

Allergy Review Series II

Recombinant allergens

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A great variety of recombinant plant, mite, mold, mammal, and insect allergens have been expressed in heterologous hosts (e.g., *Escherichia coli*), their cDNA being used as a template. The number of biologically active recombinant allergens available for experimental, diagnostic, and therapeutic purposes is increasing tremendously. Recombinant allergens have proven to be valuable tools to investigate T-cell and B-cell recognition of allergens as well as to study mechanisms of specific IgE regulation. The immunologic equivalence of many relevant recombinant allergens with their natural counterparts has been demonstrated, and the three-dimensional structures of several recombinant allergens have been described recently. As a result of extensive cross-reactivities among the relevant allergens, it appears that the number of epitopes needed for diagnosis and specific immunotherapy is less diverse than originally anticipated and might be soon covered by recombinant molecules. Recombinant allergens have been used for successful *in vitro*, as well as *in vivo*, allergy diagnosis, and work is in progress to produce recombinant allergen derivatives with reduced anaphylactic potential to improve current forms of immunotherapy.

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This review discusses progress made in the field of allergen characterization using recombinant DNA technologies. A large panel of biologically active recombinant allergens has been produced during the last few years (1, 2). Initial efforts aimed to reveal the nature of relevant allergens by analysis of their DNA sequence and to produce recombinant molecules with properties similar to their natural counterparts. The advantages and disadvantages of the strategies employed are discussed and some examples of the usefulness of recombinant allergens for basic allergy research are given. Unexpectedly, it has turned out that, due to the extensive immunologic and structural similarities of relevant allergens, the number of recombinant allergens needed for diagnosis and specific therapy is less diverse (3). From the rapid progress made in the field of molecular allergen characterization, it appears likely that the use of recombinant allergens will considerably improve the diagnosis (4) and specific therapy (5) of type I allergies in the near future.

Strategies for obtaining recombinant allergens

Several strategies have been employed to obtain cDNAs coding for allergens. Review of the various cloning attempts shows that IgE immunoscreening of expression cDNA libraries prepared from the allergen source yielded recombinant allergens which most closely mimicked their natural counterparts in terms of antibody-binding capacity and ability to stimulate specific T cells as well as to elicit allergic effector mechanisms (6, 7). DNA-based techniques, such as polymerase chain reaction amplification, frequently lead to the isolation of cDNAs coding for allergen isoforms with reduced or eliminated IgE-binding capacity and diminished biologic activity (8-10). While the first approach, i.e., IgE immunoscreening, delivered recombinant allergens that are extremely useful for antibody-based *in vitro*, as well as *in vivo*, diagnosis, the DNA-based approach allowed the identification of allergen variants with greatly reduced IgE-binding capacity and anaphylactic activity. These

