



Recombinant house dust mite allergens



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ARTICLE INFO

Article history:

Available online 31 July 2013

Keywords:

House dust mite allergy
Immunotherapy
Recombinant allergens

ABSTRACT

House dust mites (HDM) are a globally important source of allergen responsible for the sensitization of more than 50% of allergic patients. Specific immunotherapy with HDM extracts is effective but allergen extracts cannot be fully standardized and severe side-effects can occur during the protracted course of treatment. The introduction of molecular biological techniques into allergy research allowed the identification of more than 20 groups of HDM allergens. Recombinant HDM allergens can be produced in defined concentrations and consistent quality and allow the development of vaccines for HDM allergy with reduced allergenic activity and retained immunogenicity. The immunotherapy trials in pollen allergic patients with recombinant pollen allergens/hypoallergenic allergen derivatives have shown that this treatment is effective and indicated that recombinant HDM vaccines might improve immunotherapy of HDM allergic patients. Here we report the steps for the development of vaccines for HDM allergy. After selection of the most prevalent HDM species, the panel of allergens to be included into a therapeutic vaccine for HDM allergy needs to be determined. HDM allergens with high IgE-binding frequency and clinical relevance will be modified into hypoallergenic variants and evaluated for their allergenic activity and immunogenicity. Derivatives with reduced allergenic activity but with retained immunogenicity would be good candidates for a HDM vaccine for safe and efficient immunotherapy.

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1. Immunotherapy for HDM allergy

The most frequent triggers for asthma attacks in subjects with house dust mite (HDM) allergy are thought to be non-allergenic noxious insults acting on inflamed airway such as infections and irritants [1]. This differs from pollen-induced rhinitis, venom-induced anaphylaxis and cat allergy where the symptoms are most frequently caused by hypersensitivity reactions directly triggered by the allergen. It is therefore probable that the mechanism for optimal immunotherapy will be different. Although increasing attention is being paid to events that precede allergic sensitization in asthma it should nevertheless be appreciated that the probability of HDM-allergic children developing asthma is proportional to their anti-HDM IgE titre [2,3] and poor prognosis is associated with the early development of the IgE antibody and early sensitization for anti-HDM Th2 cytokine responses [4,5]. Interference with the sensitization either by HDM allergen immunotherapy or by the administration of anti-IgE monoclonal antibody omalizumab markedly ameliorates the disease for most people. Indeed oma-

lizumab was found to be most effective for HDM-allergic children living in HDM infested homes and for cockroach-allergic children living in cockroach infested homes showing a specific anti-allergy action [6]. Subcutaneous injections of allergen extracts can ameliorate the three major diseases associated with HDM allergy, namely asthma, rhinitis and atopic dermatitis. To take examples, the double blind placebo controlled trial of Pifferi et al. [7] showed that the number of exacerbations of asthmatic children declined from 8 to 2 per year, β -blocker use dropped from 40 to 20 days per year and corticosteroid use from 20 to 5 days with very significant improvements in bronchial hyperreactivity to an average within the normal range. Similarly the double blind placebo controlled trial of Garcia-Robaina and colleagues [8] resulted in 50% reduction in symptom scores and medication use and bronchospasm induced by inhalation of HDM extract needing twice the dose of extract for a given drop in lung function. A similar degree of benefit for nasal symptoms was reported for patients with allergic rhinitis [9] and immunotherapy has been shown to decrease the symptoms of atopic dermatitis patients from moderate to mild with reductions in erythema, secondary skin infection, itch, sleep disturbance and lichenification [10,11].

Sublingual immunotherapy with HDM extract has also been examined and has the potential to reduce the anaphylactic side ef-

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fects produced by injected allergen and the need for the protracted course of injections. Several double blind placebo controlled trials have demonstrated that HDM sensitized subjects gain an improvement similar to found for subcutaneous immunotherapy [12–14] although there are reports to the contrary. Scrutiny of one these, which examined small treatment groups for 6 only months, did in fact show improvements of a similar magnitude to other studies but there was a large variation in the placebo [15] and another, not only examined rhinitis patients with few symptoms, but concomitantly instigated HDM avoidance and optimisation of corticosteroid treatment throughout the trial [16].

