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Test Procedures for Allergoids and Hypoallergenic Recombinant Allergens: Immunological Characterization

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Introduction

Allergen specific immunotherapy with hypoallergenic preparations was introduced more than 20 years ago using allergoids produced by aldehyde treatment of allergen extracts [1,2]. This chemical modification causes both intra- and intermolecular cross-linking with the result that the IgE-binding activity of such allergoids is substantially reduced by comparison with the native allergen. The potential benefits of such allergoids are that they possess a reduced risk for inducing IgE-mediated anaphylactic reactions, and the maintenance dose can be achieved after a relatively short course of injections [3]. The immunogenic activity of such hypoallergenic allergen preparations was clearly demonstrated by their ability to induce strong IgG4- and IgG1-antibody response to epitopes of the native allergens in patients during immunotherapy. The therapeutic efficacy of allergoids prepared from different allergen sources has been shown in several clinical trials [4-7].

Recombinant DNA technology now allows us to determine whether or not the complex natural allergen extracts can be replaced by recombinant allergens. The results of the basic research with recombinant allergens in the last decade have shown that hypoallergenic variants of different allergens, can be designed by gene or protein engineering [8-12]. These hypoallergenic recombinant allergens, like the chemically modified allergoids, possess reduced IgE-binding, diminished skin test activity and retained T-cell reactivity [9-13]. First clinical trials have shown that such hypoallergenic recombinant allergens induce strong IgG4- and IgG1-antibody responses [14,15].

Methods

Allergen strip test

Natural and recombinant proteins were blotted on nitrocellulose membranes as described previously [16]. The samples were adjusted to 100 µg/ml and incubated

with the membranes for 30 min. The tested sera were diluted 1:10, and after washing the bound IgE was made visible by alkaline phosphatase-conjugated monoclonal anti-human IgE and the substrate NBT/BCIP (Invitrogen, Karlsruhe, Germany). Mouse monoclonal binding was detected using alkaline phosphatase labelled goat anti-mouse IgG/M (Dianova, Hamburg, Germany).

EAST-inhibition assay

The IgE-reactivities of native and modified allergens/extracts were evaluated with an EAST-inhibition assay as previously described [9]. Microtiter plates were coated with 1 µg/ml purified allergen or 10 µg/ml allergen extract in NaHCO₃ buffer, pH 9.6, overnight. After washing with phosphate buffered saline (PBS) containing 0.05 % Tween 20 (PBST), the plates were blocked with PBST containing 1 % BSA. Sera were diluted with PBST containing 0.1 % BSA and were pre-incubated with serial dilutions of inhibitors for 1 h. The samples were applied to the washed plates and incubated for 2 h at room temperature. After further washing steps, an anti-human IgE alkaline phosphatase conjugate (Allergopharma) was allowed to bind for 1 h at room temperature. Detection was performed by incubation with *p*-nitrophenylphosphate (Sigma, Deisenhofen, Germany). Optical density was measured with an ELISA plate reader (ICN, Meckenheim, Germany) at 405 nm. The inhibitory capacities of the tested molecules were expressed as percent inhibition according to:

$$\{(\text{OD [BO]} - \text{OD [sample]}) / (\text{OD [BO]} - \text{OD [NSB]})\} \times 100 = \text{inhibition [\%]}$$

BO: max. binding (serum without inhibitor)

NBS: Blank (negative control without serum)

Relative potency (P_{rel}) was calculated as ratio of the concentrations of reference and sample which were estimated to reach 50 % inhibition.

Basophil activation test

Basophil activation using CD 203c-expression measurement was performed as previously described [17,18] in heparinized whole blood. Non-stimulated controls and a positive control (anti-IgE mAb 8B3, 1 µg/ml) were performed with each blood sample. The measurement of CD 203c-expression was performed with anti-CD 203c-PE (mAb 97A6; Beckman Coulter, Krefeld, Germany). Lysis of red blood cells was achieved with the whole blood lysing kit (Beckman Coulter). Flow-cytometric analysis of CD 203c-expression was performed with a Coulter EPICS XL-MCL ADC (Beckman Coulter).

Generation of T-cell lines on T-cell clones

Allergen specific TCL and TCC were obtained according to a procedure described previously in detail [19].

T-cell proliferation assay

For the proliferation assay T-cells (TCL, TCC) were seeded at 2×10^4 cells/well of 96-well culture plates in triplicate under stimulation with the allergen (0.3 µM natural or rBet v 1) and 5×10^4 mitomycin-c treated autologous PBMC. After 48 h in the incubator 1 µCi ³H-thymidine was added to each well and the plates were further incubated for 16 h. Cells were then harvested on Microbeta filter mats with a 96-well cell harvester (PerkinElmer, Rodgau-Jügesheim, Germany). After drying, the filter

mats were prepared for counting by melting the solid scintillator Meltilex into them using a microplate heat sealer (PerkinElmer). Counting was performed in a Micro-beta scintillation counter (PerkinElmer). The stimulation index (SI) was calculated as the quotient of counts per minute (cpm) of the allergen stimulated cells (T-cells, mitomycin-c treated autologous PBMC and allergen) and the unstimulated control cells (T-cells and mitomycin-c treated autologous PBMC without allergen).