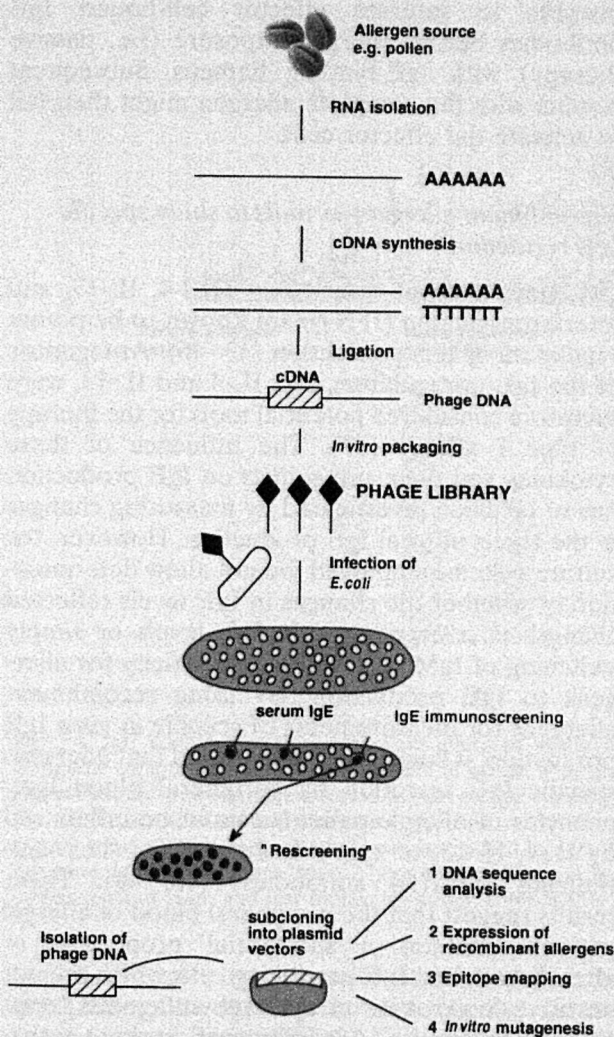


Fig. 1. Isolation of recombinant allergens by IgE immunoscreening.

variants might be considered candidates for specific immunotherapy.

For the isolation of allergen-encoding cDNAs with antibody probes, the construction of cDNA expression libraries is required as a crucial first step. Whether bacterial or eukaryotic expression systems can be utilized will depend on the involvement of post-translational allergen modifications (e.g., glycosylation) in IgE recognition. The question of whether post-translational modifications either represent targets for IgE antibodies or are involved in the stabilization of peptide epitopes needs to be addressed before construction of the library by *in vitro* translation of RNA from the allergen source (11, 12) and by experiments dedicated to their influence on IgE recognition (e.g., deglycosylation). Many relevant plant allergens could be expressed as correctly folded and biologically active recombinant allergens in bacterial

systems (reviewed in refs. 1, 2). Therefore, the construction of expression cDNA libraries in phage vectors and the technique of IgE immunoscreening are outlined in Fig. 1.

In the first step, messenger RNA is isolated from the allergen source and reversely transcribed into cDNA. The cDNA molecules obtained are thought to contain the information for all those proteins which were expressed in the allergen source during RNA isolation. It is therefore crucial to ensure that the desired allergen is actually expressed in the source chosen for RNA extraction. In the next step, cDNA is ligated into the phage DNA that is packaged *in vitro*. Expression of the inserted cDNA, mostly as β -galactosidase fusion protein, is achieved after infection of *Escherichia coli* cells. Recombinant phage are used to infect plated *E. coli* cells, and synthesis of recombinant proteins is induced by overlaying the bacterial lawn with isopropyl- β -thiogalactoside-soaked filter membranes. Phage clones expressing allergens can then be located via the filter replica with serum IgE antibodies from allergic patients. These phage clones are then purified by several rounds of "rescreening", and the allergen-encoding cDNAs can then be transported in other vector systems for further manipulation.

Recombinant allergens produced in high-level expression systems (e.g., T7 promoter-based systems [13]) are then purified, evaluated for their biologic activity, and compared with the natural allergen. The evaluation of recombinant allergens should ideally demonstrate

- 1) IgE-binding capacity (14, 15)
- 2) ability to stimulate specific T cells (16)
- 3) capacity to induce specific basophil degranulation (17)
- 4) induction of IgE responses in experimental animal systems (18–20)
- 5) induction of skin reactivity in man (21–26).

Recombinant allergens as research tools

Determination of B-cell and T-cell epitopes

The determination of B-cell and T-cell epitopes of allergens requires precise knowledge of the primary structure of the allergens. It is well established that T cells recognize oligopeptides which can be synthesized according to the deduced amino-acid sequence of the respective allergen. By the use of overlapping peptides prepared according to the deduced amino-acid sequences, T-cell recognition of major allergens could be easily studied in the recent past (16, 27–29). It could be shown that T cells of allergic patients recognize a great variety

of different T-cell epitopes on the same allergen. T cells from nonatopic subjects who mounted allergen-specific IgG responses seemed to recognize the same epitopes as T cells from atopic subjects (30, 31), indicating that it is not possible to distinguish "bad" T-cell epitopes which induce IgE synthesis from "good" T-cell epitopes which support IgG synthesis. T-cell epitopes are usually not recognized by IgE antibodies, a fact which has made it an attractive idea to use nonanaphylactic T-cell-epitope-containing allergen modifications, allergen fragments, or synthetic peptides for specific immunotherapy (32, 33). While there is ample evidence that T-cell epitopes can induce tolerance, anergy, or immunodeviation *in vitro*, as well as in experimental animal models (34–36), clinical studies are required to provide formal proof that T-cell-epitope-based immunotherapy can lead to improvement of clinical symptoms.

