

Review article

Basic and practical aspects of recombinant allergens

Scheiner O, Kraft D. Basic and practical aspects of recombinant allergens. *Allergy* 1995; 50: 384–391. © Munksgaard 1995.

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Accepted for publication 4 August 1994

Allergenic extracts isolated from natural sources are complex mixtures of numerous proteins and other molecules. Their composition differs greatly in allergenic as well as nonallergenic material. Allergenicity depends on the material used, which varies according to environmental conditions in the case of pollens, according to phase of maturation in the case of molds, and according to the species of mites, types of pets, etc. Some extracts, too, may contain major allergens in insufficient concentrations, may be contaminated with unwanted components to which the patient is not allergic, or both. Furthermore, important allergens may be lost during extraction because of the high activity of proteases copurified with the allergens or the protease nature of some of the allergens. In addition, biochemical and immunochemical procedures of protein purification are relatively costly and time-consuming. For all these reasons, interest has been focused in recent years on recombinant DNA/RNA technology as an effective alternative.

During the last 6 years, an astonishing number of proteins with allergenic characteristics have been produced by recombinant methods (Table 1 and Fig. 1). In this way, not only can pure allergens be produced more easily, but these methods have also proved to be a powerful instrument in characterizing the molecular and biologic features of allergens. Whereas complete amino acid sequences of only a few allergens of low mol. mass of 5–15 kDa (*Lol p* II (1), *Lol p* III (2), and *Ole e* I (85)) have been obtained by protein sequencing, application of

complementary DNA (cDNA) cloning, followed by DNA sequencing, has yielded complete amino acid sequences for many more allergens, including those with a higher mol. mass (15–70 kDa). In particular, the method of polymerase chain reaction (PCR) led to a dramatically increased yield of recombinant allergens (RA), but not all of them showed the same immunologic properties as their natural counterparts (5, 27, 81, 83, 84). Exchanges of a few amino acids may considerably change the immunologic characteristics of allergens in their T- as well as B-cell epitopes. Therefore, molecular biology methods lead not only to full primary structures (amino acid sequences) of allergens, but also to information on their capacity to induce immune responses. Furthermore, computer-aided sequence comparisons could be used to unravel biochemical properties and biologic functions, a development which, in turn, may lead to better understanding of allergenicity. Because the list of cloned and sequenced allergens increases from month to month, new classifications of nomenclature, B- as well as T-cell epitopes, and functional properties have had to be established and should be constantly updated with new results (4).

Recombinant methods

The term "recombinant technique" may be understood as the molecular cloning of DNA that contains at least partial nucleotide sequence coding for a protein or protein fragments. The method of "cDNA-cloning" comprises the isolation of

Table 1. Complete cDNA-cloned and sequenced allergens

Allergens	Authors	Month/year of publication
Pollens		
<i>Bet v 1</i>	Breiteneder et al. (8)	07/89
<i>Lol p 1</i>	Perez et al. (57)	09/90
<i>Poa p IX</i>	Silvanovich et al. (66)	01/91
<i>Amb a I</i>	Rafnar et al. (59)	01/91
<i>Lol p I</i>	Griffith et al. (33)	02/91
<i>Lol p "lb"</i>	Singh et al. (67)	02/91
<i>Amb t V</i>	Gosh et al. (29)	05/91
<i>Bet v II</i>	Valenta et al. (80)	08/91
<i>Amb a II</i>	Rogers et al. (62)	10/91
<i>Car b 1</i>	Larsen et al. (44)	06/92
<i>Hor v 1</i>	Mena et al. (48)	10/92
<i>Aln 1</i>	Breiteneder et al. (6)	12/92
<i>Cor a 1</i>	Breiteneder et al. (5)	04/93
<i>Amb a V</i>	Gosh et al. (30)	06/93
<i>Amb p V</i>	Gosh et al.	09/93*
<i>Lol p II</i>	Sidoli et al. (65)	10/93
<i>Phi p V</i>	Vrtala et al. (86)	11/93
<i>Dac g II</i>	Roberts et al. (60)	11/93
<i>Phi p VI</i>	Peterson et al.	11/93*
<i>Hol 1</i>	Schramm et al.	11/93*
<i>Zea m XI</i>	Staiger et al. (73)	11/93
<i>Phi p II</i>	Dolecek et al. (19)	12/93
<i>Lol p V</i>	Ong et al. (56)	12/93
<i>Phi p XI</i>	Valenta et al. (94)	02/94
<i>Par j I</i>	Costa et al. (16)	03/94
<i>Cry j I</i>	Sone et al. (70)	03/94
<i>Bet v III</i>	Seiberler et al. (63)	08/94
<i>Ole e 1</i>	Lombardero et al. (45)	08/94
<i>Phi p I</i>	Laffer et al. (42)	10/94
Foods		
<i>Sin a 1</i>	Menendez-Anas et al. (49)	12/88
Arthropods		
<i>Der p 1</i>	Chua et al. (14)	01/88
<i>Dol m V</i>	Fang et al. (26)	02/88
<i>Der p V</i>	Tovey et al. (76)	10/89
<i>Der p II</i>	Chua et al. (12)	02/90
<i>Der f I</i>	Dilworth et al. (18)	01/91
<i>Der f II</i>	Trudinger et al. (77)	01/91
<i>Der f III</i>	Yuuki et al. (93)	05/91
<i>Eur m I</i>	Kent et al. (41)	11/92
<i>Api m I</i>	Dudler et al. (12)	12/92
<i>Myr p I</i>	Donovan et al. (20)	01/93
<i>Dol m I</i>	Soldatova et al. (68)	04/93
<i>Yes v V</i>	Lu et al. (46)	10/93
Vertebrates		
<i>Fel d 1</i>	Morgenstern et al. (50)	11/91
<i>Can f III</i>	Spitzauer et al. (72)	03/94
Protists		
<i>Can a I</i>	Shen et al. (64)	11/91
<i>Asp f Ia</i>	Moser et al. (51)	07/92
<i>Asp f I</i>	Arruda et al. (3)	11/92
<i>Cla h III</i>	Zhang et al.	01/94*

