

The Importance of Recombinant Allergens for Diagnosis and Therapy of IgE-Mediated Allergies

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IgE · cDNA cloning · Recombinant allergens · Immunotherapy specific

Abstract

In the past 10 years, a considerable number of cDNAs coding for allergens have been isolated and expressed. Intensive investigations showed that recombinant allergens and their respective natural counterparts possess comparable properties with respect to structure, function and interaction with the immune system. Recent studies documented that in vitro as well as in vivo diagnosis of IgE-mediated allergic diseases can be successfully improved by the application of recombinant allergens. In addition, new strategies for a safer specific immunotherapy (SIT) have been developed based on the knowledge of the primary structures of allergens. Naturally occurring isoforms of allergens as well as recombinant allergens with modified amino acid sequences show very low IgE binding capacity but strong T cell-stimulatory activity and represent possible candidates. In case of Bet v 1, the major birch pollen allergen, isoforms d, g and l and a Bet v 1a mutant, produced by site-directed mutagenesis resulting in 6 amino acid exchanges, fulfilled the above mentioned criteria. In a third approach, two adjacent peptides covering the en-

tire Bet v 1a sequence were produced in an *Escherichia coli* expression system. These peptides contained most of the relevant T cell epitopes, but lost their IgE binding capacity and, thus, their ability to activate mast cells and basophils of sensitized patients. Our results suggest that allergen variants (isoforms, mutants, T cell epitope-containing peptides) may be used as 'hypoallergenic agents' in SIT.

Introduction

Carl Prausnitz was an extraordinary character and scientist. During his university career he was a very active and well-known researcher in the field of infectious diseases [1]. Nevertheless, his most important findings for allergologists was the pioneering experiment in which he showed that immediate hypersensitivity could be transferred to his own skin by injecting himself serum from his fish-allergic colleague Heinz Küstner and subsequently boiled fish extract [2]. Sadly, Prausnitz had to leave Germany because of the Nazifascism, but it is said that his following work as devoted country physician on the Isle of Wight gave him the greatest satisfaction during his whole professional career.

Table 1. Tests comparing RA and natural allergens

IgE-binding assays
Inhibition of IgE binding to natural allergens with RA
Histamine release assays
Skin tests and other provocation tests
T cell proliferation assays

Important milestones in the characterization of allergens were established in the last 15 years. Immunoblotting to detect specific IgE and the usage of monoclonal antibodies led to the identification and isolation of numerous allergens. But extensive knowledge of the molecular characteristics of allergens could only be obtained by the application of recombinant DNA techniques [3–7]. When we started our work on recombinant allergens (RA) in 1984, our goals were to introduce cDNA cloning to discover the complete deduced amino acid sequences of allergens, to define B as well as T cell epitopes and to develop experimental in vitro and in vivo models of enhanced IgE synthesis. After long discussions in our research group and also based on results published by Ipsen and Loewenstein [8], we decided to focus on the molecular characterization of birch pollen allergens, since birch pollen contains only one major allergen, Bet v 1.

From fall 1984 to spring 1988 we tried to clone Bet v 1 cDNA without success. At that time, no protocols, no abstracts and no papers were published in this field and we worked for nearly 3.5 years without even knowing our competitors. In summer 1988, we finally succeeded in cloning the gene of Bet v 1 and thus obtained the complete deduced amino acid sequence [9]. It was the first pollen allergen ever cloned. At that time, our Australian colleagues, Thomas and collaborators [10] had already cloned the cDNA of a major mite allergen, Der p 1, and published the deduced amino acid sequence.

According to sequence comparisons, Bet v 1 and the other major pollen allergens from hazel, alder and hornbeam belong to a family of so-called pathogenesis-related proteins [9]. This pathogenesis-related proteins family comprises a group of pollen allergens, but also a number of proteins from somatic tissue of various plants. The biochemical function of these proteins is still under discussion but RNase-like activity was reported [11, 12] and certain sequence features suggest that these proteins behave as nucleotide-binding proteins [13]. In addition to Bet v 1, another birch pollen allergen with Mr 14 kD was cloned and sequenced in our laboratories [14]. Bet v 2 belongs to the family of profilins which are intracellular, actin-associated

molecules involved in cell division and cell movement. Extensive studies showed that profilins could not only be found in pollens but also in vegetable food [15–17] and was, therefore, called a plant pan allergen. About 20% of pollen-sensitive patients react with profilins from pollens of trees, grasses, weeds and vegetable food. The basis of their cross-reactivity is the high similarity of sequences in the plant profilin family. Interestingly, IgE cross-reactivity with human profilin was also shown and gave evidence for IgE autoreactivity in cases of severe atopic diseases [14].

Recombinant DNA technology offers the possibility of producing well-defined allergens. There are three main areas of interest.