In contrast to the T-cell receptor, IgE antibodies bind to surface-exposed areas which are available to B cells during initial sensitization. The allergen recognition by antibodies requires mainly conformational epitopes assembled either by a stretch of continuous amino acids (i.e., continuous epitopes) or by different areas of the molecule brought together by proper folding (i.e., discontinuous epitopes). Because conformations are frequently not correctly assembled by synthetic peptides, more sophisticated methods of B-cell epitope mapping need to be employed. We found that the expression of allergen portions fused to β -galactosidase or a similar partner protein allowed more adequate epitope mapping than peptide technology (37). IgE-binding fragments for several allergens were indeed determined by random expression of cDNA fragments (37–39). Very recently, it has become possible to align the experimentally determined IgE epitopes to the three-dimensional structure of allergens. For birch profilin, continuous IgE epitopes were superimposed on the allergen structure, which had been determined by x-ray crystallography (40). The determination of the three-dimensional structure will be particularly valuable for the epitope mapping of allergens that mainly assemble discontinuous IgE epitopes; e.g., Bet v 1, the major birch pollen allergen (41, 42). Because only certain portions of a molecule are available on its surface, we may assume that IgE epitopes are less diverse than T-cell epitopes. It is therefore likely that therapeutic strategies can be developed which aim at interference in the IgE–allergen interaction. Among these strategies, active and passive concepts may be considered. The induction of blocking antibodies may be attempted with cocktails of nonanaphylactic allergen fragments (i.e., active therapy). It should also be

possible to saturate effector cell-bound IgE antibodies before allergen exposure (i.e., passive therapy) with IgE-binding haptens. Subsequent contact with the complete allergen might then fail to activate the effector cells.

Recombinant allergens as tools to study specific IgE regulation

Cytokines such as interleukin (IL)-4, IL-13, and interferon-gamma (IFN- γ) are known to be potent regulators of IgE production (43–46). Antagonists of the IgE upregulators, e.g., IL-4 and IL-13, were therefore considered potential tools for the therapy of type I allergy (47). The influence of these cytokines and their antagonists on IgE production has so far been investigated by measuring changes in the levels of total IgE production. However, the culture systems employed did not allow determination of whether the changes in IgE levels reflected changes in allergen-specific IgE levels or simply switching of IgM cells without specificity for allergens to IgE production. By using recombinant allergens for measurements of specific *in vitro* IgE production, it could be demonstrated that allergen-specific IgE secretion by peripheral blood lymphocytes of allergic patients cannot be influenced by IL-4, IL-13, or cytokine antagonists (i.e., neutralizing anti-IL-4 antibodies) (48, 49). These results suggest that the peripheral blood of allergic patients contains a substantial proportion of already switched IgE-producing cells which are not sensitive to cytokine or cytokine-antagonist treatment. If therapeutic application of cytokine antagonists is considered for therapy of type I allergy, it should be kept in mind that only IgM- to IgE-switching cells, but not IgE-committed B cells, can be influenced.

Recombinant allergens as tools to reveal cross-reactive structures in various allergen sources

It is known that, due to the presence of cross-reactive structures in various allergen sources (e.g., pollen and plant-derived food, hair dander from various mammals), allergic patients suffer from symptoms after contact with any of the sources. While it was possible to obtain circumstantial evidence of the presence of cross-reactive allergens in various sources by IgE-inhibition studies with crude allergen extracts, the cross-reactive structures could not be defined precisely. With recombinant allergens (e.g., the major birch pollen allergen Bet v 1, birch profilin, Bet v 2) for IgE-inhibition studies, the presence of immunologically identical molecules in the different sources could be unambiguously demonstrated (50–54). Figs. 2A