* From "Genebank database".

messenger RNA (mRNA) from the organism under investigation, and the transcription of the RNA into complementary DNA (cDNA) by the retroviral enzyme reverse transcriptase. After cloning in suitable vector systems, allergen-encoding clones can be

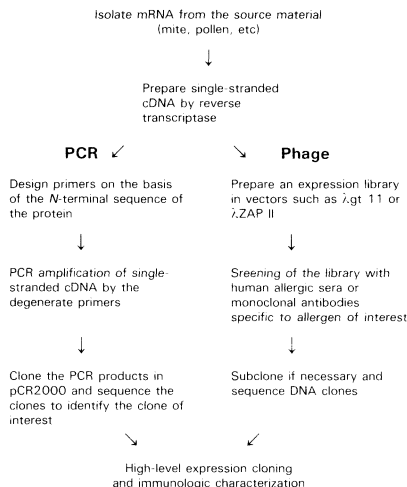


Fig. 1. Schematic representation of methods involved in molecular cloning and sequencing of allergens.

identified by hybridization, or by antibody screening of cDNA libraries with IgE-containing sera from allergic patients or monoclonal antibodies. The nucleotide sequences of the inserted cDNAs can be used to deduce the amino acid sequences of the cDNA-encoded proteins (Fig. 1). cDNA cloning methods have produced several deduced amino acid sequences of allergens, as listed in Table 1.

For the construction of cDNA libraries, the isolation of mRNA is of great importance. In the case of pollen allergens, pollens, but, surprisingly, not male inflorescences, roots, or leaves, are the tissues chosen for mRNA isolation, despite the fact that it is sometimes difficult to get an adequate yield and to avoid copurification of nonnucleic acid material(s) interfering with the cDNA synthesis (58). mRNA encoding allergens from mites, molds, and pets must be prepared from somatic tissues. The quality of the isolated RNA has to be controlled carefully. This can be done by Northern blot hybridization with DNA probes known to contain sequences encoding the protein required. If such a probe is not available, as frequently is the case, the mRNA can be subjected to *in vitro* translation (7, 74); the products can be detected by immunoblotting methods, using allergen-specific IgE antibodies from allergic patients or monoclonal antibodies (8, 12, 13, 39, 66, 67, 76). The immunoscreening method with IgE antibodies has been shown to yield good results and seems to

be a prerequisite when epitopes are believed to be of conformational nature. In the case of a single conformational epitope, *in vitro* translation methods can prove the presence of full-length, allergen-encoding mRNAs.

cDNA can be synthesized with commercially available kits. Before construction of cDNA libraries, the amount of DNA can be amplified by PCR (Fig. 1). However, this method requires knowledge of the partial sequences of the protein requested for construction of the oligonucleotide primers. PCR-amplified allergen-specific DNA can then be cloned and sequenced, and the identity of the cloned protein can be verified by comparison of the deduced amino acid sequence with the N-terminal amino acids of the protein in question. Alternatively, the cDNA can be cloned into an expression vector, and the IgE-binding properties of the recombinant allergen can be studied by comparing it with its natural counterpart. This method of cloning has been successfully applied to many allergens.

