

Antibody-based Methods for Standardization of Allergoids and Recombinant Hypoallergens

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Introduction

Allergen specific immunotherapy with hypoallergenic preparations was introduced more than three decades ago using allergoids produced by aldehyde treatment of allergen extracts [1,2]. Such allergoids were prepared by chemical modification of standardized allergen extracts from pollen of grasses, trees, and weeds [1–3] as well as mites [4,5].

This chemical modification of allergens introduces both intra- and intermolecular cross-linking, conformational changes and/or polymerization, with the result that the IgE-binding capacity of such allergoids is substantially reduced by comparison with the native allergen. The potential benefits of such allergoids for specific immunotherapy 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 retained T cell reactivity of the allergoids [6], their immunogenic activity and therapeutic efficacy has been shown in several clinical trials [5,7–9]. Recombinant DNA technology now allows us to determine whether or not complex natural allergen extracts can be replaced by recombinant allergens. By gene or protein engineering it is possible to design hypoallergenic recombinant allergens [10–17]. Such hypoallergenic recombinant allergens, like the allergoids, possess reduced IgE-reactivity, reduced specific IgE-mediated effector cell activation and mediator release, diminished skin test activity and retained T cell reactivity [4,10–22]. Clinical trials have shown that such hypoallergenic recombinant *rBet v 1* derivatives induce strong IgG4- and IgG1-antibody responses [23–26]. The characterization and control of allergen products for diagnostic and therapeutic purposes includes the determination of potency in addition to the quantification of the protein content, the determination of protein and allergen profiles, and the quantification of major allergen content [27]. It is accepted that the total allergenic activity of an allergen prepa-

ration reflects the potency or biological activity, and that this total allergenic activity can be determined by a competitive binding test [27]. In view of the fact that allergoids and hypoallergenic recombinant allergen derivatives possess a reduced IgE-reactivity, such IgE-based immunoassays can not be used as potency assays for determination of the biological activity. Therefore it is necessary to develop assays for the determination of the biological activity which can be used for assessment of potency.

Results and Discussions

Antibody-based assays for the determination of the biological activity of allergoids

The biological activity of native allergen preparations is measured by determination of their total allergenic activity. Competitive IgE-binding tests like RAST- or EAST inhibition assays are used for determination of this total allergenic activity of native allergen preparations for diagnostic application. It is clear, that such *in vitro* determined total allergenic activity reflects very well the biological activity of allergen preparations for skin or provocation testing in allergic subjects. It is also clear, that the allergenic activity of native allergen preparations for therapeutic use correlates with the risk for inducing unwanted allergenic reactions (side effects of the specific immunotherapy). Chemically modified allergen extracts (allergoids) and hypoallergenic recombinant allergen derivatives possess a reduced IgE-reactivity, and therefore it is not possible to use IgE-based inhibition assays for the determination of the biological activity. Clinical studies with allergoids and hypoallergenic recombinant allergen derivatives have shown their therapeutic efficacy in spite of their reduced allergenic activity [5,7–9,23,25,26]. Therefore, we must investigate, if other immunological properties can be used for the characterization of the biological activity of hypoallergenic preparations for their application in specific immunotherapy. Our recent knowledge about the mechanism of action of the allergen-specific immunotherapy shows that the main biological activity of an allergen preparation is its capacity to interact with the components of the immune system and to modulate the allergic immune response [28–30]. The most important effects of the allergen specific immunotherapy are the induction of a regulatory T cell responses, the induction of anergy in the T-helper cell population, a shift from the TH2 dominated allergic response to a TH0/TH1 response and the induction of a strong humoral immune response with IgG4 and IgG1 antibodies. All these immunological responses are based on the immunogenic and antigenic properties of the therapeutic preparations. The retained T cell reactivity of allergoids was demonstrated by their capacity to stimulate T cell lines and clones established from allergic subjects to proliferation and cytokine synthesis [6,16,17]. Clinical studies with grass pollen allergoids have shown that such hypoallergenic preparations are clinically efficacious and that they induce a strong IgG antibody response against epitopes on both allergoids and allergens [9]. That means, a typical biological activity of a therapeutic allergen or allergoid preparation in the cause of the specific immunotherapy is the stimulation of IgG antibodies reacting with epitopes expressed on allergoids as well as on unmodified allergens.