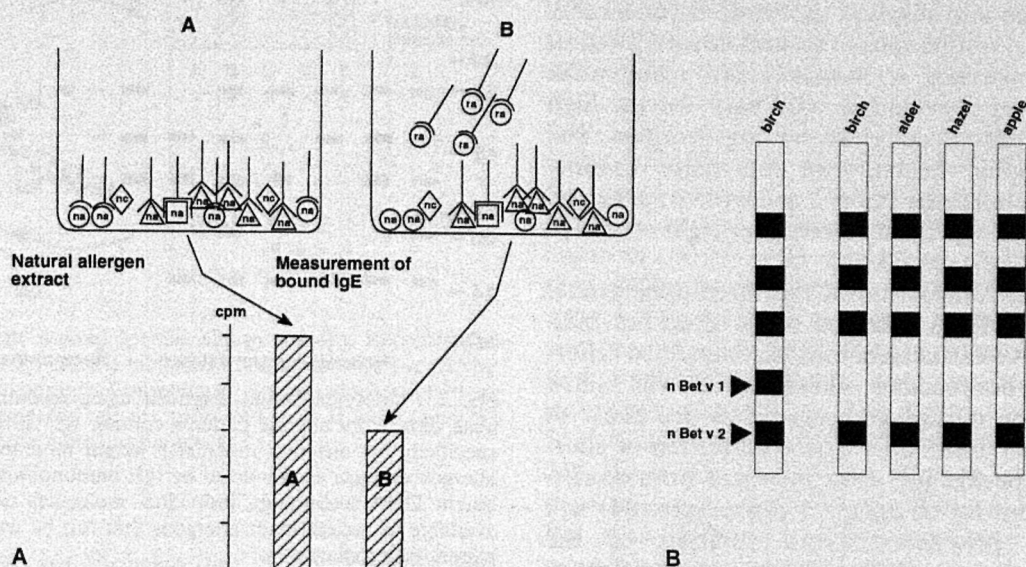


Fig. 2A. IgE inhibition with recombinant allergens. Natural allergen extracts contain different natural allergens (na) and non-allergenic components (nc). Preincubation of serum with certain recombinant allergen (ra) leads to reduction of IgE binding to natural extract that can be expressed as part of IgE binding.

Fig. 2B. Presence of natural allergens (e.g., nBet v 1, nBet v 2) in different allergen sources that share IgE epitopes with certain recombinant allergen (e.g., rBet v 1) can be studied by IgE-immunoblot inhibitions. Preincubation of serum with rBet v 1 inhibits IgE binding to Bet v 1 and Bet v 2 homologous allergens in alder and hazel pollen, as well as in apple extract. Binding to other components of high- and low-molecular-weight components is not affected.

and B illustrate the experimental setup for the estimation of the IgE levels directed against certain extract components (Fig. 2A), as well as for the identification of cross-reactive structures in other allergen extracts (Fig. 2B) with recombinant allergens. Sera are preadsorbed with a given recombinant allergen, and the reduction of IgE binding to the extract allows the estimation of IgE epitopes shared by the extract and the recombinant allergen (55, 56). Inhibition of IgE binding of the preadsorbed serum to certain components present in other extracts allows the identification of cross-reactive structures on the immunoblot (57, 58). The cross-reactive allergens which harbor most of the relevant epitopes may replace others for diagnostic and therapeutic purposes.

The presence of only a few cross-reactive IgE epitopes among allergens may provide interesting information on the originally sensitizing molecule. For example, if, in a birch-pollen- and apple-allergic patient, symptoms are due to cross-sensitization but pollen-derived Bet v 1 contains more IgE epitopes than apple-derived Mal d 1, it appears reasonable to assume that the patient was originally sensitized to pollen (54). The associated food intolerance would then be due to the presence of

pollen-specific IgE antibodies that cross-react with food allergens. Likewise, if Can f 1, the major dog allergen, more completely inhibits IgE binding to Fel d 1, the major cat allergen, it might be concluded that the patient was initially sensitized to dog dander (59).