Results and Discussion

Established methods for characterization and standardization of allergen products

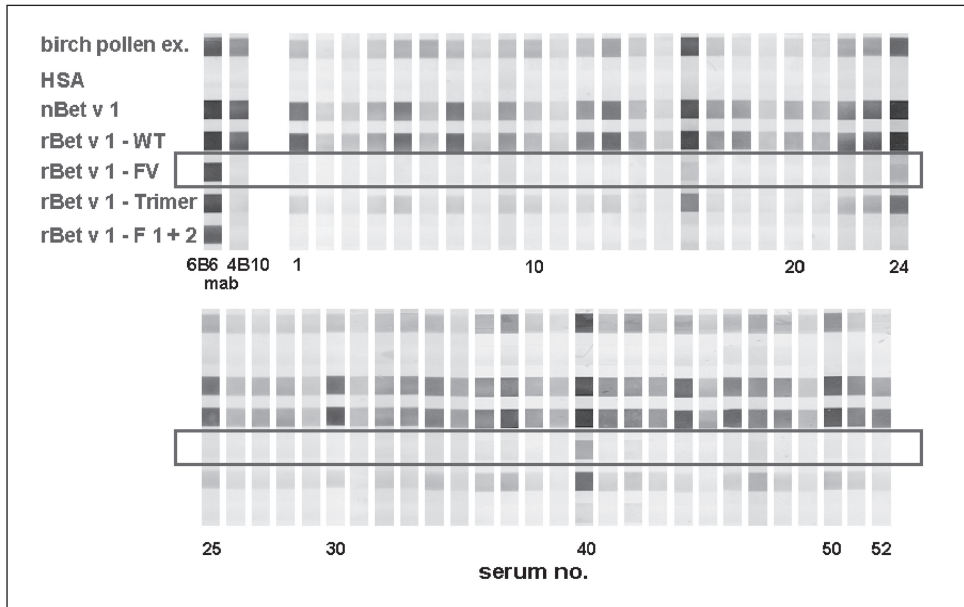
Native allergen extracts are characterized by measurement of total protein and single major allergen content, determination of the protein and allergen profiles and total allergenic activity. The biological activity of allergen preparations can be determined by skin testing. For standardization of the therapeutic preparations the biological activity is determined by RAST-inhibition using a biologically standardized in-house reference preparation (IHRP). The protein profiles are normally determined by gel electrophoresis (SDS-PAGE) or high performance liquid chromatography (HPLC), and the allergen profiles can be obtained by immunoblotting using IgE-antibodies from defined allergic donors. The same methods can be applied to chemical modified allergen extracts. However, with the exception of the total protein determination, the allergoids show differences by comparison with native extracts. The protein profiles of allergoids show shifts in their isoelectric point ranges and the size exclusion profiles are also changed.

Hypoallergenic recombinant allergens are produced mostly in bacterial expression systems and are purified by a combination of different chromatographic techniques, like affinity chromatography, ion exchange chromatography, hydrophobic interaction chromatography and size exclusion chromatography [20]. The identity, purity and homogeneity of recombinant allergens can be examined by different physiochemical and biochemical methods [20]. In contrast to native allergen extracts and wild-type recombinant allergens, the IgE-binding of chemically modified allergen extracts (allergoids) or genetically modified hypoallergenic allergen variants is reduced and therefore RAST-inhibition cannot be used for standardization.

Testing of the IgE-reactivity of modified allergens and recombinant allergen variants

The IgE-reactivity of allergoids or recombinant allergen variants can be investigated *in vitro* by different methods. A simple semi-quantitative assay to assess the IgE-reactivity of such hypoallergenic candidates is the allergen dot test using nitrocellulose strips with bound candidates and reference samples. For the testing of the IgE-binding of different variants of the birch major allergen Bet v 1 nitrocellulose strips containing areas with adsorbed natural Bet v 1, recombinant wild-type Bet v1 and 3 different hypoallergenic candidates were used (Figure 1). Birch pollen extract and human serum albumin served as controls. Bet v 1-FV is a special folding-variant of Bet v 1 expressed as a soluble molecule in *E. coli* [21]. This folding-variant was purified by hydrophobic interaction chromatography, anion-exchange chromatography and size exclusion chromatography using alkaline inorganic running solution. A titration with weak hydrochloric acid after the final gel filtration step yielded a neu-