The immunization of animals with allergoid preparations results also in a strong humoral immune response with IgG antibodies and we have investigated if such antibodies can be used for the construction of potency assays. For allergoids, potency tests should consist of a discriminator test or a combination of immunological tests to distinguish between native and modified molecules [27]. Therefore, we have investigated the binding properties of antisera raised by immunization of goats with allergoids prepared from grass pollen (mixture of 6-grasses), birch pollen and house dust mite allergen extracts. The goat antisera raised by immunization with 6-grass pollen allergoid contain antibodies which bind not only to solid phase bound 6-grass allergoid, but also to native 6-grass pollen allergen extract and to the purified native major allergens *Phl p 1* and *Phl p 5* (Fig. 1a). Antisera elicited by immunization with birch pollen allergoid react with surface-bound allergoid, native birch pollen allergen extract and with purified *Bet v 1* (data not shown). Comparable binding properties were also found for house dust mite (*Dermatophagoides pteronyssinus*) allergoid induced antisera which contains antibodies reacting with the allergoid, the native mite extract and the purified native allergens *Der p 1* and *Der p 2* (Fig. 1b).

We have used these goat anti-allergoid sera to establish immunoassays based on the inhibition of binding of IgG-antibodies to microtiter plate-adsorbed allergoids by pre-incubation of the antisera with soluble allergoids or native allergen extracts (Fig. 2 and 3). The binding curves show that the 6-grass allergoid (Fig. 2) as well as the house dust mite allergoid (Fig. 3) are very effective inhibitors of the IgG antibody binding. The inhibition curve with native allergen extracts demonstrate that

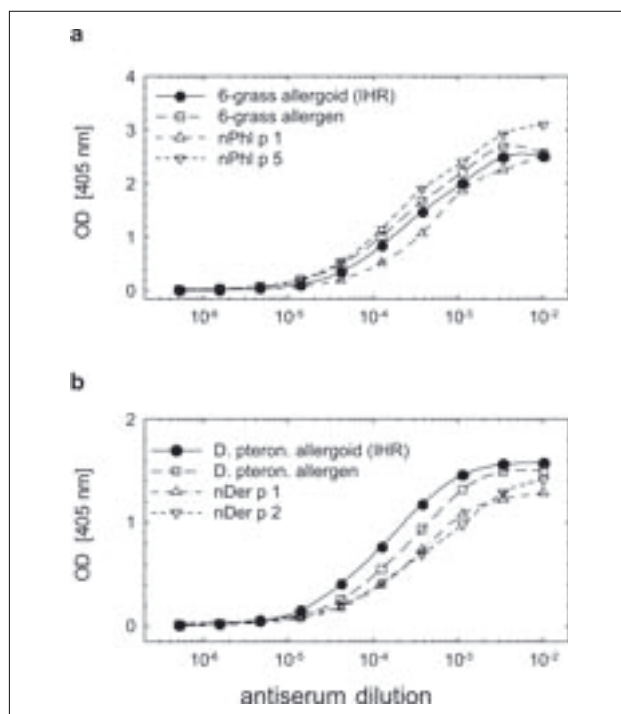


Fig. 1. Specificity of goat antisera raised by immunization with 6-grass pollen allergoid (a) and house dust mite (*Dermatophagoides pteronyssinus*) allergoid (b). Allergoid induced antisera react with the immunogen, native allergen extracts and purified major allergens.

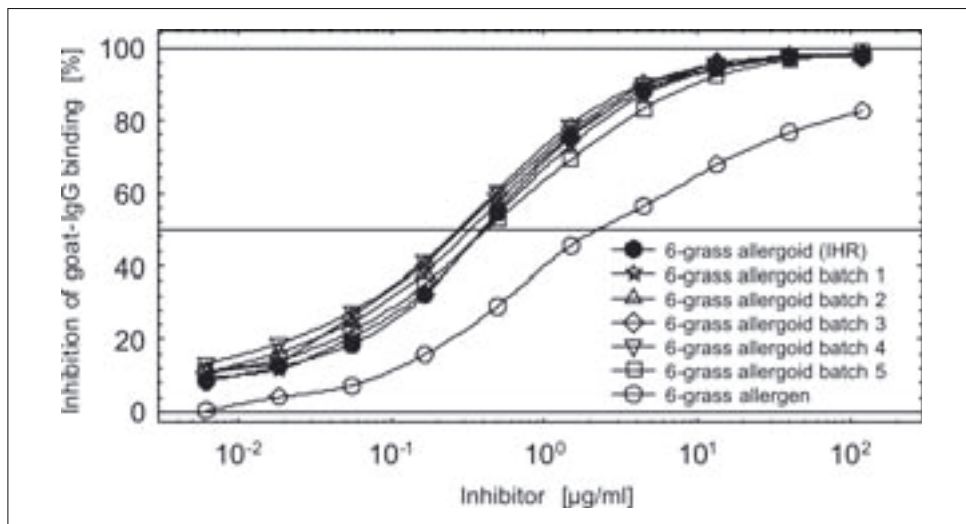


Fig. 2. Comparison of different batches of 6-grass allergoid with the in-house reference preparation by the positive discriminatory IgG-binding inhibition immunoassay for 6-grass allergoid. Native 6-grass allergen extract has a lower capacity to inhibit the IgG-binding to solid phase 6-grass allergoid.