Structure/Function Relationship

By the use of RA the relationship of the molecular structure of allergens with a putative function can be investigated in order to understand why some molecules are produced by plants, mites, pets or moulds, and may then become an allergen. Successful cloning and sequencing of allergen-encoding cDNAs led to the deduced amino acid sequences and allowed to compare these sequences with those of already described proteins with known functions. Similarities and identities with such proteins introduced new ideas as to why a biochemical or biological function predisposed a protein to become an allergen. For example, the deduced and later shown proteolytic activity of major mite allergens may be crucial for the penetration through mucous membranes, which may then be responsible for enhanced sensitization [18].

Clinical Application of RA in Diagnostic Test Systems

A great advantage of RA is that they can be produced in unlimited quantity at high quality and at reasonable costs. If the expression system and the purification steps are established, there are no differences in quality as usually observed with natural extracts, which show a high batch to batch variation. But RA will improve the diagnostic test repertoire only in case these molecules behave in the same manner as their natural counterparts, at least in immunological terms. This has to be tested by several assays (table 1). At present, there is no doubt that only a few RA are sufficient for diagnosis of pollen-, mite-, mould-, pet- and insect venom-induced Type I allergies. However, there may be also some disadvantages connected with the use of RA in

diagnostic tests. It has been reported that some RA show less IgE binding than the corresponding natural allergens caused by the existence of many isoforms of allergens. Isoforms differ in few amino acids, obviously leading to variations in secondary and tertiary structures of the allergenic molecules with less or increased IgE binding capacity. In diagnostic tests it is, therefore necessary to use only RA which possess the same immunological characteristics as their natural counterparts.

Using RA, allergy diagnosis can be extended to measure IgE levels against individual components which allows to define the patients' personal allergogram (table 2). With RA it is possible to find out how many patients react with a specific allergen. After a detailed diagnosis a patient would not be defined as grass pollen- or mite-allergic but being sensitized to group 1 or group 5 grass pollen allergens or to Der p 1, one of the major mite allergens. In addition, by the use of one single allergen it is possible to measure the quantity of specific antibodies and to get knowledge of the allergenic potential of a particular allergen. Furthermore, antibody reactivities against well-defined RA can be followed up during the course of disease and treatment. This may add information on the mechanism of sensitization in children. Contact with allergens or avoidance of allergens may be established on the basis of increased or decreased antibody levels to single allergens. Having established the patient's allergogram, sensitization to new allergens or loss of reactivity to distinct allergens can be monitored. Finally, using RA for IgE inhibition studies, the presence of immunologically very similar molecules in the different sources of allergens could undoubtedly be shown. Especially Bet v 1 and Bet v 2 homologues and a cluster of not yet completely identified allergens of approximately Mr 60 kD were found in various pollens and vegetable food [19–23]. Since there is a constantly increasing number of pollen-allergic patients, clinically significant cross-reactivity due to similar molecules in vegetable food is more frequently observed and in vitro tests with a few RA should be sufficient to diagnose most of the hypersensitivity reactions to the relevant food.

Concepts for Improved Allergen-Specific Immunotherapy Using Recombinant Allergens

The use of natural allergens for specific immunotherapy (SIT) has always been hampered by their allergenic activity, which is a desired property for a sensitive diagnosis but occasionally leads to clinical side effects during treatment. Recent investigations focused on the generation of an aller-

Table 2. Clinical application of RA in diagnostic tests

Patients' allergogram regarding component-specific IgE
Monitoring of antibodies against distinct allergens in childhood
Follow-up studies of antibodies during disease and treatment
Characterization of allergenic cross-reactivities

Table 3. Immunological properties of Bet v 1 variants

	T cell reactivity relative %	Skin prick test wheal areas (mm ²)	IgE binding relative %
nBet v 1	100	n.d.	100
rBet v1a	41	70	100
rBet v1e	67	10	83
rBet v1b	92	19	60
rBet v1d	100	7	5
rBet v1l	87	10	3
1–6 mutant	41	4	7

n.d. = Not done.

gen vaccine with reduced anaphylactic activity. Such a vaccine may consist of recombinant 'hypoallergenic' isoforms or mutants, or parts of allergens produced by site-directed mutagenesis.

Allergen Variants

We have previously shown that Bet v 1 isolated from pollen consists of a mixture of isoforms [24]. The existence of many isoallergenic forms of Bet v 1 in nature might have important clinical implications. It is possible that distinct isoforms are associated with differential T and B cell recognition and consequently different allergenic potential. Such a situation would definitely have to be taken into account for the development of new reagents for SIT of IgE-mediated allergies.