Fig. 1. *IgE-reactivity of hypoallergenic recombinant Bet v 1 variants. Binding of IgE-antibodies from birch pollen allergic subjects to nitrocellulose strips bound hypoallergenic variants rBet v 1-FV, mixture of Bet v 1-fragments F1 + F2, and rBet v 1-trimer in comparison with natural and recombinant wild-type Bet v 1. The IgE-antibodies from the allergic donors don't react or show only weak binding to the hypoallergenic Bet v 1 variants.*



tral and physiologic protein solution. The other two candidates were a trimer [11] and a mixture of 2 fragments containing the whole sequence of Bet v 1 [10]. The allergen strips were prepared with the same concentrations of natural and recombinant Bet v 1, and Bet v 1 derivatives. The presence of native and modified Bet v 1 molecules, on the membrane, was shown by the strong staining with the Bet v 1 specific mouse monoclonal antibody 6B6. The binding of this monoclonal to the derivatives shows that the recognized epitope is conserved on the folding-variant and the other derivatives. In contrast, the monoclonal 4B10, another Bet v 1 specific antibody, does not react with the three Bet v 1 derivatives. This monoclonal antibody binds to an epitope, which is dependent upon the native conformation of the Bet v 1 molecule. The IgE antibodies in the sera from different birch pollen allergic subjects stain the natural Bet v 1 and the recombinant wild-type molecule with the same intensity. This shows that the recombinant and the purified natural Bet v 1 express the same or very similar epitopes. The IgE antibodies of the sera from the investigated allergic subjects do not or only very weakly bind to Bet v 1-FV and to the mixture of the Bet v 1 half-molecules. The IgE-binding capacity of the Bet v 1 trimer is reduced also, but some sera contain a fraction of antibodies, which react with this modified molecule. All three Bet v 1 derivatives contain the whole amino acid sequence of Bet v 1, but do not have the native folding. The differences in the conformation between native Bet v 1 and the derivatives could be shown by circular dichroism spectrometry [10,11,21]. The binding profiles of IgE antibodies from dif-

ferent allergic donors with the native Bet v 1 and the three derivatives show that all or the great majority of the Bet v 1 specific IgE antibodies react with epitopes which are dependent on native conformation of the molecules. The experience with allergen strips prepared with major allergens from grass pollen or house dust mites has shown that this simple assay is an excellent screening test for the assessment of the IgE-reactivities of hypoallergenic recombinant allergen derivatives or chemically modified allergens (allergoids) during the development of such therapeutics.

The best *in vitro* method for assessment of the reduced IgE-reactivity of hypoallergenic recombinant allergen variants or allergoids prepared by chemical treatment of allergen extracts is the RAST- or EAST-inhibition assay. Such an IgE-binding inhibition assay has been used originally for potency determination of different allergen batches by comparing the capacity of a test sample (hypoallergen or allergoid) and of a reference preparation (native allergen or allergen extract) to inhibit the binding of IgE antibodies to a solid-phase bound native reference material. The IgE-binding inhibition assay can be made with standardized serum pools specific for the corresponding allergen or with single sera from allergic individuals. With this inhibition assay it is possible to determine the relative potency (P_{rel}) of allergoid preparations (Figure 2). Comparison of the inhibition curves of the native birch pollen extract (reference preparation) with the allergoid prepared by formaldehyde treatment of the allergen extract showed an approximately 1,000-fold reduction in