they do not express all epitopes which are present on the allergoids and are detected by the allergoid-induced IgG antibodies. These IgG immunoassays can distinguish between the hypoallergenic allergoids and their unmodified native allergen extracts. Such assays are positive discriminatory assays for the allergoids. These IgG-inhibi-

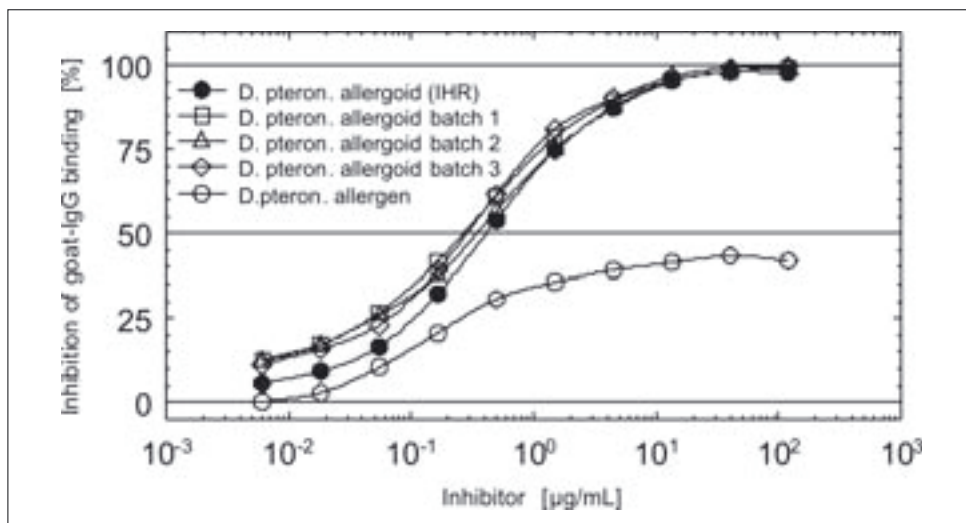


Fig. 3. Comparison of different batches of house dust mite (*Dermatophagoides pteronyssinus*) allergoid with the in-house reference preparation by the positive discriminatory IgG-binding inhibition immunoassay for house dust mite allergoid. Native house dust mite allergen extract has a lower capacity to inhibit the IgG-binding to solid phase 6-grass allergoid.

tion assays are potency assays which can be used for the assessment of antigenic properties of different batches of allergoids in relation to an in-house-reference preparation (IHR). We have used as IHR the batch which was used for the immunization of the goats. The comparison of the capacity of different batches of 6-grass allergoid (Fig. 2) or house dust mite allergoid (Fig. 3) with their in-house-reference preparations demonstrates the good batch-to-batch consistency of the production process for allergoids.

The antigenic activity of allergoids determined with such IgG-inhibition assays reflects the biological activity of therapeutic allergoid preparations, as do IgE-inhibition assays for native allergen preparations for diagnostic use.

Monoclonal antibody-based potency assays for a hypoallergenic recombinant Bet v 1 derivative