2. Allergen specificity

The objective of specific immunotherapy is to determine the allergen responsible for the sensitization of patient and then to administer it in a way that stimulates the patient's immune system to turn off or counter-act harmful response to the allergen. Evidence that the therapy used today, such as the injection of a succession of progressively increasing doses of allergen, is mediated by allergen-specific changes in the responses of allergen reactive antibodies and T cells is surprisingly scant. There are reports that describe specific immunological changes [17] but others show a lack of specificity [18]. There are also conflicting reports on the specificity of clinical efficacy. Successful ragweed immunotherapy for subjects with dual grass and ragweed sensitivity has been reported to only alleviate symptoms in the ragweed and not the earlier grass pollination season [19]. Conversely however a trial with sublingual immunotherapy of dual birch and grass pollen allergic patients found therapy with either allergen reduced symptoms during both the grass and birch pollen [20]. The treatment was however most effective for the homologous allergen and allergy combinations and a mixture of allergens was the most effective. Reports showing that children given immunotherapy with an extract do not develop new sensitizations to other sources of allergen also point to non-specific mechanisms [21,22]. Repeatedly administering allergens to sensitized people with the attendant cytokine and chemokine cascades could elicit all manner of specific and non-specific effects while the action of blocking antibody and allergen-specific regulatory cells would be specific. The clinical specificity of the different types of immunotherapy could reflect their mechanism of action and help identify immunological or inflammatory changes that could be monitored when developing new strategies.

3. Advantages from recombinant allergens

Broadly the advantages would be (1) the use of defined reproducible formulations, (2) the use of balanced formulations, (3) access to large amounts of allergen, (4) removal of non-allergen inflammatory stimuli and (5) entree to genetically modified allergens.

The current standardisation procedures for extracts measure their ability to induce skin test responses not their composition. A recent survey found that ratio of the two major allergens from *Dermatophagoides pteronyssinus* Der p 1 & 2 differed at the extreme by 16-fold between extracts and not uncommonly from 3–5-fold [23]. Only 4/13 extracts contained detectable quantities of all of the mid-tier allergens Der p 5, 7 and 21 (to be described below) and it was not uncommon to be unable to detect any of them [23]. The use of formulations with known and therapeutic doses of the important allergen would not only improve the prospect for efficacy but would allow reproducible investigations to made to establish optimal protocols. The poor balance of the concentrations of different allergens creates the inherent problem of trying to achieve therapeutic doses with an allergen present in low con-

centration when allergens at higher concentrations have the ability to cause anaphylactic side effects. The sometimes large discrepancy in the group 1 and 2 concentrations in *D. pteronyssinus* extracts, usually in favour of Der p 1, would not help achieve optimal desensitization to Der p 2 and some mid-tier allergens have been reported to be present in 100 fold less concentrations [24]. It should be noted that the concentration of the allergens in the extracts does not reflect the quantities made by the HDM, Der p 7 for example being made in similar quantity to Der p 2 [25]. In fact the culture conditions used to make HDM extracts are optimised to produce high amounts of the group 1 and 2 allergens and do not represent the growth conditions found in homes. Industrial scale recombinant technology has the ability to provide large quantities of allergen and this may have special application for modified allergens designed to be administered in large quantity without side effects and sublingual therapies where, as best studied for pollen allergy, about 10-fold more allergen than the current subcutaneous regimens is required. Extracts contain inflammatory molecules including enzymes such as kallikreins [26] and although not documented certainly ceramides and other immunomodulatory lipids as well as β -glucans [27] and the ever popular endotoxin [28]. Immunotherapy might be improved if pre-determined and known immunomodulators were added to the allergens but the uncontrolled and varying presence of unknown modulators will not help establish a reproducible medicament. As will be discussed below recombinant allergens provide one the avenues to construct modified allergens for new types of immunotherapy and this might be the most important application.

4. The important allergens

The allergenic potential of different HDM allergens has been assessed by quantitative IgE binding with panels and purified and recombinant allergens [29–32] and by absorption of the IgE staining moieties on 2-D western blotting [33]. The IgE binding to Der p 1 and Der p 2 has been found to constitute 50–60% of the IgE binding to all of the HDM allergens for essentially all HDM allergic subjects with the summated titres to Der p 1 and 2 tightly correlating with the binding to extracts. The mid-tier allergens Der p 4, 5, 7 and 21, each of which only bind IgE in about 50% of patients, bind individually and collectively in proportion to the major allergens constituting over 30% of the total titre. This consistent proportional pattern provides an excellent platform for selecting recombinant allergens. Importantly the IgE binding pattern was the same in spectrum and proportion for children admitted to a hospital emergency department for asthma compared to children and adults with controlled and mild disease [30] and was the same for subjects with persistent and frequent disease compared intermittent asthmatics [34]. The formulation required for severe disease is therefore the same as that required for milder allergy. Results consistent with the IgE binding analyses have been obtained with skin test in several European countries [35].