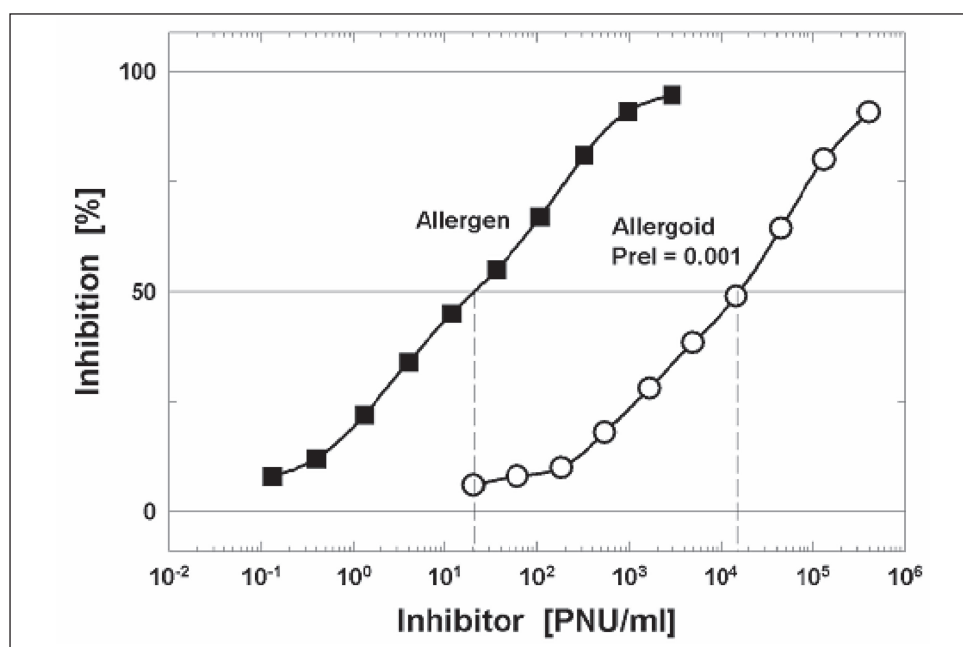
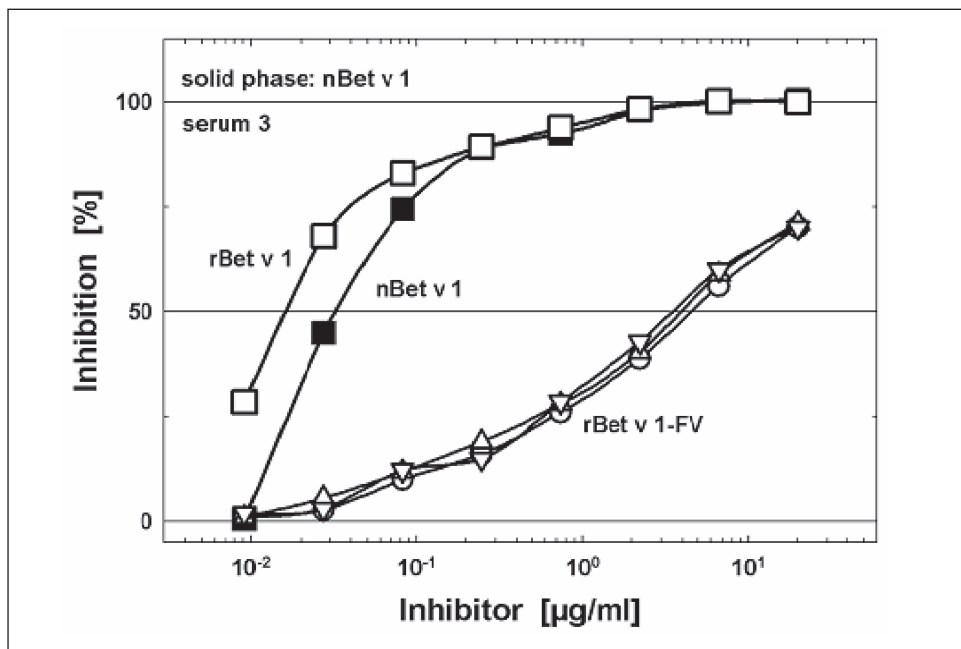


Fig. 2. IgE-binding inhibition by birch pollen allergen extract (■) and allergoid (○). Birch pollen allergoid is characterized by significant lower capacity to inhibit the IgE-binding to solid-phase bound native birch pollen allergen than the native birch allergen extract. Relative potency of the allergoid: $P_{rel} = 0.001$.

Fig. 3. IgE-binding inhibition assay with natural Bet v 1 (■), recombinant wild-type Bet v 1 (□), and the hypoallergenic folding-variant rBet v 1-FV (3 different batches: ○, △, ▽). The IgE-inhibitory capacity of rBet v 1-FV is at least 100 fold lower than those of natural and recombinant wild-type Bet v 1.



IgE-binding. The degree of the reduction judged at the 50 % inhibition level varies dependent upon the quality of the serum used. The shape of binding curves is dependent on affinity and epitope specificity of the antibody populations in individual sera or in different serum pools. The RAST- or EAST-inhibition assay can be used for quality control of allergoid preparations. In this application of the assay it is necessary to use standardized serum pools, standardized allergen solid phase (for example, allergen paper discs), and an in-house allergen reference preparation. The relative potency (P_{rel}) of different allergoid preparations cannot be used for standardization in terms of biological activity. The inhibition assay can be used also to assess the IgE-reactivity of recombinant allergen variants. A comparison of the inhibition curves of the natural Bet v 1, recombinant wild-type Bet v 1 and hypoallergenic folding-variant rBet v 1-FV is shown in Figure 3. The folding-variant is characterized by a substantial loss of IgE-reactivity, which was shown with a representative serum pool and with 25 single sera from different allergic donors (shown only serum no. 3). The reduction in the IgE-reactivity as judged at the 50 % inhibition is more than one hundred-fold using serum no. 3. The degree of inhibition and the shape of the curves vary between the individual serum reflecting differences in epitope specificity and affinity of antibody populations. The very similar shapes of the inhibition curves from 3 different batches of recombinant variant rBet v 1-FV demonstrate the consistency of the recombinant product. The reduced IgE-reactivity of rBet v 1-FV and its good solubility make this molecule a hopeful candidate for immunotherapy