Recombinant allergens are useful tools for allergen component-resolved diagnosis and candidates for a future specific immunotherapy [31,32]. The first clinical studies with recombinant allergens have shown the therapeutic efficacy of recombinant timothy grass pollen allergens (cocktail of *rPhl p 1*, 2, 5a, 5b, and 6) for specific immunotherapy of grass pollen allergic patients [33] and of recombinant *Bet v 1* for birch pollen allergies [34]. Hypoallergenic derivatives of recombinant allergens have been constructed by site-directed mutations [10,11], by preparation of unfolded allergen fragments [12] or by construction of oligomeric forms [13]. An alternative approach for developing a hypoallergenic derivative of the birch pollen major allergen *Bet v 1* came from the observation that during the purification procedure, recombinant *Bet v 1* can be induced to adopt a stable random coil structure which can be clearly distinguished from secondary structure of the native molecule by circular dichroism spectroscopy [17]. This folding variant, designated *rBet v 1-FV*, exhibits hypoallergenic properties as judged by IgE-inhibition test and basophil activation assays [17]. The retained T cell reactivity of this *rBet v 1-FV* could be shown by stimulation of T cell lines and clones derived from birch pollen allergic donors to proliferate and synthesise cytokines [17]. A first open, randomised comparative clinical study with *rBet v 1-FV* and a natural birch pollen extract preparation has shown that this hypoallergenic molecule is safe and clinically efficacious [25]. The reduced IgE-binding capacity of this modified *rBet v 1-FV* can be examined by IgE-inhibition assay [4,17]. This assay is a suitable tool for the demonstration of the reduced IgE-reactivity for each batch during the production process, but can not be used for determination of the biological activity. The most important biological activities of therapeutic allergen preparations during specific immunotherapy are their reactivity with T cells of treated patients and immunogenicity. The T cell reactivity of *Bet v 1-FV* was shown with T cell clones and lines from birch pollen allergies [4,17], but such a cellular assay is not practicable for routine testing of biological activity.

The immunogenicity of the hypoallergenic *rBet v 1-FV* was tested by immunization of mice, rabbits and goats. These animals species developed a strong IgG-antibody response against epitopes expressed on the immunogen (*rBet v 1-FV*) as well as on the native *rBet v 1* or natural *Bet v 1* purified from birch pollen extract.

The retained immunogenicity of *rBet v 1-FV* in man could be shown in the first clinical study. All *rBet v 1-FV* treated patients developed strong IgG4- and IgG1-antibody responses [25,26]. These IgG-antibodies react with epitopes expressed on the immunogen as well as on the native *Bet v 1* [25,26]. The strong IgG-antibody response in different species demonstrates the retained immunogenicity of hypoallergenic *rBet v 1-FV* and represents an important aspect of the biological activity of this molecule. Such induced IgG-antibodies can be used for the development of assays for the determination of the IgG-binding capacity. This antigenic property of the hypoallergenic *rBet v 1-FV* can be measured with IgG-antibody based potency assays and used for the standardization of the modified allergen derivatives. However, the IgG-antibody based inhibition assays developed with polyclonal antisera from immunized animals have a limited availability. An alternative approach is the use of monoclonal antibodies.

Therefore, we have prepared monoclonal antibodies in mice by immunization with native *Bet v 1*, purified from birch pollen extract, and with the hypoallergenic *rBet v 1-FV*. The binding properties of these monoclonals were characterized by direct binding to solid phase bound natural and recombinant *Bet v 1*, the hypoallergenic *rBet v 1-FV* as well as unfolded *Bet v 1* fragments F1 and F2 (amino acid residues 1–73 and 74–159). The 12 monoclonals raised by immunization with native *Bet v 1* react with birch pollen extract, purified natural *Bet v 1* and native *rBet v 1*. Seven of these monoclonals react also with the hypoallergenic *rBet v 1-FV* and with either the N-terminal fragment F1 or the C-terminal fragment F2. Two monoclonals show a reduced reactivity with *rBet v 1-FV*, and three other antibodies do not bind to this hypoallergenic derivative. Two of these *rBet v 1-FV* non-reactive antibodies (1–1D6, 1–10D9) can also not react with the unfolded *Bet v 1* fragments (F1, F2) and with any of the overlapping synthetic dodecapeptides covering the whole sequence of *Bet v 1*. It is likely that these monoclonals recognize conformation-dependent epitopes which are destroyed on the hypoallergenic *rBet v 1-FV* and on the fragments. The monoclonal 1–4B10, which also does not react with *rBet v 1-FV*, binds to the C-terminal fragment F2 and to 3 overlapping dodecapeptides with the common amino acid residues 118–123. This sequence-dependent epitope 118–123 is surface expressed on the native *Bet v 1*, but hidden on the hypoallergenic *rBet v 1-FV*.

A panel of 46 monoclonals were selected after immunization and primary screening with the hypoallergenic *rBet v 1-FV*, and 45 of these bind not only to the immunogen, but also to the solid-phase adsorbed native *Bet v 1* (recombinant or purified from extract). These monoclonals bind also to adsorbed F1 or F2 fragments of *Bet v 1*. Binding experiments with overlapping dodecapeptides of *Bet v 1* have shown that these monoclonals recognized 13 different sequence dependent epitopes scattered over the whole sequence of *Bet v 1*. Because these antibodies bind in the direct binding assay to both hypoallergenic and native *Bet v 1*, it can be concluded that these sequence dependent epitopes are surface expressed on hypoallergenic as well as native *Bet v 1* molecules.