Biochemical and biologic properties of allergens

Successful cloning and sequencing of allergen-encoding cDNAs have enabled us to deduce the respective amino acid sequences and to compare these sequences with those of already described proteins with known functions. In some cases, significant sequence homologies with well-described proteins or protein families have been discovered. The attractive assumption that the allergens possess functions similar to those described for the homologous proteins has led to some new ideas as to why a biochemical or biologic property might predispose a protein to become an allergen. As suggested by Stewart et al. (75), the presumed proteolytic activity of the major mite allergens *Der p* I and *Der f* I may be crucial for the penetration of mucous membranes by these molecules, a penetration which then may lead to sensitization.

Amino acid sequence homologies of more than 80% were found among the major tree pollen allergen family, *Ber v* I, *Ahi g* I, *Cor a* I, and *Car b* I (5, 6, 37, 44, 78), and a distinct family of "pathogenesis-related proteins" encoded by "disease-resistance response genes" (8) from the bean (88), parsley (69), pea (28), and potato (47). Whereas in pollens these proteins are always expressed, the synthesis of the pathogenesis-related proteins in plants is induced by wounding, microbial or fungal infections, heat shock, and chemical or other stress signals (89). It may be of interest that molecules similar to *Ber v* I and to the other major tree pollen allergens are not uncommon and can be detected in fruits (apple (22), pear, kiwi), nuts (hazelnut and walnut (36)), vegetables (carrot,

celeriac), and spices (aniseed and caraway seed (Hirschwehr et al., in preparation)). Although proteins of the other pathogenesis-related protein families show clear functions (chitinases, glucanases, proteases, protease-inhibitors, etc.), at present, no biologic properties of the 17-kDa allergen homologues are known. However, provisional data (40) indicate that environmental pollution, especially car-exhaust fumes, might lead to enhanced expression of *Ber v* I in birch pollen and other tissues of birch. This molecule could then act as a stress protein, but, unfortunately, also induce enhanced IgE synthesis in genetically predisposed persons. Observations in Japan (38) that the incidence of pollen allergies is connected with car-exhaust fumes support this hypothesis (91).

A cross-reactive allergen with mol. mass of 14 kDa, was cloned from birch pollen (79, 80) and later from grass pollen (82). This 14-kDa allergen was identified as a cytoskeleton-associated protein, profilin. It is a highly conserved protein, obviously present in all eukaryotic cells, and, by interaction with actin, it is involved in cell structure and movement, and cell division. In man, high concentrations of profilin can be found in platelets, macrophages, and mast cells. Under normal conditions, no profilin can be detected in the circulation, but in the course of inflammatory processes, including those of allergic origin, relevant amounts of profilin may be released and may act as an antigen capable of driving the immune response against exogenous as well as endogenous profilins. Although the amino acid sequence homology between birch and human profilin, albeit significant, is not high, this molecule could be one of those which might initiate IgE autoreactivity (80).

Epitopes of allergens

Epitopes of antigens are classified as either B- or T-cell reactive epitopes. Each antigen seen by the immune system is recognized by its three-dimensional (3-D) structure by immunoglobulins (B-cell epitopes), or, after ingestion and processing by antigen-presenting cells (APC), as small peptide fragments bonded with major histocompatibility complex (MHC) class II molecules by T cells (T-cell epitopes). T-cell epitopes are not conformational, but sequential; as such, they do not usually react with allergen-specific IgE antibodies. A prerequisite for the identification of epitopes is knowledge of the primary structure (amino acid sequence) of the allergen in question. Overlapping peptides can then easily be produced by recombinant DNA methods, i.e., by expression cloning of overlapping cDNAs, produced by either sequential deletions or random fragmentation of the cDNAs, and, after that, by the

synthesis of the corresponding peptides in *Escherichia coli*, yeast, or mammalian cells. Alternatively, small or larger peptides may be chemically synthesized. Epitopes can then be detected *in vitro* by antibody assays (direct or inhibition ELISA, RAST, histamine-release assays), by T-cell proliferation, or by tests *in vivo* (skin tests, other provocation tests, or immunogenicity in animals).

