undergo such chemical modification – specifically, arginine residues show no, or very low, reactivity with glutaraldehyde [3,13].

Most globular proteins have several lysine residues dispersed over the molecular surface, and therefore most IgE binding epitopes are effectively modified by aldehyde treatment. Der p 1, however, is peculiar in this aspect as only two lysine residues are exposed on the molecular surface.

In this report, we present data on commercial and experimental allergoids with or without alum and with particular focus on house dust mite vaccines.

Methods

Allergen extracts and products

Allergen extracts were produced by aqueous extraction of allergenic source materials and freeze-dried essentially as described [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 hrs before the concentration of glutaraldehyde was increased and incubation at room temperature continued for another 18 hrs. Reactions with glutaraldehyde were stopped by addition of glycine in excess and the preparations subsequently purified by size exclusion chromatography and concentrated as described [11]. Aqueous allergen extracts and allergoids were coupled to aluminium hydroxide (investigational vaccines) essentially as described [14].

Allergen products were obtained from commercial suppliers as follows: Alutard SQ[®] (ALK-Abelló, Hørsholm, Denmark) ‘intact allergen vaccine 1’: intact allergen products derived from birch pollen (*Betula verrucosa*, Bet v), grass pollen (6-grass mix + secale; and *Phleum pratense*, Phl p), and HDM (*Dermatophagoides pteronyssinus*, Der p).

Pangramin Plus[®] (ALK-Abelló, Hørsholm, Denmark) ‘intact allergen vaccine 2’: intact allergen products derived from grass pollen (5-grass mix) and HDM (Der p).

Allergovit[®] and Acaroid[®] (Allergopharma, Reinbek, Germany) ‘allergoid vaccine A’: allergoids derived from birch pollen (Bet v), grass pollen (6-grass mix), and HDM (Der p). Purethal[®] (HAL Allergy, Leiden, the Netherlands) ‘allergoid vaccine B’: allergoids derived from birch pollen (Bet v), grass pollen (10-grass mix), and HDM (Der p).

Depigoid® (Leti, Madrid, Spain) ‘allergoid vaccine C’: allergoids derived from birch pollen (*Betula alba*, Bet a), grass pollen (5-grass mix), and HDM (Der p).

All commercial products were formulated in aluminium hydroxide and they were kept at the recommended storage condition and used prior to the day of expiry. Proteins from commercial products were eluted from aluminium hydroxide using 50 mM phosphate buffer pH 7.2 as described [15] and analysed.

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, only allergen extract was used for immunization instead of purified major allergen.

SDS-PAGE and immunoblot

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

Crossed immunoelectrophoresis (CIE)

Crossed immunoelectrophoresis (CIE) was performed as described [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, Tarrytown, NY, USA). Pools of sera from at least 10 sensitised individuals with specific IgE levels to the relevant allergen of at least class 3 in Magic Lite assay, were used. The assays were performed essentially as described [6].

Enzyme assays

Cysteine protease assays

Commercial HDM products were assessed for proteolytic activity in triplicate using the Z-Leu-Leu-Glu-AMC substrate (Bachem, Bubendorf, Switzerland), which is cleaved by cysteine proteases, but not serine proteases and therefore represents a Der p 1 specific assay in HDM extracts.

When the tripeptide is bound to the fluorogenic group AMC (7-amino-4-methylcoumarin), the fluorescence is quenched. Upon cleavage by a protease, AMC is released and fluorescence increases. The measurement of the increase in fluorescence is thus a measurement of the proteolytic activity of the enzyme. To confirm the identity of the protease activity, parallel assays were performed, where E64 (a cysteine protease inhibitor) was added.

Substrate hydrolysis was measured by a continuous monitoring of the release of AMC over 30 minutes, using a SpectraMax Gemini XS fluorimeter (Molecular Devices, Sunnyvale, CA, USA), with $\lambda_{ex} = 350$ nm, $\lambda_{em} = 450$ nm. The rate of hydrolysis was determined from the maximal slope of the curve, fitted by the SOFTmax PRO 4.3 LS software.