Only the *rBet v 1-FV* reactive monoclonal antibody 2–1G6 does not bind to the native *Bet v 1*. This antibody recognizes a conformational epitope specific for the

hypoallergenic derivative *rBet v 1-FV*. The weak binding of this antibody to the F2 fragment and to dodecapeptides covering the sequence between amino acids 112 and 129 is an indication that amino acid residues in this range are involved in the binding to the antibody.

We have tested different combinations of these well characterized monoclonals in 2-site binding ELISA systems with native or hypoallergenic *Bet v 1-FV*. The first type of ELISA uses the monoclonals 1-4B10 as capture and 1-2E10 as detector antibody. The monoclonal 1-4B10 reacts with an epitope which is surface expressed on the native *Bet v 1*, but not accessible on the hypoallergenic *rBet v 1-FV* molecule. This antibody binds to the unfolded C-terminal fragment F2 and to the dodecapeptides covering the positions 112–129 (common core sequence 118–123). The detector monoclonal 1-2E10 binds the 2 overlapping dodecapeptides with amino acids 55–66 and 58–69. The 1-2E10 reactive epitope is accessible on N-terminal fragment F1, on native and hypoallergenic *Bet v 1* molecules. This combination 1-4B10/1-2E10 can be used for the quantification of native *rBet v 1* and purified natural *Bet v 1* (Fig.4). This ELISA system can not detect hypoallergenic *rBet v 1-FV*. Therefore, this assay can be used as a negative discriminatory assay for the folding variant. On the other hand, this ELISA system is an excellent tool for detection and quantification of correctly folded *rBet v 1* in *rBet v 1-FV* preparations. This negative discriminatory assay can be used for the demonstration of the absence of correctly folded *rBet v 1* molecules during the production (batch-to-batch consistency control) and stability studies with the hypoallergenic *rBet v 1-FV*.

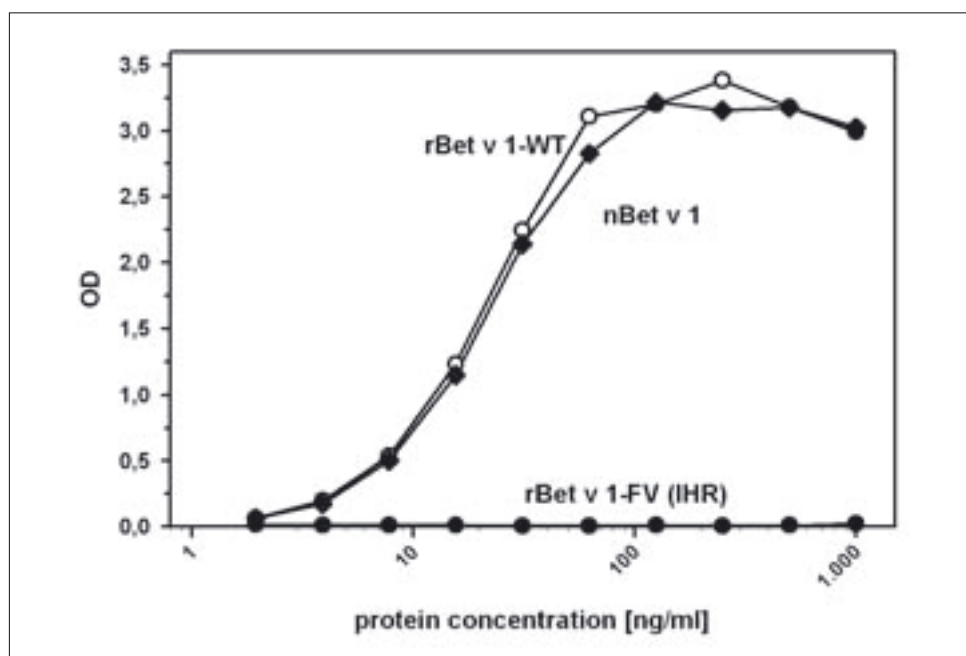


Fig. 4. Negative discriminatory ELISA for hypoallergenic *rBet v 1-FV*. Monoclonal antibodies: 1-4B10 (capture) and 1-2E10 (detector).