of birch pollen allergic patients [15,21]. Another strategy has been used to create hypoallergenic recombinant allergen variants of the grass pollen major allergens Phl p 5a and 5b [9,22]. The hypoallergenic variants of both allergens have two short deletions outside of the dominant T-cell epitopes. The IgE-reactivities of the Phl p 5a variant DMbc and of Phl p 5b variant DM4 are substantially reduced when assessed using a representative serum pool from allergic donors (Figure 4). The reductions in the IgE-reactivities are caused by small deletions directly in IgE-binding sites in the C-terminal parts of both molecules or by influence on conformation through a deletion outside the recognized IgE- and T-cell binding regions as with the more N-terminal deletions in both molecules. These examples demonstrate that IgE-binding inhibition assays are useful tools to assess the IgE-reactivity of chemically modified allergen extracts (allergoids) and hypoallergenic recombinant allergen derivatives.

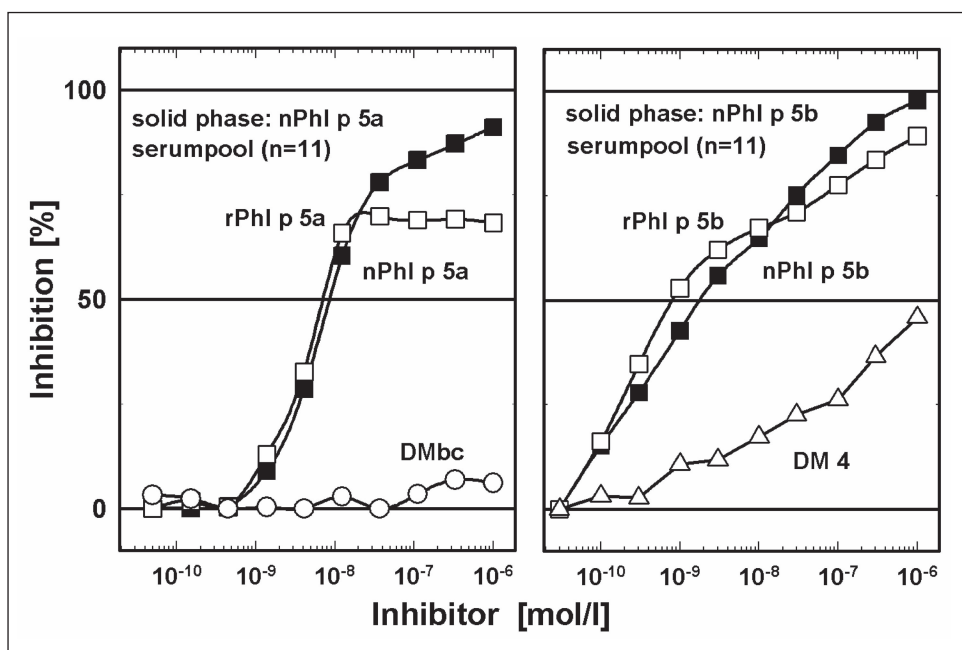


Fig. 4. IgE-binding inhibition assay with hypoallergenic deletion variants Phl p 5a-DMbc (○) and Phl p 5b-DM4 (△). Both deletion variants show a weaker capacity to inhibit the IgE-binding to the purified natural Phl p 5 isoforms a and b than the natural (■) and recombinant (□) Phl p 5 isoforms a and b.

IgE-mediated effector cell reactivity

The further test procedure for allergoids and hypoallergenic recombinant allergen variants is the assessment of their hypoallergenic features at the level of allergic effector cells. The biological reactivity of hypoallergenic candidates can be assessed *ex vivo* by testing the IgE-mediated activation or histamine release from blood basophils [9,10,17] or *in vivo* by skin testing in allergic subjects [9,13,23]. The allergen induced IgE-mediated activation of basophils can be measured by flow cytome-

try using the expression of the activation markers CD 63 [24] or CD 203c [17,18,25,26]. The measurement of CD 203c-expression by flow cytometry provides a rapid and sensitive method for the estimation of the allergenicity of complex allergen extracts and allergoids as well as purified major allergens and hypoallergenic derivatives of recombinant allergens [17,18,25,26]. The comparison of grass pollen allergen with allergoid shows substantial reduction in the activity of the latter (Figure 5). The reactivity patterns with allergen extract and allergoid vary with the individual allergic subjects, as demonstrated for 6 examples. Previous studies had shown that different grass pollen allergoid batches have a 10- to 30,000-fold reduced allergenicity by comparison with their corresponding native extracts when comparing concentrations leading to 50 % specific activation [17]. The median values achieved with patients in the study amount to 440- to 660-fold reductions of the basophil activating capacity for the grass pollen allergoids [17]. A similar reduction of the basophil activation capacity was found for birch pollen allergoid, the variation between different allergic donors being 5- to 1,000-fold [26]. Recombinant allergen derivatives with reduced IgE-binding activate human basophils from allergic donors substantially lesser than the recombinant wild-type proteins or purified natural counterparts. The Phl p 5b variant DM4 shows a marked reduction in the capacity to activate basophils from allergic donors as compared with natural and recombinant wild-type Phl p 5b (Figure 6). The degree of reactivity varies between donors reflecting differences in the sensitization of the cells and in quantity, affinity and epitope specificity of surface bound specific IgE. The deletion variant DM4 reveals strong hypoallergenic features, its basophil activating capacity is 50- to 10,000-fold reduced