Three-dimensional structures of allergens

The three-dimensional structure of the major ragweed allergen Amb t 5 has already been determined by nuclear magnetic resonance (NMR) spectroscopy using purified natural allergen (60). The allergen consisted of a C-terminal α helix and two to three β -strands. The ability to obtain large amounts of homogenous recombinant allergens has now led to the determination of the three-dimensional structures of several relevant allergens. The structure of the major birch pollen allergen, Bet v 1, determined by NMR and x-ray crystallography, presented a seven-strand, antiparallel β -sheet that wrapped around a C-terminal α helix (42). A partial structure of the major timothy grass pollen allergen Phl p 5 has been reported (61), and the three-dimensional structure of birch pollen and *Arabidopsis* profilin was determined by x-ray

crystallography (40, 62). The structure of birch profilin comprises three α helices, seven β -strands, and 10 turns and displays the same seven-strand, incomplete, antiparallel, up-and-down β -barrel structure observed in *Amoeba* and mammalian profilins, thus providing a solid basis for the high degree of cross-reactivities among profilins. The crystallographic structure of the major timothy grass pollen allergen Phl p 2 is devoid of α helical elements and represents a compact eight-strand β -barrel (63).

While the comparison of the three-dimensional allergen structures obtained so far does not indicate conserved structural motifs common to different non-cross-reactive allergens, it will allow identification of surface areas which are likely to interact with antibodies. Cocrystallization of allergens and specific IgE Fabs obtained from combinatorial libraries of patient lymphocytes (64) will allow the precise structural analysis of the allergen-IgE interactions. From these structural data, it may be possible to construct peptide mimetics which may interfere with the allergen-IgE interaction and hence be useful for active as well as passive therapy.

Recombinant allergens for *in vitro* and *in vivo* diagnosis

So far, *in vitro* diagnosis of type I allergy has been performed with crude allergen extracts prepared from the allergen sources. Crude allergen extracts contain in addition to the desired allergens a large variety of proteins, carbohydrates, and nucleic acids which are not allergenic. Certain allergens are either not well represented or degraded during the preparation of the extract. In addition, it is difficult to standardize crude allergen extracts for their allergen contents. Despite these difficulties, crude allergen extracts are certainly useful to determine the allergen source to which a patient is sensitized and to estimate the amount of IgE antibodies against the cocktail of allergens present in the extract. However, allergen extracts will allow neither identification of reactivities to the individual allergens nor measurement of the amount of IgE antibodies against certain allergens (4). Therefore, allergy diagnosis might start with the identification of the relevant allergen sources by extract-based diagnosis or with a cocktail of recombinant allergens that can replace the extract. For further accuracy, diagnosis should then be extended by the use of recombinant allergens to measure IgE levels directed against the individual components (Fig. 3). This will allow the establishment of the patient's individual IgE-reactivity profile (65). Our view that future allergy diagnosis can be improved by a

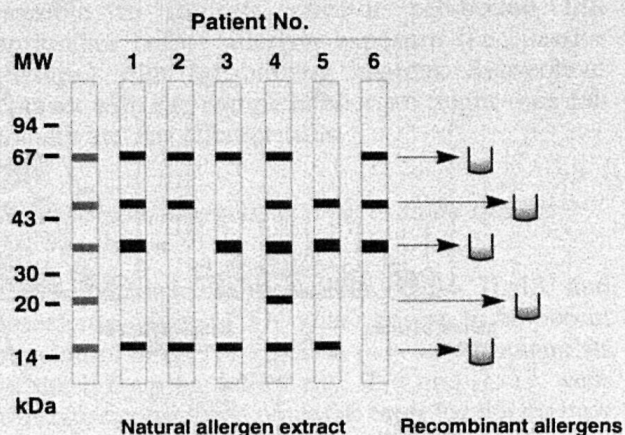


Fig. 3. Component-based diagnosis using recombinant allergens. Sera from allergic patients contain IgE antibodies with specificity for different molecular weight moieties in natural allergen extracts as visualized by IgE immunoblot. By recombinant DNA technology, individual molecules can be made available as recombinant allergens that can be used for component-based diagnosis.

component-based system using recombinant allergens is supported by a series of experimental data which show that recombinant allergens can be used for both *in vitro* and *in vivo* allergy diagnosis (14, 15, 22–26, 66, 67).