Positive discriminatory ELISA systems for the hypoallergenic *rBet v 1*-FV could be established with several combinations of monoclonals which react with different epitopes. The first type of ELISA system is based on the folding variant-specific antibody 2-1G6 as capture antibody and different detector monoclonals reacting with various sequence-dependent epitopes expressed on native *Bet v 1* as well as on the hypoallergenic folding variant, e.g. 1-6B6 (epitope 4-18), 1-6D10 (epitope 19-33), 5-13D9 (epitope 22-39), and 5-2F2 (epitope 82-99, core sequence 88-93). These antibody combinations are capable of detecting the hypoallergenic *rBet v 1*-FV, but not the native *rBet v 1*. The combination 2-1G6/5-2F2 detects hypoallergenic *rBet v 1*-FV with the highest sensitivity (Fig. 5). This ELISA system is an excellent tool for the detection and quantification of the hypoallergenic *rBet v 1* folding variant and can be used as a potency assay. The comparison of the curves of different *rBet v 1*-FV batches (A-I) demonstrates the similarity of different batches of *rBet v 1*-FV in respect of their immunological reactivity with these IgG antibodies.

The second type of ELISA system is based on monoclonals which react with sequence-dependent epitopes expressed on solid phase bound *rBet v 1*-FV as well as native *rBet v 1*. We have selected several combinations of such monoclonals which react with the hypoallergenic *rBet v 1*-FV in a two-site ELISA, but do not detect the correctly folded *rBet v 1*. The ELISA using the monoclonal 5-2F2 reacting with amino acid residues 88-93 (core sequence) as capture antibody and the detector monoclonal 1-6D10 reacting with the amino acid residues 19-33, detects the hypoallergenic folding variant, but not the correctly folded *rBet v 1* (Fig. 6). The reason for this difference could be due to different steric orientation of these epitopes on

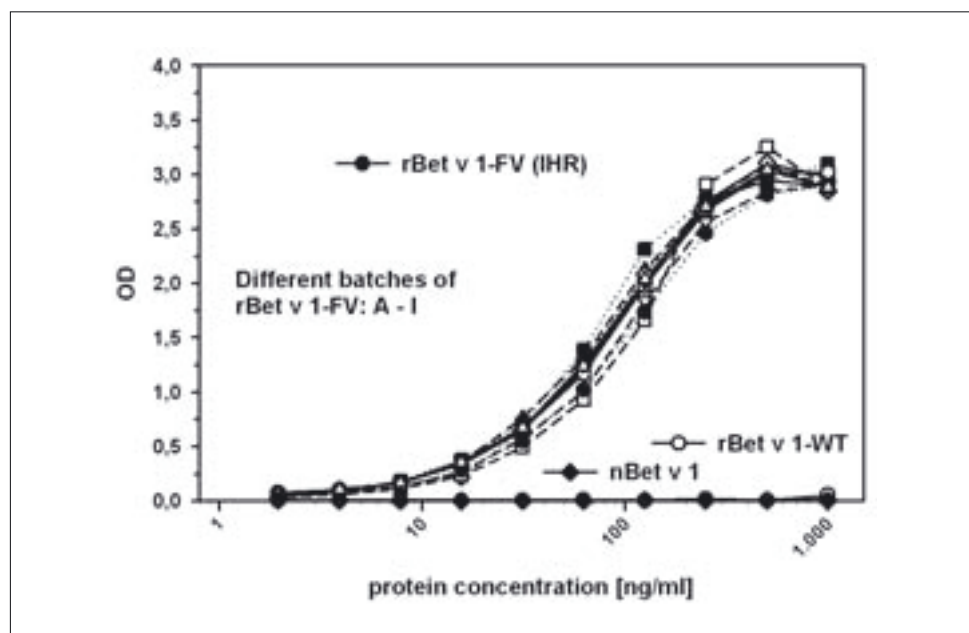


Fig. 5. Positive discriminatory ELISA for hypoallergenic *rBet v 1*-FV: comparison of different batches of *rBet v 1*-FV with the in-house reference preparation. Monoclonal antibodies: 2-1G6 (capture) and 5-2F2 (detector).