T-cell epitopes

T helper (TH) cells have a crucial role in the control of the IgE synthesis (55, 90). The allergen-specific immune response starts with the uptake and processing of antigen by APCs and with the binding of peptide/MHC class II complexes on APCs by T-cell receptors (TCR) on allergen-specific TH cells. The TH cells become activated to proliferate and secrete lymphokines when costimulatory signals are provided by the APC. In contrast, T-cell nonresponsiveness can be secured if costimulatory signals are absent, or by suprainmunogenic doses of antigenic peptides. Because administration of peptides corresponding to T-cell epitopes (minimum 10–12 AA) led to T-cell tolerance to the complete antigens in mice (9, 15), and similar observations (i.e., T-cell nonresponsiveness) were made in the case of human T-cell clones (31, 34, 43, 54), it is currently believed that immunodominant T-cell epitopes of an allergen could also function as strong inducers of T-cell nonresponsiveness in man (87). Therefore, much attention has been focused on the delineation of functional regions of an allergen with respect to their capacity to stimulate T-cell proliferation *in vitro*. During the last 4 years, peptides containing T-cell-reactive epitopes have been determined for the major allergens of grass pollens (10, 57, 71), tree pollens (23–25), weed pollens (62), mites (35, 52, 92), cat (32, 53, 61), and bee-venom phospholipase A₂ (11, 17). Most typically, allergen-specific T-cell clones from allergic patients were produced with natural allergens and then used to map the epitopes with overlapping peptides covering the whole amino acid sequence of the allergen. Other approaches, however, have focused on the total potential T-cell repertoire in the circulating lymphocyte pool, and T-cell lines and peripheral blood mononuclear cells (PBMC) have been cultured with dodekamer- or eikosamer-overlapping synthetic peptides instead of T-cell clones (10). It turned out that most, if not all, allergens contain multiple T-cell epitopes (11, 17, 23–25, 35, 52, 53, 61, 92), although some of them are more frequently recognized. Furthermore, recognition of these epitopes may be restricted to various MHC class II molecules, making the situation even more complicated. These findings imply that, for each patient, all individual T-cell-dependent epitopes

of an allergen should be identified and included in peptide immunotherapy (IT). However, it is tempting to speculate that some so-called immunodominant epitopes may be sufficient to tolerize TH2 cells, activate TH1 or T suppressor (TS) cells, or both tolerize and activate. It is of interest that three studies, although conducted in two countries with different populations, located a single immunodominant T-cell epitope on *Lol p I*, a major allergen of the grass *Lolium perenne*, at AA 193–206 (10, 57, 71).

B-cell epitopes

Despite the rapidly increasing number of recombinant allergens (RA), far fewer IgE-reactive B-cell than T-cell epitopes have been established, although some B-cell epitopes on allergens have been discovered to react with monoclonal antibodies. The epitope-mapping by monoclonal antibodies may be valuable for the detection and quantification of allergens in allergen extracts and for the characterization of isoallergens (molecules with similar molecular size and function and >67% identity of amino acid sequences). Only in the case of monoclonal antibodies that recognize the same epitopes as IgE antibodies would their reactivity pattern be of interest to researchers. Knowledge of IgE-binding B-cell epitopes may be of great interest for several reasons: to increase the specificity and sensitivity of diagnostic tests by including molecules with such epitopes, to distinguish B-cell from T-cell epitopes, to understand the trigger mechanisms of IgE-sensitized mast cells and basophils, and, finally, to test the hypothesis that some diseases, such as intrinsic bronchial asthma, are caused by IgE-reactive autoantigens. As mentioned above, IgE antibodies characteristically recognize allergens by their 3-D structure (conformational epitopes). Some allergens might even constitute an epitope *in toto*; this seems to be true in the case of *Bet v I* (variant 1 of isoallergen *Bet v I*). Only cDNAs encoding the entire open reading frame could be isolated by screening expression libraries with patients' IgE. Furthermore, cutting the *Bet v I* encoding cDNA with *Bam*HI in the middle of the cDNA and subcloning the fragments in expression vectors did not lead to expression of IgE-binding peptides. This corresponds to data showing that cleavage of *Bet v I* with trypsin or thrombin destroys its IgE-binding capacity (unpublished results from our laboratory). Although, in theory, T-cell epitopes do not bind to IgE, recent data have shown that small peptides can bind to IgE and subsequently block (as monovalent haptens) or trigger basophils or platelets. In addition, T cells can produce histamine-releasing factors, thus priming basophils and mast cells for IgE-mediated reactions.