The structures of these allergens are well defined [36]. The group 1 allergens are cysteine proteases similar to papain. The group 2 allergens are structurally the same as the myeloid differentiation (MD) antigen-like lipid binding proteins (ML domain proteins). Indeed there is evidence that Der p 2 can mimic the action of MD-2, which loads LPS onto toll-like receptor (TLR)-4 to activate an innate inflammatory cascade [37] and Der f 2 binds lipopolysaccharide (LPS) with high affinity in a manner similar to MD-2 [38]. The group 4 allergens are typical α -amylases [39] and the group 7 allergens are structurally related to the LPS binding bactericidal permeability increasing protein (LPB/BPI) family of proteins [40] that include the major horse allergen Equ c 3 and the cat allergen Fel d 8 [41]. The group 5 and 21 allergens are re-

lated proteins with 40% sequence identity and so far appear unique to mites. Their function is unknown but the crystal structure of Der p 5 shows a bundle of coiled coils [42] that can polymerise to create a cage with a hydrophobic cavity.

A corollary elaborated elsewhere is that the binding to the other HDM allergens, including to some favourites like the serine proteases and glutathione-S-transferase, is low. Recent studies of the chitinase-like and chitin-binding domain proteins Der p 15 and 18 have demonstrated low IgE binding [32]. The only known allergens that need further IgE binding analysis are the unstable group 11 and 14, paramyosin and apolipoprotein-like proteins respectively. While it is unlikely that the low-tier allergens would be useful for immunotherapy they still have a place in diagnosis of part of arrays for quantitative component resolved diagnosis. This can resolve issues with cross reactivities and, as shown for Australian aboriginals [43] previously-unstudied populations can have very unusual responses and sensitization in new populations is likely to increase as allergy moves hand in hand with economic development.

The cross-reactive group 10 tropomyosin allergens are important for diagnosis and as recently identified with a very large study patients, anti tropomyosin IgE is found in patients with more cross reactive and diverse allergic responses [44]. The frequent IgE anti-tropomyosin antibodies detected in Japan and Zimbabwe still needs clarification [45,46]. They are not simple cross reactivities because they are confined to sera that bind other HDM allergens.

Niederberger et al. [47] demonstrate with recombinant grass allergens that IgE binding and activity in skin test and nasal provocation can differ. Specifically the allergenicity of Phl p 2 when measured by skin test and nasal provocation was higher than expected from its IgE binding titres. The skin test reactivity of the group 1 and 2 HDM allergens are similar [48] and although not titrated, Lynch et al. found the skin test reactivity of Der p 5, 7 and 21 to be commensurate with their IgE binding activity [49]. There are no studies that compare the skin test reactivities of HDM allergens other than the major and mid-tier categories but it should be noted that for the grass example above the IgE binding titres to Phl p 2 were not low. They were on average 25% of the titres of major allergens so they would still be important IgE-binding specificities.

5. Mite species and variant allergens

Most HDM allergy is caused by sensitization to the pyroglyphid mite species *Dermatophagoides pteronyssinus* and *D. farinae*. There are defined regions where they co-exist in homes with the glycyphagoid mite *Blomia tropicalis* which because of their genetic disparity and differences in allergen production are a separate source of allergen. As recently reviewed [50] *D. pteronyssinus* is the most prevalent species in maritime western and southern Europe, South America, Africa, and Australasia. In northern America the western maritime regions are biased to *D. pteronyssinus* although Los Angeles and Vancouver have both species. The mid western regions have few HDM because of the continental climate and since *D. farinae* compete better in regions of low humidity and low temperature they are dominant species. This bias continues across to the northeast extending to Toronto. For Asian countries, Japan and many regions of China have mixed populations, Singapore has *D. pteronyssinus*, Thailand has mainly *D. pteronyssinus*, Taiwan has a *D. pteronyssinus* bias and Korea, except for southern coastal regions, has *D. farinae*. Sweden and Poland have biases to *D. farinae* but other European and Scandinavian countries have both species. There are however variations, a very interesting one being the dominance of *D. farinae* in Italy where extensive research has been conducted with *D. pteronyssinus*.