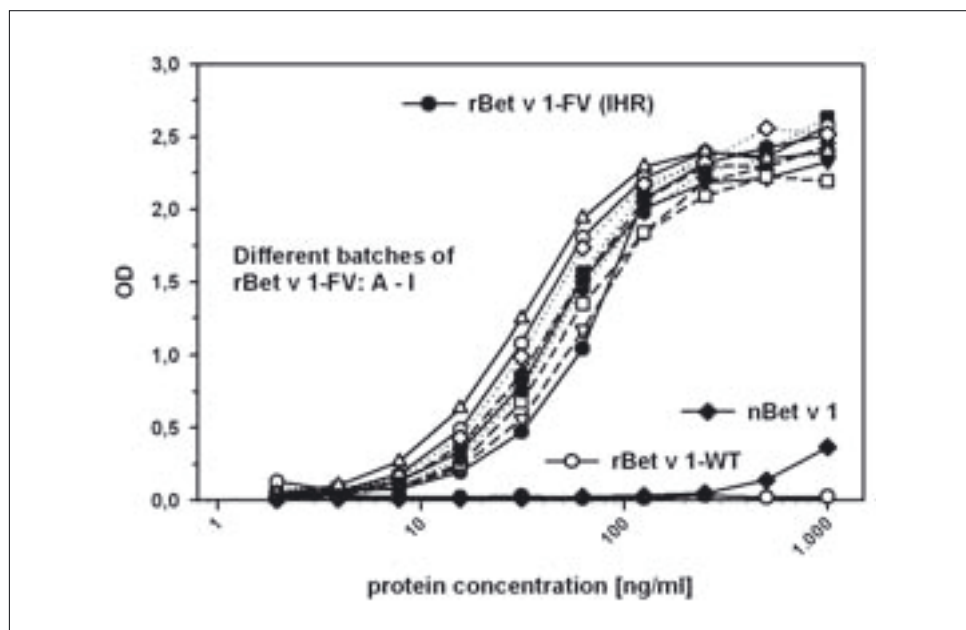


Fig. 6. Positive discriminatory ELISA for hypoallergenic *rBet v 1-FV*: comparison of different batches of *rBet v 1-FV* with the in-house reference preparation. Monoclonal antibodies: 5-2F2 (capture) and 1-6D10 (detector).

the native and hypoallergenic *rBet v 1*. Both epitopes are accessible for a two-site binding on hypoallergenic *rBet v 1-FV*, but not on the native molecule. The similar binding curves of different *rBet v 1-FV* batches demonstrate the batch-to-batch consistency of the production process.

Another type of ELISA with the capture antibody 1-1G7 and the detector monoclonal 1-6D10 detects also only the hypoallergenic *rBet v 1-FV* and does not react with native *rBet v 1* (Fig. 7). The capture antibody 1-1G7 binds to a sequence epitope with the core amino acid residues 76–81, which form the turn between the β -strands 4 and 5, and the detector antibody 1-6D10 reacts with the amino acids 19–33 which include the small non-helical part between alpha 1 and alpha 2 helices. Both epitopes are localized in close proximity on the surface of the native *rBet v 1* using the 3D model of *Bet v 1* [35] and the binding of the detector antibody is excluded by steric hindrance. The two-site binding of the hypoallergenic *rBet v 1-FV* by these antibodies shows that these epitopes are arranged in positions which allow the binding of both antibodies. This demonstrates very clearly that the 3D-structure of the folding variant is quite different from that of the native *rBet v 1*.

Conclusion

Immunoassays based on polyclonal animal IgG antibodies raised against allergoids have been shown to be suitable for determination of biological activity (potency) of modified allergen preparations (allergoids). Furthermore these assays are able

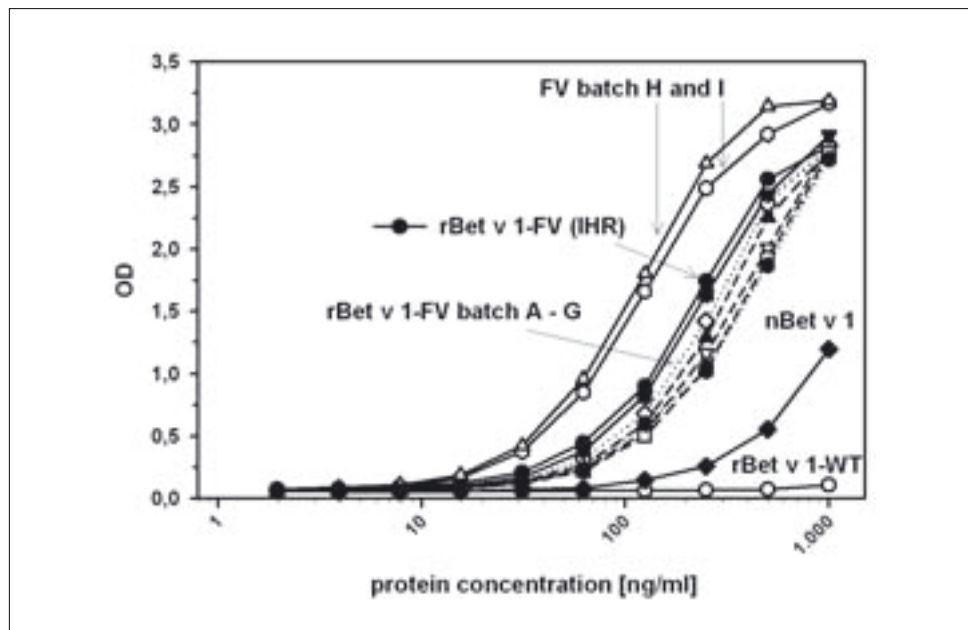


Fig. 7. Positive discriminatory ELISA for hypoallergenic rBet v 1-FV: comparison of different batches of rBet v 1-FV with the in-house reference preparation. Monoclonal antibodies: 1-1G7 (capture) and 1-6D10 (detector).