The opportunity to use RAs for epitope mapping has shown that the natural allergens and the RAs share IgE-binding B-cell as well as T-cell epitopes. Further investigation will be necessary to detect all relevant epitopes for the improvement of diagnostic as well as therapeutic procedures.

Use of recombinant allergens for diagnostic and therapeutic purposes

DNA technology offers the possibility of producing well-defined allergens in large quantities for use as diagnostic and therapeutic agents. At present, there is no doubt that only a few recombinant molecules are sufficient for diagnosis of pollen-, mite-, mold-, pet-, and Hymenoptera-induced Type I allergies. The accuracy of tests with RAs will improve significantly in the near future, because not only defined mixtures of allergens, but also major as well as important minor allergens are separately available for IgE-binding assays, skin tests, and basophil degranulation tests. The diagnosis of allergic conditions will certainly be much more reliable, because some natural allergens are rapidly denatured during their extraction and storage (e.g., apple) and are not available in the diagnostic test systems. Other natural allergens may exist only in very low concentrations in the extracts (e.g., cypress pollen allergens), and some allergens are destroyed because of the enzymatic activity of proteins in the allergen extracts. Furthermore, the use of single RAs, such as the profilins, may function as an effective marker of severe atopy or provide a useful tool to characterize a patient's sensitivity to single allergens (e.g., *Ber v 1* or *Ber v II* homologues in fruits, vegetables, nuts, and spices). Finally, the use of single RAs, as opposed to whole natural allergen extract, can give important information before IT is started, because it has been shown that patients allergic to several allergens are poor responders to IT (3b). However, there are some disadvantages connected with the use of RAs. It has been shown that some RAs show less IgE-binding capacity than those of the corresponding natural allergens. This is quite clear because of the existence of many isoforms of allergens with different IgE-binding capacity. Therefore, it is necessary to use only RAs which have been shown to possess the same immunologic characteristics as their natural counterparts. Instead of cloning many more isoallergens, only those detected by immunoscreening and established in their allergenicity by histamine-release assays and T-cell proliferation tests should be used for diagnostic purposes.

Although IT with RAs cannot be envisaged at present, major allergens produced by cDNA technology may be added to commonly used natural extracts to certify standardized quality. In addition,

RAs allow the application of tailor-made allergen preparations in IT. This concept seems to be of particular interest, because, at present, patients receive during IT large numbers of proteins (including allergens) to which they are not allergic. In this way, they might even become sensitized to new allergens. Moreover, RAs could be polymerized more easily in order to reduce the IgE-binding capacity but still retain the T-cell receptor activity. These modifications were impractical in the past because several allergens and impurities were present in varying concentrations in each extract, making it difficult to polymerize all molecules to a similar degree. Furthermore, although oral IT has been used for decades, evidence of the efficacy of this therapy could not be established. By means of pure RAs as whole or partial molecules, reevaluation of this type of therapy seems to be possible. RAs may be useful here because very large doses can be easily produced, and specific delivery systems may help to target the allergen on the gut-associated lymphoid tissue thought to be important in the generation of orally induced tolerance.

In addition, there is the opportunity to construct live vaccines: cDNAs encoding for allergens can be inserted into nonpathogenic microorganisms and orally applied. Allergens produced by these microorganisms could be released at immunocompetent sites in the gut and influence the immune response by selecting TH1-like responses in man. However, the risk of IgE-mediated anaphylactic reactions has to be considered if B-cell epitopes are released and recognized by mast-cell-bound IgE antibodies. Once again, cDNA technology using cDNAs encoding only T-cell epitopes could overcome these difficulties.

Lastly, IT with T-cell-reactive peptides derived from allergens seems to provide another form of treatment to induce peripheral T-cell nonresponsiveness. However, it must be taken into account that a specific T-cell epitope vaccine might provide help for TH2 cells, possibly by inducing the costimulatory signals of APC, and actually boost IgE production. Additionally, an excessive DTH (delayed-type hypersensitivity) reaction could be started by activation of TH1 cells. Clinical trials will have to prove the usefulness of this type of treatment, especially whether a limited number of allergen-specific T-cell-reactive epitopes is sufficient to turn off T-cell responsiveness to the complete allergen, and whether this loss of T-cell responsiveness would lead to a significant reduction of synthesis of allergen-specific IgE antibodies. If this proves to be the case, a new era of IT may begin.

Acknowledgment

This paper was supported by grants from the Austrian Science Foundation (FWF) projects S06703-MED, S06704-MED, and S06707-MED.

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