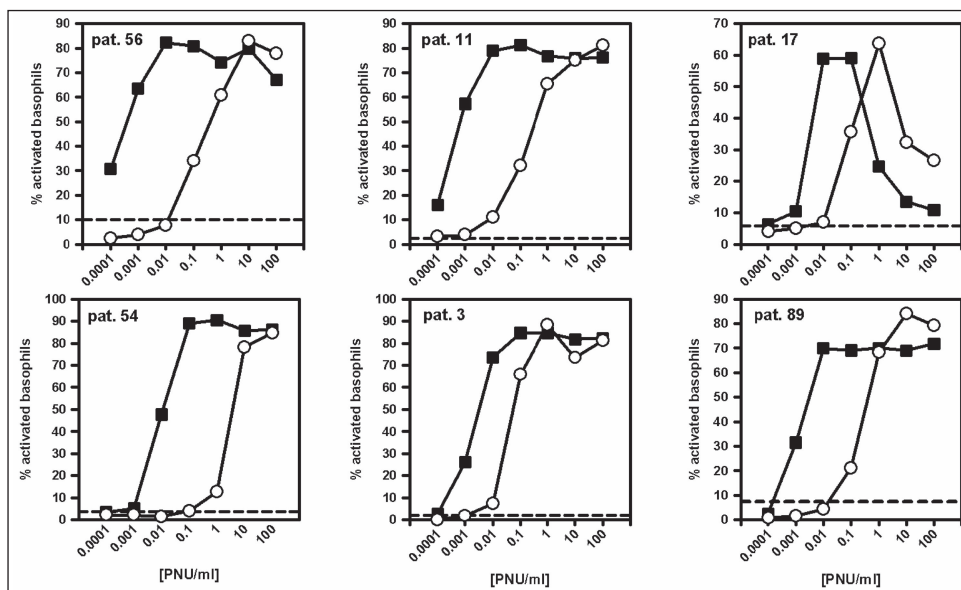
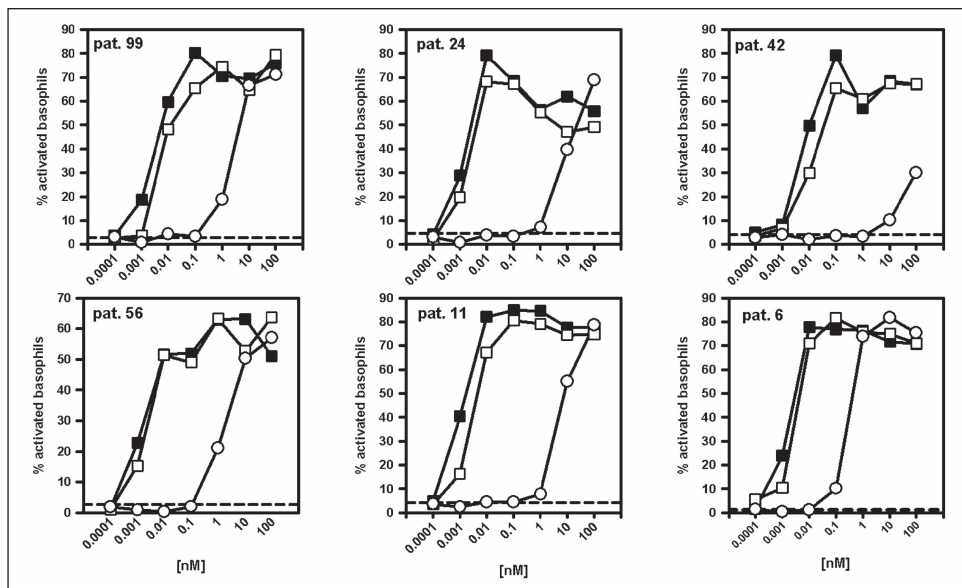


Fig. 5. Activation of basophils from grass pollen allergic donors with grass pollen allergen extract (■) and the corresponding allergoid (○). The activation is measured by the expression of the activation marker CD 203c on basophils. Response to the buffer control is shown as a broken line.