Serine protease assays

Serine protease activity assays were performed using the same procedure but applying the serine protease substrate Boc-Gln-Ala-Arg-AMC (Bachem, Bubendorf, Switzerland). This substrate is cleaved both by cysteine and serine proteases; however, the cysteine protease activity is dependent on the presence of a reducing agent, such as DTT, therefore the assay in the absence of DTT allowed 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 immunisation and antibody analyses (ELISA)

SJL mice, 8 per group, were immunised 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 Ethical Committee under the Ministry of Justice. All necessary legal, regulatory and ethical permissions were obtained. Animals were handled by educated 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 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 three investigational allergoids compared with the three 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 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 precipitates after modification 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).

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, non-parallel hill slopes for all three 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), while the commercial grass and HDM allergoids did not contain a sufficient amount of protein to perform this analysis.

For all three investigated species, the IgE-binding epitopes of the allergoids appeared significantly affected by the aldehyde modification process, which therefore have altered the allergenicity/immunogenicity of the vaccines (see discussion). These considerations are relevant for the allergen extracts, whereas conclusions for individual allergen molecules, such as the Der p 1 and Der p 2 major allergens, cannot be drawn from these data.

Enzymatic activity of house dust mite vaccines

Several house dust mite allergens are proteolytic enzymes secreted into the mite intestine and present in faecal 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 analysed HDM vaccines (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 (i.e. Der p 3, 6 and 9).

IgG antibody responses to house dust mite vaccines

Mice immunised with the commercial HDM ‘intact allergen vaccine 1’ and investigational HDM allergoid vaccine showed similar IgG responses to Der p 1 (Figure 7), whereas the response to Der p 2 decreased and was delayed in mice immunised 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 as the chemical cross-linking would produce larger molecules with better capacity to stimulate immune responses [2]. However, more recent studies have shown that commercial allergoids in some cases are 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 [6,11] and grass [12] allergen extracts. 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 non-registered allergen products on the German market for the frequent allergen groups, such as grass and birch pollen, house dust mites 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 currently marketed doses (Table 2).

Overall, analyses on investigational and commercial birch and grass allergoids extract and vaccines in comparison with intact allergen extracts and vaccines 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).

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 much less effect of modification (SDS-PAGE, immunoblot, CIE, protease activity and mouse immunisation 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 three such amino groups, whereas Der p 2 contains 15 modifiable groups, meaning that Der p 2 is more extensively modified by aldehyde treatment as compared to Der p 1. In addition, it is clear from the crystal structures shown in Figure 1 that the three Der p 1 primary amino groups (i.e. amino terminal plus two 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.

Another important HDM allergen is Der p 23. As Der p 23 was discovered not so long ago, specific reagents to analyse Der p 23 are not available, but as the molecule, which is very small, approx. 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 allergen were found in a modified extract, but 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, which indicates that the missing enzymatic activity is not due to lack of the protein, but most likely the chemical modification 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 standardised 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 a slower kinetic of induction of Der p 2 specific IgG responses in mice as compared to the corresponding commercial intact allergen vaccine. This indicates that Der p 2 immunogenicity was not fully retained after the chemical modification in agreement with published studies on birch and grass allergen extract vaccines that 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 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 fulfil the theoretical concept of reduced allergenicity and enhanced immunogenicity laid out by David Marsh in 1970.

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Conflict of interests

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

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TABLES

Table 1. Biochemical properties and lysine content of house dust mite allergens. The exact number of lysine residues may vary for some isoallergens

Allergen	Primary accession number	Biochemical name	Molecular weight (kDa)	No. lysine residues	% lysine
Der p 1* (mature)	P08176	Cysteine protease	24	2	0.9
Der p 2*	P49278	NPC2 family [no known protease activity]	15	14	10.9
Der p 3	P39675	Trypsin-like [serine protease]	31	19	8.2
Der f 6**	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*	L7N6F8	Peritrophin-like protein domain	8	4	5.8

*Major allergens

**Considered equivalent to Der p 6, for which there is only a sequence fragment published.

Table 2. Outcome of double blind placebo controlled phase III SCIT studies with allergoid products and a primary endpoint of symptom and/or medication score and registered in the clinical trial databases, clinicaltrials.gov or clinicaltrialsregister.eu. as of 07-12-2021.