to discriminate between native and modified allergen. A large panel of monoclonal antibodies has provided the means to establish discriminatory assays for a hypoallergenic folding variant of *rBet v 1* and the native form of the allergen. These can be used to detect fine differences in the 3D-structure and to confirm the correct folding of *rBet v 1*-FV, as well as for detecting the presence of residual *rBet v 1* in a preparation.

These antibody-based discriminatory assays are suitable tools for quantifying biological activity of hypoallergenic derivatives as well as for demonstrating batch to batch consistency.

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Discussion

Esch: I have two related questions regarding allergoids. One is to your observation that new antigens or new epitopes are created during production. Have you looked at monoclonal antibodies that recognize these new epitopes but do not recognize the native allergen. Second question, do you have an opinion as to what clinical implications these new antigens or epitopes might have with respect to either safety or efficacy?

Fiebig: As for the first question, I believe it is clear that if you have a modified allergen or antigen you must have new epitopes. There is no chance for the animal not to recognize these epitopes. We do not find monoclonal antibodies against these new epitopes of the allergoid because we have a lot of monoclonal antibodies that recognize the grass pollen or mite allergens. A fraction of these monoclonal antibodies can be used for the detection of the native epitopes in the allergoid. Unfortunately, however, in most cases we do not have two monoclonal antibodies which work together in a two-site ELISA. We have such an ELISA for *Phl p 5*, but the modification of the allergoid – we have a little bit of dimers and oligomers – might lead to incorrect results with such an ELISA. The interpretation of the results can be very hard. Secondly, I believe there is no clinical implication of these observations. The antibodies against these new epitopes are of the IgG isotype, i.e. IgG4 and IgG1; we have never seen them in IgE. It results from subcutaneous injection and I believe if we induced such IgE which is reactive with the new epitopes, they have no physiological relevance because pollens do not have these epitopes. And therefore I believe it is a safe preparation.

van Ree: In your first experiments where you did the inhibition studies with the allergoids, did you also try to do that with the purified allergens as a coating? So can you in some way get an impression whether that is reproducible as well when you have coated your plates with purified *Phl p 1* or *5* in addition to extracts or allergoids?

Fiebig: No, we have not done these experiments. It is very easy to perform such experiments. However, we have to do an evaluation of the whole allergoid and not of a single allergen in the allergoid.

van Ree: Well, but one of your questions you of course have is whether your major allergens are represented in a reproducible way in your allergoid as well.

Fiebig: Oh, we know this. We have shown that T cells are reactive with the allergoids three years ago. And we have established T cell lines against the major allergens of *Phl p 5*, *Phl p 1*, and now also against *Phl p 6* and *Phl p 2*. And we can detect the allergens in the allergoid preparation and we can use this assay also for batch release. However, this assay is too complicated for the routine.

van Ree: One short question about your last slide. Are batches H and I, which have a different behavior, thrown away?

Fiebig: Yes, they are thrown away, they will not go into the final product. However, we need these batches for comparison and for the validation of other assays. Therefore, it is very important for us to have such preparations.

Vieths: Congratulation to this nice piece of work. I have one comment and one short question. The comment is that the text of the guideline regarding a discriminatory test is very strict. It is possible that it will be slightly rephrased and that it says discriminatory test or a combination of tests, which probably makes life a little easier when you are allowed to combine different methods that can distinguish between the different forms. We acknowledge that it is very difficult to raise antibodies that really discriminate between the different strategies.

Fiebig: I believe the guideline is not precise in this point. Therefore we developed positive and negative discriminatory tests. This is a way to go ahead.

Vieths: The question is did you also try to use your monoclonal assays for the folding variant of *Bet v 1* to test birch allergoids?

Fiebig: No.

Ferreira: Helmut, just a very short technical question. How do you quantify allergoids?

Fiebig: The quantification of allergoids is routinely done in the company with the so-called PNU method, that is, the estimation of the protein nitrogen.