The allergens from the 2 species cross react so it is not possible to determine the species distribution with skin tests. The se-

quences of the allergens from *D. pteronyssinus* and *D. farinae* share 80–85% identity which, as shown for pollen allergens, should be sufficient for a large degree of IgE antibody cross reactivity but with significant species-specific reactivity [51]. Lind et al. showed with sera from Belgium that the group 1 allergen cross reactivity is very variable amongst individuals ranging from complete cross reactivity to 1000 fold less of IgE binding [52]. Large and variable differences with a bias to Der p 1 binding been well documented in Japan [53]. Studies in regions with mixed species have shown a very high correspondence of titres for Der p 2 and Der f 2 [48,53]. As shown in Australia where the exposure of subjects to *D. farinae* is limited, extensive T-cell cross reactivity between Der p 1 and Der f 1 can be demonstrated using purified natural allergens [54] although it is now known the allergen preparations used contained sufficient LPS to enhance small responses. Studies with synthetic peptides have however also showed shared T-cell epitopes but with higher responses to peptides representing the prevailing Der p 1 allergen [55]. With respect to the species required for immunotherapy there are no direct comparisons but for current extract-injection regimens the efficacies using *D. pteronyssinus* in *D. farinae*-infested Italy [7,56] have been similar to those from England using *D. pteronyssinus* for *D. pteronyssinus* sensitization [9] and those from South Korea using *D. farinae* for *D. farinae* sensitization [57] or mixtures of *D. pteronyssinus* and *D. farinae* in Italy [58].

HDM allergens are simpler than most pollen allergens because the dominant allergens are encoded by single genes and not gene families. The sequences are however very polymorphic [59] with approximately half of 48 sequences of Der p 1 analysed by Piboonpocanun et al. being different [60]. The sequences differed from each by 1–5 residues but mostly with sporadic substitutions so that the predominant core sequence can easily be represented by a single sequence. A consistent substitution of with alanine and valine each being found in about 50% of mites was however found and since this profoundly affects T-cell responses in mice [61] and is in a region frequently recognised by T cells in humans [55], it should be taken into account. It has been suggested that Der p 1.0102 and Der p 1.0105 which only differ in residue 124 would be suitable for therapy. Initial observations from Thailand [60] indicated that Der f 1 might be less polymorphic but recent entries into the IUIS database show that it has the same degree of variation as Der p 1.

There are also many variants of the group 2 allergens, which can have 5% sequence variation [59,60]. In contrast to the group 1 allergen they have an evolutionary pattern of sequence substitution where, for Der p 2, the valine, threonine, methionine and aspartate amino acids at positions 40, 47, 111 and 114 for Der p 2.0101 become substituted to varying degrees with the leucine, serine, leucine and asparagine found in Der p 2.0104. Antigenic differences between the variants (Der p 2.0101-like and Der p 2.0104-like) have been demonstrated with monoclonal antibodies [62] but more importantly humans not only have different T-cell responses to the variants [63] but IgE binding that can differ by as much as 50% with higher binding to Der p 2.0104 [63,64]. Since the prevalence of the variants can differ in different geographical regions as shown for Thailand and Australia, which have a respective predominance of Der p 2.0104 and Der p 2.0101-type sequences [60], the most efficient formulation for recombinant Der p 2 should represent both types of sequence. At least one culture of *D. pteronyssinus* used for the manufacture of extracts for clinical medicine has been found to contain about 50% Der p 2.0101 plus Der p 2.0104-like variants [65].

Recombinant variants of Der p 2 have shown differences in their IgE-binding capacity and cloned human antibodies that bind to different variants appear commonplace [66]. While the variation of IgE binding of the variants is not sufficient to affect the degranulation of basophils armed with polyclonal antibodies from mite-allergic subjects its role in the induction of sensitization might

be significant in other ways, for example by increasing clonal diversity or CD23-mediated allergen presentation. It might also be very important for immunotherapy strategies that rely on IgG blocking to cover all epitopes bound by IgE.

Der f 2 from *D. farinae* in Asia [60,67–69] and Europe [70] falls into two groups [60]. Der f 2.0101 sequences show conservative uncharged substitutions in residues 88, 11 and 125. Another group that can be described as Der f 2.0107-like have characteristic changes in amino acids 57, 58 and 59 including substitution of the surface accessible aspartate 59. In the one letter code the Der f 2.0107-like variants change SLD to NIN in amino acids 57–59. About 30% of the sequences described from Germany were of the Der f 2.0101 type and 70% the 0107 type while the proportion was reversed in Bangkok. Like Der p 2 the variants vary in different locations. Four sequences from Japan [67] and those from Korea [68] were of the Der f 20101-type and sequences from China showed both groups [69].

The exposition of the variants of major allergens raises a number of important points. The first is the dearth of information from many populous countries and the second is the small amount of follow-up on the published research. If there is 50% difference in IgE binding then especially for blocking antibodies it might be important for vaccine to cover the best possible representation of specificities. A third imperative that vanguard-researchers have appreciated is that publications should provide information to define the variant(s) of recombinant allergen(s) being studied.