Recombinant allergens and their modifications for specific treatment of allergic diseases

The concept of patient-tailored specific therapy

Specific immunotherapy of type I allergy is based on the systemic application of increasing doses of the allergens to which the patient is sensitized (68, 69). Currently, immunotherapy is performed with crude allergen extracts which contain mixtures of allergens as well as nonallergenic materials. Although the basic mechanisms of specific immunotherapy are not yet completely understood, the basic intention is to reduce allergic reactions to those allergens which are recognized by the patients' IgE antibodies. Presentation of allergens that are not recognized and other components in an immunologically relevant fashion (e.g., specific immunotherapy) might even lead to *de novo* sensitization and worsening of the disease. Therefore, we strongly maintain that current forms of immunotherapy may be substantially improved by injection of only those allergens against which substantial amounts of IgE antibodies are produced (Fig. 4). The idea is that, according to the patient's IgE-reactivity profile established with recombinant allergens, those components are selected against which a substantial IgE response is mounted

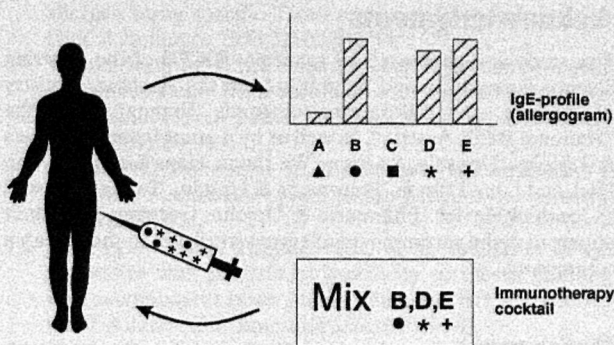


Fig. 4. Patient-tailored specific allergy therapy. Recombinant allergens (A–E) allow establishment of patient's IgE-reactivity profile (allergogram). Components against which substantial amounts of specific IgE are produced (e.g., B, D, E) may be used for specific immunotherapy.

(3). Support for the idea that immunotherapy can be performed with selected recombinant allergens that harbor most of the relevant epitopes comes from earlier immunotherapy studies showing, for example, that tree-pollen allergy was treated with equal effectiveness with a mixture of different tree pollens or with birch-pollen extract alone (70). While recombinant allergens that possess biologic activity comparable to their natural counterparts may be considered as such for immunotherapy, recombinant DNA technology offers various ways to produce derivatives with greatly reduced, or no, anaphylactic activity. Some of these therapy strategies, which are still experimental, are outlined below.

IgE-binding haptens from major allergens and allergen-specific antibody fragments: tools for local passive therapy

The cross-linking of effector cell (i.e., mast-cell and basophil)-bound IgE antibodies with specificity for at least two different IgE epitopes is required to trigger release of inflammatory mediators. Therefore, precise knowledge of IgE-binding sites is crucial to analyze the allergic effector response. This can be achieved by mapping immunodominant IgE epitopes, as was recently demonstrated for the major timothy grass pollen allergen, Phl p 1 (37). The epitope mapping is then supplemented by structural analysis so that antibody-binding sites can be superimposed on the three-dimensional allergen structure, as demonstrated for birch profilin (40). Nonanaphylactic IgE-binding haptens might be used for local saturation of effector cells to prevent subsequent activation by exposure to the complete allergen or for active immunotherapy to induce blocking IgG antibodies exactly against the IgE epitopes.