Fig. 6. Activation of basophils from grass pollen allergic donors with natural Phl p 5b (■), recombinant wild-type Phl p 5b (□), and the hypoallergenic deletion variant rPhl p 5b-DM4. The activation is measured by the expression of the activation marker CD 203c on basophils. Response to the buffer control is shown as a broken line.



by comparison with nPhl p 5b or the recombinant wild-type molecule [17,25]. Similar reduced basophil activating capacities have been found for other hypoallergenic recombinant allergen variants, such as rPhl p 5a-DMbc [22], rBet v 1 fragments and trimer [18], and rBet v 1-FV [21]. In conclusion, the measurement of the degree of CD 203c- expression on basophils has proved to be a very valuable tool for the evaluation of the allergen variants as possible candidates for a future application in specific immunotherapy.

T-cell reactivity

Insight into the mechanism of allergen specific immunotherapy has shown that the reaction of the allergen-specific T-cells with allergen-derived peptides in complex with the MHC-molecules on the antigen presenting cells is an essential step for the modulation of the allergic immune response. The T-cell reactivity of allergen preparations for specific immunotherapy is a very important property for their biological activity and therapeutic efficacy. The estimation of T-cell reactivity is not included in the established test procedures for characterization and standardization of native or modified allergen extracts. The provision of evidence of T-cell reactivity of modified allergen extracts or engineered hypoallergenic recombinant allergens should be an essential step in the development program.

Some years ago it was shown that allergoids prepared by formaldehyde treatment of grass pollen allergen extracts and the corresponding native allergen extracts stim-

Table 1. Proliferative response of T-cell clones and T-cell lines stimulated with natural Bet v 1, recombinant wild-type Bet v 1, and hypoallergenic variant rBet v 1-FV.

	Epitope	Proliferation [SI] ¹			
		nBet v 1	rBet v 1	rBet v 1-FV	
				batch A	batch B
TCC²					
25A9	10-24	4.7	2.2	2.9	4.0
21D10	49-63	2.2	4.2	2.7	2.3
13B5	49-63	2.8	n.t. ⁴	n.t.	2.4
14B4	85-96	9.5	n.t.	n.t.	18.1
16D6	88-99	17.8	n.t.	14.7	16.1
TCL³					
51.11	10-24	5.2	2.7	7.5	6.3
51.02	16-27	7.4	2.7	15.4	13.2
36.15	49-63	166.6	178.9	215.0	220.8
54.06	124-135	10.4	4.4	23.2	25.4
54.04	85-96	5.2	4.8	11.8	12.9
68.19	88-99	20.3	13.3	40.1	38.2
43.03	88-102	111.8	38.6	235.7	223.6
92.05	112-123	12.9	6.5	12.2	12.2
52.12	142-153	30.0	13.6	n.t.	44.7
36.10	136-159	6.0	7.2	11.4	9.4

¹ SI: stimulation index² TCC: T cell clones³ TCL: T cell lines⁴ n.t.: not tested

ulated peripheral blood mononuclear cells (PBMC) to the same extent in a dose-dependent fashion [19]. Similar results were found for allergoids prepared with other pollen or house dust mite allergens (unpublished data). Additionally, high doses of grass pollen allergoids shift the typical TH2-cytokine response of PBMC from allergic donors *in vitro* towards a TH0/TH1-phenotype [19]. The grass pollen allergoid stimulates T-cell lines (TCL) and T-cell clones (TCC) selected with the grass pollen major allergen Phl p 5 using PBMC or dendritic cells for antigen presentation [27]. Both allergoids and allergen extracts stimulate dose-dependent proliferation of the TCL [19]. The great majority of the studied TCC were induced to proliferate by both allergen extract and allergoid [19], indicating that the formaldehyde treatment of the allergoid had only a minimal effect on the function of the sequential T-cell epitopes.

TCL and TCC selected with purified natural or recombinant allergens are the best tools to prove that hypoallergenic recombinant allergen variants retain T-cell reactivity. It was previously shown that the deletion variants of Phl p 5, including the variant DM4 and of the rPhl p 5a-DMbc, retain T-cell reactivity [9,22], as do other

hypoallergenic recombinant allergen variants such as the trimer and fragment mixture of Bet v 1 [28], and the folding-variant rBet v 1-FV. In contrast to the Phl p 5 deletion mutants, the hypoallergenic rBet v 1-FV has the same primary structure as the wild-type molecule. Therefore it is not surprising that all TCC and TCL with known epitope specificities were stimulated to proliferate with the natural and recombinant Bet v 1 as well as with the hypoallergenic variant (Table 1). The comparison of the stimulation indices obtained with two different batches (A and B) of the hypoallergenic folding-variant which were produced at an interval of several months shows the good batch to batch consistency of this recombinant product.