6. Immunotherapy with major allergens

It would be advantageous to conduct immunotherapy with one or two allergens. This would not only have the obvious advantage of only having to make 2 antigens (with variants) but it also might be more effective than administering a more complex mixture. Therapy with fewer allergens would both elicit fewer allergic side effects and reduce the production of inflammatory mediators that activate dendritic cells and thereby antagonise the induction of energy [71]. This could be the mechanism behind intranasal-tolerisation experiments in mice showing that three proteins administered together were ineffective whereas administration of less-antigenic peptides representing the allergens was effective [72]. For immunotherapy strategies aimed at redirecting the anti-allergen response, for example to induce blocking antibody, the principles from antigenic competition, that cause complex vaccines to be ineffective [73] would also apply.

The existence of tolerising regimens that induce regulatory mechanisms to suppress responses to bystander allergens is well established. Targeting these might be the most effective therapy to apply with recombinant allergens. As demonstrated in mice the mucosal application of allergens is particularly adept at this [74–77] including HDM allergens for intra- [74] and intermolecular [76] suppression. Intradermal injection of a peptide containing a T-cell epitope from Fel d 1 has also been shown to suppress responses to bystander epitopes and markedly reduce hypersensitivity responses to Fel d 1 in HLA transgenic mouse model [78]. Most excitingly T-cells from patients given immunotherapy with Fel d 1 peptides had reduced *invitro* stimulation responses to Fel d 1 peptides that were not in the vaccine formulation [78]. Bystander help for allergen sensitization has been demonstrated in experimental animals [79,80] and although its manifestations in humans is conjectural this might be the mechanism by which immunotherapy can reduce the development of new sensitizations [21,22].

Immunotherapy with recombinant Bet v 1 alone has been shown to be as efficacious as therapy with birch extract [81]. However since Bet v 1 contributes 80–90% of the IgE binding activity of birch pollen this would not be unexpected. Studies with ragweed

would be more relevant because the major allergen Amb a 1 contributes 50–60% of the IgE binding of pollen [82]. Norman et al. showed that immunotherapy with purified natural Amb a 1 produced clinical improvement but required three or four years of treatment [83]. Reports that are largely unpublished have shown a varied efficacy for immunotherapy conducted with a CpG-conjugated purified natural Amb a 1 but these were conducted with a novel short protocol [84] and the procedure only induced transient increases of IgG antibody of an unspecified magnitude [84].

7. Production of recombinant HDM allergens

Recombinant group 1 allergens must be produced with its 80 amino acid residue proenzyme sequence that then needs to be cleaved to create the mature protein. This has most efficiently been accomplished with the host *Pichia pastoris* and the removal of the single N-glycosylation site of the mature enzyme [85,86]. Der f 1 has either been produced as a mature protein with the pro-region self cleaved during its synthesis by *P. pastoris* [85] or as proenzyme that then had to be cleaved by an acid incubation step [86]. Recombinant Der p 1 has also been made but it was more difficult at least partially due to the presence of an N-glycosylation site in the pro- as well as the mature region [87]. Also the acid conditions, required for maturation and preventing the proenzyme sequence from inhibiting the self-catalysed cleavage of the protein, can also deleteriously induce the unfolding of the mature sequence [88]. Although the process is difficult [88–90] recombinant Der p 1 has nevertheless been crystallised to show a similar structure to natural Der f 1 as well as enzymatic activity [92,93]. From a recent report [94] careful downstream processing to isolate only the correctly cleaved and folded protein is essential and there can be difficulty in making mutant allergens. The proenzyme can be very readily produced in high yield and with good structure [90].

Der p 2 can readily be produced as a high-IgE-binding recombinant molecule and the recombinant group 2 allergens have been used for high resolution NMR and for X-crystallography [95–97]. They have been produced either by the denaturation and solubilisation of a polypeptide produced in the inclusion bodies of *Escherichia coli* [95,96,98] or by secretion from *P. pastoris* [65,97]. A point of note however is that Der p 2 expressed from *P. pastoris* and isolated directly by size exclusion chromatography did not assume its ML-domain structure until it was salt precipitated and resuspended [97]. The binding of the allergen to a cationic matrix and its elution with 500 mM salt also appears sufficient for folding [65]. Here Der p 2 produced in *P. pastoris* was superior to the *E. coli* product because it had the high thermal stability of natural Der p 2 and did not contain mis-paired cysteine bridges that were quite prevalent in the *E. coli*-produced and refolded Der p 2.