The occurrence of allergen-specific antibodies that do not belong to the "reagin" class was reported as early as 1935 (71). It was later demonstrated that certain allergen-specific IgG antibodies can inhibit anaphylactic reactions (72, 73). Moreover, it was suggested that blocking antibodies, particularly those belonging to the IgG4 subclass, might be induced by specific immunotherapy (74). However, several studies failed to correlate the presence and/or increase of allergen-specific IgG antibody levels with successful immunotherapy (75). While previous studies on the role of allergen-specific IgG antibody responses were hampered by the lack of well-defined allergen preparations, purified recombinant allergens have now become available for measurements with defined components. Studies performed with recombinant Bet v 1 (23, 24), the major birch pollen allergen, recombinant timothy grass-pollen allergens (Phl p 1, Phl p 2, and Phl p 5) (76), and recombinant Der f 2 (77, 78) established the following points:

- 1) sera from allergic and nonallergic subjects contained allergen-specific IgG antibodies belonging to different subclasses
- 2) levels of allergen-specific IgE are poorly correlated with IgG subclass responses
- 3) with epitopes prepared from major allergens by recombinant DNA technology, it could be shown that IgE and IgG antibodies of allergic patients bind to the same and also different epitopes (78).

This indicates that IgE and IgG responses in allergic patients may also evolve in a nonsequential way, and that IgE and IgG antibodies may possess different affinities or fine specificities for certain epitopes.

Thus, it appears that the allergen-specific IgE and IgG response in allergic patients is poorly synchronized for epitope recognition and perhaps affinity. These differences in epitope recognition of IgE and IgG antibodies could recently be demonstrated for monoclonal human IgG antibodies specific for the major birch pollen allergen Bet v 1 (79). However, the demonstration that certain Bet v 1-specific IgG antibodies can block IgE binding to Bet v 1 and inhibit the Bet v 1-induced histamine release in several donors, clearly indicated that blocking IgG antibodies effectively reduces anaphylactic effects. We suggest the use of recombinant allergen-specific Fabs for passive (blocking) therapy of type I allergy. Recombinant Fabs can be easily produced in bacterial expression systems and thus obviate tissue culture (80–82). Bacterially expressed Fabs can be modified by standard molecular biologic techniques and engineered according to the desired application. To reduce the immunogenicity of an antibody fragment which is intended

for passive therapy, one can reduce the size of the molecule, as well as to cut out immunogenic parts. Alternatively, mouse monoclonal antibodies can be humanized to reduce their immunogenicity. By *in vitro* maturation with mutagenesis or complementary determining regions rearrangements, it is possible to increase the affinity of a recombinant Fab for its antigen (83). Such a manipulation might even increase the blocking activity of allergen-specific Fabs.

Nonanaphylactic derivatives of allergens for specific active immunotherapy

Recent data suggest that a key mechanism of successful conventional immunotherapy is to alter T-cell function, either by diminishing production of both Th1 and Th2-like cytokines (84, 85), or by inducing "immune deviation" from a Th2-like cytokine pattern to a Th1-like pattern (86, 87). To increase the dose of allergens which can be applied to the patient with reduced anaphylactic side-effects, the use of nonanaphylactic allergen derivatives has been suggested. Isoforms of major allergens were found which showed no or very low IgE-binding capacity but possessed strong T-cell-stimulating sequences. Such hypoallergenic isoforms were described for major tree pollen allergens (10) and could be artificially produced by site-directed mutagenesis of major mite allergens (88, 89). In the case of allergens that harbor conformational epitopes, disruption of the three-dimensional structure of the allergens may result in nonanaphylactic derivatives that contain all relevant T-cell epitopes. Expression of two halves of the major birch pollen allergen Bet v 1 resulted in a T-cell epitope containing fragments which, due to disruption of the three-dimensional structure of the molecule, failed to display anaphylactic activity (41).

A similar approach makes use of allergen-derived peptides. It could be shown that human T-cells become nonresponsive when antigen presentation occurs in the absence of costimulatory signals (34). Furthermore, mice administered peptides that represented major T-cell epitopes of allergens became tolerant of the complete allergen (35, 36), and the first clinical studies are underway to determine whether peptide immunotherapy may represent a low-risk alternative to traditional immunotherapy (90).

It is known from vaccination in infectious disease trials that immunization with pDNA can induce cytotoxic T cells which display a preferential Th1-like phenotype. It is expected that cells which are transfected by the DNA vaccine will continue to produce small amounts of allergen that may modulate the immune response in a Th1 direction (91, 92).

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