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
	2008-002264-34 ¹	
	2007-004255-10 ¹	
	2006-005868-10	
	2006-000602-23	
	2014-004431-38	
	2012-004916-79	
	2013-002129-43	

¹ Wrong study reported.

FIGURES

Figure 1. Crystal structures of Der p major allergens Der p 1 and Der p 2.

The molecular surfaces of Der p 1 (PDB ID 1XKG) and Der p 2 (PDB ID 1KTJ) are shown in orange. The lysine side chain amino groups and the N-terminal amino group are coloured green. Der p 1 contains two lysines and one terminal amino group, whereas Der p 2 contains 14 lysines and one terminal amino group.

Figure 2. Silver stained SDS-PAGE performed under reducing conditions showing intact allergen extracts and investigational allergoid , both without alum, as well as commercial vaccines

Lane 1: MW marker; Lane 2: intact allergen extract (-alum); Lane 3: investigational allergoid Lane 4: Mw marker; Lane 5: intact allergen vaccine 1; Lane 6: investigational allergoid vaccine; Lane 7: 'allergoid vaccine A' ; Lane 8: 'allergoid vaccine B' ; Lane 9: 'allergoid vaccine C'

In all vaccines, investigational as well as commercial, the allergen extracts were eluted from alum with phosphate buffer prior to analysis.

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 visualised using polyclonal rabbit antibodies raised towards either Der p extract (αDer p), purified Der p 1 (αDer p 1), purified Der p 2 (αDer p 2) Bet v extract (αBet v) or purified Bet v 1 (αBet v 1)

Figure 4. Immunoblots of Der p investigational allergoid vaccine and Der p commercial vaccines using a rabbit polyclonal anti-Der p 1

Lane 1: MW marker; Lane 2: Der p intact allergen extract; Lane 3: 'intact allergen vaccine 1'; Lane 4: investigational Der p allergoid vaccine; Lane 5: 'intact allergen vaccine 2'; Lane 6: 'allergoid vaccine A'; Lane 7: 'allergoid vaccine B'; Lane 8: 'allergoid vaccine C'

In all vaccines, the active ingredients were eluted from alum with phosphate buffer.

Figure 5. IgE inhibition curves of intact allergen extract, ‘intact allergen vaccine 1’, and investigational allergoid extract and vaccines

The inhibition of the interaction between biotinylated Bet v (left top graph)/Phl (right top graph p/Der p (left bottom graph) and IgE by various inhibitors was examined. The hill slope was compared directly in the fitting procedure. ■ = Intact allergen extract, ▲ = intact allergen vaccine, □ = investigational allergoids Δ = investigational allergoid vaccines.

Figure 6: Protease activity in commercial Der p allergen vaccines

Serine (green columns) and cysteine (red columns) protease activity in commercial Der p intact allergen vaccines and Der p allergoid vaccines were measured as described in Methods. 95% confidence intervals indicate uncertainty levels of triplicate determinations. Serine protease activity of allergoid vaccines is less or equal to levels measured in intact allergen vaccines with inhibitor aprotinin added.

Figure 7: Kinetics of Der p 1- and Der p 2-specific IgG antibody responses following mouse immunisation

Immunisation was performed on days 0, 14, 28, 42, 56, and 70. Blood samples were analysed after 2, 3, 5 and 6 immunisations (corresponding to weeks 3, 5, 9 and 11). Results are shown for commercial HDM ‘intact allergen vaccine 1’ (+alum) (■) and investigational HDM allergoid vaccine (+alum) (▽). Statistical difference between points on the two curves is marked with *($P < 0.05$) or ***($P < 0.001$) confidence limit.