

Recombinant Allergens—gateway to efficient Diagnosis and Therapy for Atopic Asthma and Rhinitis

Prabhanshu Tripathi, B.P.Singh, Naveen Arora

Institute of Genomics and Integrative Biology, Mall Road, Delhi-7, INDIA

Abstract

Current allergy diagnosis and therapy have problem with regard to quality of allergens. This can be overcome by availability of recombinant allergens. Over the past 15 years, many allergens from mites, pollens, fungi, animal danders, insects, and foods have been cloned, sequenced, and protein expressed using high level expression systems such as bacteria or yeast. Three-dimensional structure of allergens has been determined and B-cell/T-cell epitopes have been mapped for some proteins. Most of the recombinant allergens showed comparable IgE binding to their natural counterparts by in vivo and in vitro tests. Cocktails of recombinant allergens with predetermined and uniform allergen levels offer innovative, patient-based therapy of allergy replacing natural allergens. There is extensive progress in developing new forms of allergens such as hypoallergenic variants, synthetic peptides, DNA vaccines, T-cell epitope vaccines, allergens coupled to IgE suppressive adjuvants etc. to improve immunotherapy. Synthesis and characterization of recombinant allergens and their variants will bring valuable change in diagnosis and therapy of allergy.

Keywords: Recombinant allergen, Allergy diagnosis, Immunotherapy, T cell epitope vaccine, DNA vaccine

INTRODUCTION

Type 1 allergy affects more than 25% of the population worldwide¹. Allergen specific immunotherapy is the only treatment which changes the immunological profile² of individual from T_H2 to T_H1 . A large number of proteins from different sources are identified as allergens, purified and characterized. But the diagnosis and immunotherapy is practiced with commercially available allergen extracts from natural sources, which contain varying

amounts of individual allergens in different batches. The allergen sources usually contain multiple major and minor allergenic proteins and it is difficult to standardize mixture of proteins in each extract³. The quality and quantity of individual allergens varies in natural extract due to change in source material, extraction protocol and/or storage conditions⁴. Recent reports suggest that some allergen extracts contain toxins that may influence allergenic response in undefined manner and such extracts can not be used for immunotherapy⁵⁻⁷.

Previous studies demonstrated that allergens have diverse biologic functions. They may act as enzymes, enzyme inhibitors, lipocalins, structural proteins etc. The allergen's biologic function may be related or unrelated to their ability to cause or promote IgE

Address for correspondence: Dr. Naveen Arora, Room 509, Allergy and Immunology Section, Institute of Genomics and Integrative Biology, Delhi University Campus, Mall Road (Near Jubilee Hall), Delhi 110007, INDIA; Phones: 91-11-27666156, 27666157; Fax: 91-11-27667471; E-Mail: naveen@igib.res.in
IJAAl, 2006, XX (1) p 41-51.

antibody responses. But their mixing with other allergens affect efficacy in diagnosis and therapy. The application of recombinant DNA technology has facilitated enormously in analysis of allergenic proteins and production of purified allergenic proteins during the past decade. Many allergens were identified and their cDNA cloned and expressed in bacterial and eukaryotic systems (yeast). Complete amino acid sequences of only a few allergens of low molecular weight of 5-15 kDa have been obtained by protein sequencing⁸. Application of cDNA cloning followed by DNA sequencing has yielded complex amino acid sequences for many allergens including high molecular weight proteins (15-70 kDa).

The first allergen structure solved by X-ray crystallography and NMR was for Bet v 1 in the year 1996^{9, 10}. Later three dimensional structure of other recombinant allergens such as Der p1, Der p2, Bla g2, Equ c1, Bos d2 and Ara h1 was determined by NMR and X-ray crystallography. Recombinant DNA technology has provided sequence information about several allergens. At present more than 400 proteins or nucleotide allergen sequences are available in the Genbank and other databases¹¹.

Based on these sequences, molecular modeling, location of residues involved in IgE binding as well as epitopes involved in T-cell activation have been determined. Further B-cell and T-cell epitopes have been mapped to form allergenic mutants to develop hypoallergenic variants. Hypoallergenic variants have almost similar sequence with a little modification of some amino acid residues¹²⁻¹⁸.