The mid-tier allergens Der p 4, 5, 7 and 21 can all be produced with good structure. Der p 4 can be produced with its amylase activity in *P. pastoris* [39] and Der p 5 [33,99], 7 [33,40,100] and 21 [31] can be produced in *E. coli* hosts with near natural IgE binding activity. The crystal structures of Der p 5 [42] and Der p 7 allergens [40] have been determined.

8. Production of allergens for therapeutic trials

Recombinant biopharmaceuticals to be used in clinical trials have to be produced under the principles of cGMP (current Good Manufacturing Practice). GMP is an internationally harmonized manufacturing standard which ensures the fundamental quality criteria of pharmaceutical products (i.e. identity, purity, safety, strength, potency and stability). In the major guideline of the International Conference on Harmonisation (ICH Q7A), the fundamental GMP principles are regularized [101]. Although regulatory guide-

lines exist that specifically cover the manufacturing and quality control of recombinant allergens for specific immunotherapy [102], generally the same GMP regulations and quality standards as compared to any other biopharmaceutical have to be applied. The maintenance of an appropriate quality management system consisting of quality assurance (QA) and quality control (QC), and the operation of certified clean room facilities are essential prerequisites for GMP. Equipment for production and analytical testing needs to be qualified with respect to their intended or specified performance. Similarly, the performance of analytical methods and production processes needs to be proven by validation. Special care must be given to the issue of equipment cleaning, cleaning procedures and the respective cleaning validation, in particular in multi-product facilities representing a risk of cross-contamination. Analogous, appropriate methods for equipment sterilization and decontamination need to be specified and validated. In this context, the application of certified single-use (disposable) processing equipment is currently gaining importance. Especially smaller batches for early clinical trials are increasingly produced by using disposable materials like filters, mixing and storage containers, tubes and even analytical probes or bioreactors. For active pharmaceutical ingredients to be investigated in clinical trials, not all the requirements specified in the GMP guidelines may be appropriate [101]. Moreover, for clinical phase I investigational products, the U.S. FDA has issued a guideline covering also vaccines and recombinant and allergenic products, which in some aspects may suggest facilitated or simplified regulations for manufacturing of phase I active ingredients [103].

For manufacturing of a recombinant biopharmaceutical, particular attention must be put on the very early steps, namely the choice and construction of the expression system. Among the numerous recombinant protein expression systems currently available, *E. coli* is still a prominent work-horse, and the methylotrophic yeast *P. pastoris* is being increasingly applied. From the aforementioned information *P. pastoris* appears to be most appropriate of these hosts for the group 1 and 2 allergens. The secretion facilitates purification but the allergen needs to be modified for misglycosylation. The group 4 alpha amylase has been found to be produced as an active enzyme from recombinant *P. pastoris* but not *E. coli*. The group 5, 7 and 21 have been suitably produced by *E. coli* with a high yield of soluble intracellular product. This may be the host of choice because the cultivation of *E. coli* is less time-consuming compared to *P. pastoris*.

Irrespective of the expression system chosen, the design, construction and sequencing of the expression vector should be done carefully. With respect to the marker sequence, beta-lactam antibiotic resistance (ampicillin) should be avoided at the favour of kanamycin resistance, because severe hypersensitivity reactions to ampicillin have been reported. Once the expression construct has been finalized, a Master Cell Bank (MCB) derived from one single transformed clone should be established and frozen as glycerol stock. Such a Master Cell Bank (or its succeeding Master Working Cell Bank, MWCB) will be the starting point for all further production lots. Both the expression construct and the cell banks have to be characterized and quality-tested extensively [101,104,105]. For product expression and fermentation, high cell density cultivation based on a fed-batch protocol is currently the state of the art-method. High performance cultivation of *E. coli* may result in a cell density of more than 100 OD units, and for *P. pastoris* of more than 200 OD units, both resulting in recombinant protein titers of up to 10 g/L culture broth. Importantly, the culture media should be free of animal-derived components or antibiotics.

After cultivation of *E. coli*, the cell paste is usually separated by continuous-flow centrifugation, followed by mechanical high-pressure homogenisation. In some cases, insoluble intracellular inclusion bodies may require a product renaturation step

(refolding), whereas for *P. pastoris*, secreted product can be directly purified from the culture supernatant. Ideally, two to three chromatographic purification steps are applied for product capture, intermediate purification and polishing. Filtration is another essential and frequently applied processing technique. In particular, depth filtration, ultra/diafiltration and aseptic 0.2 µm filtration are useful processing steps. The variety of impurities to be removed can be mainly classified into product-derived inhomogeneities (e.g. mistranslated side forms, truncated, denatured or aggregated variants) and host or process-derived components (e.g. host cell DNA, host cell proteins, endotoxins, non-host contaminations, residual raw materials).