Taking these aspects into consideration, we carried out a study addressing the subject of isoallergens and their interactions with the immune system of allergic patients [25]. We dissected the T cell activation potency (antigenicity) and the IgE-binding properties of nine isoforms of Bet v 1 (table 3). Immunoblot experiments showed that Bet v 1 isoforms differ in their ability to bind IgE from birch pollen-allergic patients. Based on these experiments, we grouped Bet v 1 isoforms in three classes; molecules with high IgE-binding activity (isoforms a, e and j), intermediate IgE-binding (isoforms b, c and f), and low/no IgE-binding molecules

Table 4. Bet v 1a and 1d mutants

Bet v 1a mutants	
A1	Phe30 → Val
A2	Ser57 → Asn
A3	Ser112 → Cys
A4	Ile113 → Val
A5	Asp125 → Asn
A6	Thr10 → Pro
A1–6	Phe30 → Val; Ser57 → Asn; Ser112 → Cys; Ile113 → Val; Asp125 → Asn; Thr10 → Pro
Bet v 1d mutants	
D1	Val30 → Phe
D2	Asn57 → Ser
D3	Cys112 → Ser
D4	Val113 → Ile

(isoforms d, g and l). The potency of each isoallergen to activate T lymphocytes from birch pollen-allergic patients was assayed using peripheral blood mononuclear cells, allergen-specific T cell lines, and peptide-mapped allergen-specific T cell clones. The highest scores for T cell proliferative responses were observed with isoform d (low IgE binder) followed by isoforms b, l, e and a. In vivo (skin prick) tests showed that the potency of isoforms d and l to induce typical immediate Type I reactions in Bet v 1-allergic individuals was significantly lower than that of isoforms a, b and e. Taken together, our results indicated that hypoallergenic Bet v 1 isoforms are potent activators of allergen-specific T lymphocytes, and Bet v 1 isoforms with high in vitro IgE-binding activity and high in vivo allergenicity can display low T cell antigenicity [25].

Our findings showing that Bet v 1 and other closely related tree pollen allergens consist of a mixture of closely related isoforms displaying striking differences in their ability to bind IgE and activate allergen-specific T cell clones [25–27] led us to speculate that these differences resulted from only a few amino acid sequence differences. To analyze the patterns of amino acid exchanges in all tree pollen isoallergens and their IgE-binding activities, we used an algorithm recently developed by Casari et al. [28] to predict functional residues in proteins. The mathematical basis of this ‘sequence space’ method is a translation of sequences in vectors. Each sequence is represented as a vector point in a multidimensional space (sequence space), with residue position and residue type as the basic dimensions. At each position in the sequence a summary is given of which amino acid type occurs at what frequency in the protein family, in our case the group 1 of tree pollen allergens. The

differences between isoforms can be picked by this algorithm by defining a ‘functional direction’ in the sequence space. In this case functional axes that separate isoforms according to their allergenicity were defined manually by assigning +1 for high IgE-binding and –1 for low/no IgE-binding activity. For the multiple sequence alignment we used a total of fourteen closely related sequences of tree pollen isoallergens: Aln g 1 [29], four isoforms of Cor a 1 [26] and nine isoforms of Bet v 1 [24]. From the combined information of the IgE reactivity patterns of 9 tree pollen-allergic patients to the fourteen isoallergens, we obtained the following list of nine most common residues/amino acid positions in Bet v 1 (given in a decreasing order of ‘predictive power’) likely to influence IgE binding: positions 113, 57, 125, 112, 30, 10, 18, 133 and 82. We chose to substitute by in vitro site-directed mutagenesis the amino acid residues occurring in positions 113, 57, 125, 112, 30 and 10 of a high IgE-binding isoform (Bet v 1a) by those present in the same positions of low IgE-binding isoforms. Similarly, the amino acid residues found in these positions of a low IgE-binding isoform (Bet v 1d) were substituted by amino acid residues present in the corresponding positions of high IgE-binding isoforms.

The substituting amino acid residues were chosen using the following two criteria: (1) the residues should be present in the corresponding positions of naturally occurring isoforms, and (2) the residues should have a high correlation with either high IgE-binding or with low/no IgE-binding activity. By using this approach, which mimics ‘in vivo site-directed mutagenesis’, we expected that disturbances of the protein fold and structural characteristics caused by residue substitutions would be kept to a minimum. This in turn is essential to properly evaluate the contribution of each amino acid residue in IgE recognition. Altogether, eleven mutants of tree pollen isoallergens were generated and tested for their IgE-binding properties [38]. All isoallergen mutants presented here and their corresponding amino acid substitutions are listed in table 4.