In conclusion, the results have shown that it is possible to create hypoallergenic preparations both by chemical modification of natural allergen extracts and by protein and genetic engineering of recombinant major allergens. Their reduced IgE-reactivity can be examined with solid-phase binding techniques and liquid-phase IgE-binding inhibition assays. The basophil activation assay is a useful method to measure the reduced allergenicity *ex vivo* before skin testing in allergic subjects. It is necessary to demonstrate the conserved T-cell reactivity of allergoids and hypoallergenic recombinant allergen variants, with proliferation assays using T-cell lines and clones derived from allergic donors. The conserved T-cell reactivity of hypoallergenic preparations is a prerequisite for their biological activity to modulate the allergic immune response.

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Discussion

Vieths: Your company is probably one of the most active ones in regard to clinical trials with recombinant allergens. So you may be close to submission of an application for marketing authorization in Europe. With this application you have to provide the agencies with a reliable potency test for the activity of your preparation. Which of those tests that you have just described would you suggest as a potency test, e.g. for the folding variant for Bet v 1?

Fiebig: I believe we can resolve this problem of the biological potency by the introduction of mass units in this recombinant system. We do not have this problem, we only have to test the preparation for safety. This can be carried out by dose-response studies of the allergenic preparations in the clinical studies. Maybe the second step of this development is to find the upper limit of the dose which we can apply in a patient without the risk of anaphylactic reactions. I will not go into the details of the data from the clinical study because Oliver Cromwell will do this tomorrow. However, the quantity of this folding variant, and the other hypoallergenic variants constructed by Rudolf Valenta, in the clinical study also had a very high maintenance dose.

Vieths: I understand this, but the point I wanted to make is that in my understanding the EMEA guideline on biotechnologically-derived products demands to provide a potency test, whatever that means. We have to discuss and to think about this problem. But the potency is in the guidelines, mass units will not be sufficient.

Fiebig: I believe I have shown that the real potency test for such hypoallergenic variants or allergoids is the reactivity with T cells because the main action of this allergen preparation is on the T cell level. And therefore, we must demonstrate that these variants can react with T cells. However, for the production control, I believe potency tests are not possible.

André: You presented us comparison with skin prick test. Did you also perform similar comparison about the late-phase reaction that can be observed following intradermal injection?

Fiebig: No, we have not.

Larsen: The idea behind the allergoids as originally proposed by David Marsh in the 1970s was to reduce IgE-binding but to maintain or enhance immunogenicity. You showed us a lot of data on IgE binding and that allergoids do have reduced IgE binding. I believe that this is not very difficult to achieve, you can just reduce the dose and then that will reduce IgE binding. But it is the ratio between the ability to reduce IgE binding and the ability to induce a beneficial immune response in the patient that is interesting. Do you have any data to support that you have a better ratio here with the allergoids as compared to the unmodified allergens? The second question is concerning the T cell reactivity of the allergoids. I believe that you are aware of the literature where some papers state that there is indeed a reduced ability of the allergoids to stimulate T cells. Do you have any comments on that?

Fiebig: I will start with the last question. We also know these data and it is clear that, e.g. for birch allergoid, that PBMCs or T cells stimulated with PBMCs as antigen-presenting cells show this behaviour. Some years ago we had a publication showing that this is dependent on the type of the antigen-presenting cell. Because in the peripheral blood of the allergic donors you have a high amount of antigen-presenting cells loaded with IgE. And these B cells or monocytes can capture and present the allergen better than an allergoid. Therefore, the disadvantage of the allergoid with PBMCs as antigen-presenting cell is on the level of the capturing the allergoid because this is not facilitated by the IgE. When you use dendritic cells for instance, which you have cultured in Petri dishes, you nearly obtain the same stimulation indices with the allergoid and the allergen. That means the advantage of the IgE-mediated uptake by the antigen-processing cell is not working in this system. What was the first question?

Larsen: The first question was that it is not very difficult to reduce IgE binding but the level of the immune response.

Fiebig: We have no direct data because we did not study conventional allergens and allergoids in a direct comparison. However, what we can say is that with allergoids we reach the maintenance dose earlier in a shorter time and we are able to introduce more protein into the patient. And this higher amount of protein induces a higher immune response. After this short time of immunotherapy, we observed the same or higher IgG4 or IgG1 responses with allergoids as compared with a normal unmodified depot preparation.

Jacobsen: This may be more a comment to the discussion about the potency testing for new products. I think that in that relation it is very important to understand that for every new product like hypoallergenic proteins, a potency application should be related to the expected use of the product. And in my opinion there will be no products that come into use without having proven their own benefits and their own worth by doing dose-response studies. There is no way to transfer potency testing from one product to new products like this. So I just recommend that the activities ought to be established and that each product has to show its own value in the way it is recommended for use.