In order to ensure stability of the active pharmaceutical ingredient (API, drug substance), rational formulation development and long-term stability monitoring are essential. For mixtures of recombinant allergens – which would be likely the case for a HDM-vaccine – sufficient storage stability for the individual components has to be demonstrated. Finally, the clinical drug product has to be filled, labelled and packaged in a suitable container closure system, which will be sterilized glass vials in most cases. GMP manufacturing is generally based on testing and release of individual lots (or batches). The application of well established and documented quality control methods is a basic GMP requirement. However, it is an important GMP principle that quality cannot be tested with the final product. The fundamental equation “the process is the product” means that product quality has to be ensured by controlling, monitoring and analyzing critical parameters and acceptance criteria during the manufacturing process as well (in-process controls).

9. Strategies for modified allergens

Recombinant technology is ideally suited to provide modified allergens that will enable new treatments. The modifications can be aimed at several strategies. They can be modified to reduce their binding to IgE antibodies and thus allow higher doses to be delivered without anaphylactoid side effects or they can be modified to preferentially target control mechanisms such as the induction of blocking IgG antibodies [106], immune deviation away from a Th2 response [107,108], anergy [109,110] or T-regulatory cells [111,112].

Recent observations for long-term pollen immunotherapy have pointed to the ameliorating effects of blocking antibodies [113]. Blocking antibodies and their ability to inhibit anaphylactic reactions were recognised by 1935 [114,115] and identified as IgG antibodies, [116] but since the importance of T regulatory cell early in immunotherapy [113] was not known the poor correlation with the clinical efficacy at the initiation of immunotherapy was puzzling [117]. The blocking effect of IgG antibody on CD23 mediated presentation of allergens to T cells [118] was also unknown. The induction of IgG antibodies with recombinant pollen allergens and their associations with successful immunotherapy has now been repeatedly shown [119–125].

Allergen cloning has provided the amino acid sequence information to use synthetic chemistry to construct peptides that contain sequences known to contain either T-cell epitopes [126,127] or B-cell epitopes [128,129], the latter only being capable of low affinity interactions with antibody. As trialled for cat and venom allergy the T-cell peptides aim to induce tolerance or anergy in allergen-specific T-cells whereas the B-cell epitope containing peptides, as shown possible in experimental animals, aim to induce blocking IgG antibodies.

Recombinant DNA technology can similarly manipulate epitopes. It has been used to destroy conformational IgE epitopes by site-directed mutagenesis [130–132] and gene fragmentation [133] or to remove IgE-epitopes by deletion [134,135]. These genetic manipulations essentially leave all the potential T-cell

epitopes and can still conduct low affinity interactions with B-cells to induce IgG blocking antibody.

Other strategies for modified allergens are to increase their antigenicity. One example called modular antigen-translocation (MAT) has shown efficacy in animals [136]. Here a membrane translocating domain and a major histocompatibility complex invariant chain sequence were genetically fused to the cat allergen to improve T-cell presentation. Another amenable to recombinant technology and *in vivo* administration [137] is to genetically fuse part of the invariant chain (the Ii-Key sequence) to an allergen construct to enhance responses.

10. Modified house dust mite allergens

So far, hypoallergenic derivatives have only been produced from the group 1 and 2 allergens (Table 1).

10.1. Group 2 allergens

Hypoallergenic derivatives of Der p 2 and Der f 2 were developed by site-directed mutagenesis of cysteine residues to prevent the formation of disulfide bonds and thus to destroy the beta-barrel structure of the molecule [130,131,138,139]. The derivatives showed reduced IgE-reactivity but whereas IgE reactivity to Der p 2 was most efficiently reduced by disruption of the C73–C78 bond, for Der f 2, the C8–C119 bond seemed to be most important for the stabilization of the secondary structure [130,131,139]. Site-directed mutagenesis was also used to disrupt IgE-binding sites without affecting the three-dimensional structure [140,141]. The mutants showed reduced IgE-reactivity but only slightly reduced histamine-releasing activity. N- and C-terminal deletion variants of Der p 2 produced by molecular biological techniques showed reduced IgE-binding activity and did not induce *in vivo* skin reactions in mite allergic patients [142]. Fragments of Der p 2 and a hybrid molecule, where the two fragments were recombined in inverse order by PCR-based gene-SOEing [143,144], showed reduced IgE-reactivity, allergenic activity, allergenicity and induced blocking IgG antibodies in animal models [144] (Table 1). Synthetic peptides of Der p 2 which lacked IgE-reactivity and the major T-cell epitopes showed reduced allergenic activity and induced blocking IgG antibodies in mice and rabbits [145] (Table 1).