Each of the single point mutations introduced on the Bet v 1a isoforms efficiently decreased IgE-binding for some, but not for all patients tested. Similar results were obtained with the Bet v 1d mutants. Each of the single amino acid substitutions could effectively restore IgE-binding activity for some, but not for all patients tested. These results indicate that although each of the single amino acid substitutions introduced by site-directed mutagenesis have dramatic effects on the IgE-binding properties of tree pollen isoallergens, the pattern of recognition of each modified isoallergen among allergic individuals is not uniform. Based on these observations, we conclude that the Bet v 1

allergen contains multiple conformation-dependent epitopes that are differently recognized by patients' IgE [30].

The A1–6 mutant combines the six amino acid exchanges that were individually tested for Bet v 1a and shown to influence IgE binding. In immunoblot experiments, all patients tested displayed extremely low IgE binding activity to A1–6 mutant. Inhibition assays showed that the A1–6 mutant required 50- to 100-fold more protein than wild-type Bet v 1a to reach 90–100% inhibition of IgE-binding to immobilized Bet v 1a. In vivo (skin prick) tests indicated that the potency of the A1–6 mutant to induce typical urticarial skin reactions (wheal and flare) in allergic individuals was dramatically reduced (100- to 1,000-fold) compared to Bet v 1a. Taken together, these results demonstrate that the amino acid positions 10, 30, 57, 112, 113 and 125 are all important for the in vitro and in vivo IgE-binding activity of the Bet v 1 allergen [30].

Bet v 1-specific T cell clones (TCC) established from the peripheral blood of birch pollen-allergic patients [25, 31] were tested for reactivity with multiple-point A1–6 mutant. All TCC reacting with Bet v 1a were also activated by the A1–6 mutant [30]. Thus, the amino acid exchanges in the multiple-point mutant had no influence on T cell recognition. Even in the case where three amino acid residues were exchanged in one T cell epitope (epitope 110–128, containing the substitutions A3, A4, and A5), no influence on the recognition of this peptide by the respective TCC was observed [30]. From these experiments, we conclude that it is possible to modulate the IgE-binding properties of allergens without significantly changing their recognition by allergen-specific T lymphocytes.

Hypoallergenic Fragments of Allergens

In a further approach we tested the concept whether 'disruption' of the conformation of Bet v 1a might abolish its IgE-binding capacity but simultaneously maintain relevant T cell epitopes [32]. Bet v 1, the major birch pollen allergen, was selected, because its IgE-binding epitope(s) are predominantly of conformational nature. In addition, Bet v 1 belongs to the most common allergens recognized by serum IgE from >95% of tree pollen-allergic patients and almost 60% of these patients react exclusively with this allergen. Two fragments, coding for the first and second half [amino acid (aa) 1–74 and aa 75–160] of Bet v 1a, were generated by PCR amplification, using cDNA of Bet v 1 [9] as template. The cDNA fragments were cloned into expression plasmid pET 17b (Novagen, Madison, Wisc., USA), expressed in *Escherichia coli* BL 21 (DE 3; Novagen) and purified to homogeneity. Both recombinant Bet v 1 fragments had almost no IgE-binding capacity and a significantly re-

duced anaphylactic activity as evaluated by histamine release experiments and skin prick testing in birch pollen-allergic patients [32]. Bet v 1-specific TCC proliferated at least with one of the recombinant Bet v 1 fragments, indicating that together both Bet v 1 fragments contained the T cell epitopes represented by complete recombinant Bet v 1a. Circular dichroism analysis of the recombinant Bet v 1 fragments and Bet v 1a indicated that the lack of IgE-binding capacity of the fragments was due to a loss of their native structure [32]. Nevertheless, immunization of mice and rabbits with recombinant Bet v 1a fragments induced high levels of IgG antibodies, which recognized recombinant Bet v 1a and strongly blocked human IgE-binding to natural Bet v 1a. We expect that – apart from their potential to modulate Bet v 1-specific T cell responses – recombinant Bet v 1a fragments will induce 'blocking antibodies' that can be assumed to protect against birch pollen allergy.

Based on the findings presented here, we propose a novel approach to SIT of Type I allergy: a treatment with high doses of recombinant molecules with no or very low IgE-binding activity, but containing all T cell-reactive domains. Molecules with low IgE-binding activity may occur naturally, as shown for certain isoforms of the major allergen Bet v 1. Alternatively, suitable molecules may be produced by means of recombinant DNA technology as demonstrated for the Bet v 1 allergen. This strategy for hyposensitization combines the advantage of low risk of IgE-mediated side effects with the possibility of downregulation of a broad repertoire of T cell specificities. It has been shown that high antigen doses lead to a modulation of the quality of the T lymphocyte response to the allergen [33–36]. Therefore, it can be presumed that this T cell-targeted therapeutic strategy will function via a reduction of the secretion of IL-4 by lymphocytes of the Th2 type and lead to an improvement of the clinical efficacy of SIT.

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