10.2. Group 1 allergens

The production of modified group 1 allergens has been difficult, because the uncertainties in producing mature allergen from the

proenzyme [85–94]. The proenzyme with its 80 amino acid pro-region however has weak IgE-reactivity and could therefore represent a hypoallergenic molecule for immunotherapy [146,147] (Table 1). In this context, it has been shown that the aggregated proform of Der p 1 induced a Th1-biased immune response in mice, characterized by the production of specific IgG2a [147,148]. Group 1 allergens expressed as recombinant proteins without the prosequence also show very low IgE-reactivity and may from this consideration be useful for immunotherapy [149] although the folding and thus quality control would not be predictable. In addition, synthetic peptides, containing the major T cell epitopes of Der p 1 were produced which induced T cell tolerance and mucosal tolerance [74,76]. Synthetic peptides containing 16 aminoacids of the allergen Der p 1 were covalently coupled to highly repetitive virus-like particles and induced high IgG responses in human subjects [150] (Table 1).

10.3. Mosaic allergens

A recent and promising development has been to produce recombinant mosaic proteins consisting of shuffled sequences of Der p 1 and Der p 2 [151, 152] (Table 1). These were produced in *E. coli* as soluble proteins in good yield. One of the constructs that lacked cysteines appeared to be a promising vaccine candidate because it did not form aggregates and thus would not only have increased safety but would be easy to produce in large scale fermentations. The shuffled recombinant fusions had defined secondary structure shown by circular dichroism but lacked the ability to bind IgE. Remarkably rabbits immunised with the constructs produced high titers of IgG antibodies to both allergens, which inhibited patients' IgE binding to Der p 1 and Der p 2 [151].

11. Immunogenicity of hypoallergens

Hypoallergenic derivatives need to be evaluated *in vitro* and *in vivo*, for their allergenic activity and their immunogenicity (Table 1). Importantly immunization of animals (e.g. mice) with hypoallergenic derivatives of HDM allergens coupled to adjuvants have to show that they can induce blocking antibodies, which recognize the wild type allergen and inhibit patients' IgE binding to the allergen. So far, this has been shown for the proform of Der p 1 [146,147], for Der p 1 peptides coupled to virus-like particles [150], for Der p 2 fragments, hybrids [144] and Der p 2 peptides coupled to KLH [145] and for Der p 1/Der p 2 mosaic proteins [151, 152] (Table 1). Allergen derivatives like these with reduced allergenic activity but with retained immunogenicity would be front line candidates for inclusion in a vaccine for safe and efficient immunotherapy of HDM allergic patients.

12. Conclusions

Recent advances using quantitative methods have consolidated the findings that for *D. pteronyssinus* the responses of most allergic subjects follow an allergen hierarchy where over half the IgE is directed to Der p 1 and 2 and most of the rest is directed to the allergens Der p 4, 5, 7 and 21. The proportionality and restricted spectrum is retained for high and low responses and for severe and mild asthmatics. This provides a sound platform for developing formulations of recombinant allergens for immunotherapy which taking the experience from other allergies might only require Der p 1 and 2. Many of the other allergens described from HDM extracts may be important for component resolved diagnosis for ascertaining cross reactivities and for the study of HDM allergy in countries with developing economies and attendant increases in atopy. Although there have been past difficulties with the group 1 allergens all the major

Table 1
Hypoallergenic derivatives of house dust mite allergens.

Modification	IgE-reactivity	Allergenic activity	Immunogenicity	Ref.
<i>Der p 1</i>				
proDer p 1	–	–	+	[146,147]
Der p 1- <i>E. coli</i>	–	ND	ND	[149]
B-cell peptides	ND	ND	+	[150]
<i>Der p 2</i>				
Mutants	–	–	ND	[130,131,138]
Mutants	–	+/-	ND	[140,141]
Fragments	–	–	ND	[142]
Fragments	–	–	+	[144]
Hybrid	–	–	+	[144]
Peptides	–	–	+	[145]
<i>Der p 1/Der p 2</i>				
Mosaic	–	–	+	[151]
Hybrid/mosaic	–	–	+	[152]

and second tier HDM allergens have been produced as recombinant proteins with verified structures and are highly amenable to large scale production under good manufacturing conditions. Methods of producing hypoallergenic major allergens have been demonstrated and a recently produced hybrid from Der p 1/Der p 2 sequences not only displays favourable immunological properties but also a potential for clinical-scale production.

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