The recombinant allergens show scope for improved allergy diagnosis and immunotherapy due to use of quality reagents. Besides, modified recombinant proteins are expected to change dose schedule, duration and safety of immunotherapy.

PURIFIED NATIVE ALLERGENS

A large number of native proteins from different sources like mites (e.g. Der p1, Der p 2), fungi (e.g. Alt a1, Alt a 13, Asp f1, Cur l1, Epi p1, Pen ch13), pollens (e.g. Bet v1, Phl p1, Phl p2, Phl p3), animal dander (e.g. Fel d1), etc are purified and characterized in recent years. Despite the knowledge available, the native proteins could not become popular for clinical

use due to certain disadvantages. The cost of protein purification is high and the yield is low with most of the methods. The purified samples from different chromatographic techniques show variation from one batch to another. The purified proteins did not show adequate biologic activity sometimes because of degradation or conformational changes due to harsh conditions used for purification. Partially purified allergens get degraded during long time storage may be due to inherent proteases¹⁹.

In our lab, clinically important allergenic proteins have been identified and isolated from tree pollens, grass pollens, fungi, insects and food materials²⁰⁻²⁶. Standardized protocols were used for purification of major allergens such as 26 kDa glutathione-S-transferase (Alt a 13) from *Alternaria alternata*, 12 kDa Cytochrome c, 47 kDa enolase and 31 kDa serine protease (Cur l 1) from *Curvularia lunata*, 33.5 kDa serine protease (Epi p 1) from *Epicoccum purpurascens* and 29 kDa serine protease from *Periplaneta americana*. Besides, 45 kDa and 65 kDa major allergens from *Fusarium solani*, 67 kDa from *Imperata cylindrica* and 66 kDa from *Prosopis juliflora* were identified and characterized²⁰⁻²⁵. Some of the proteins e.g. 26 kDa from *A. alternata* (Alt a 13), 12 kDa Cytochrome c and 47 kDa enolase from *C. lunata* have been synthesized as recombinant isoforms and compared with native counterpart for clinical use.

The details including basic characteristics like molecular weight and biological nature of proteins are available at the website <http://www.allergen.org/List.htm>. A brief data on most common allergens are given in table 1.

RECOMBINANT ALLERGENS

Recombinant allergens are genetically engineered isoforms representing molecules from different sources. For preparation of recombinant allergens, mRNA is isolated from the allergen source and converted to cDNA by Reverse transcriptase polymerase chain reaction (RT-PCR). The cDNA is cloned in suitable vector and allergen encoding clone is identified by screening with IgE containing patient's sera or monoclonal antibodies. Radio-labelled or fluorescent-labelled specific DNA probe designed by PCR using degenerate primers can also be used for

Table 1. List of important allergens derived from Allergen Nomenclature data
(International Union of Immunological Societies)

Allergen source	Allergen Name	Molecular weight(kDa)	C: cDNA P: Peptide sequence	References No. and/or Accession No.
A. weeds				
<i>Ambrosia</i>	Amb a 5	5	C	M84987
<i>artemisifolia</i>	Amb a 10	18	C	AY894659
<i>Artemisia vulgaris</i>	Art v 2	35	P	Q7M1G9
	Art v 4	14	C	AJ421030
	Art v 5	10	C	AY904434
B. Grasses				
<i>Cynodon dactylon</i>	Cyn d 1	32	C	S83343
	Cyn d 12	14	C	Y08390
<i>Lolium perenne</i>	Lol p 1	27	C	M57474.
	Lol p 2	11	C	X73363
<i>Phleum pratense</i>	Phl p 5	32	C	Z82986
	Phl p 6		C	Z27082
	Phl p 12		C	X77583
<i>Imperata cylindrica</i>		67		24
C. Trees				
<i>Betula verrucosa</i>	Bet v 1	17	C	Z72436
	Bet v 2	15	C	M65179
<i>Castanea sativa</i>	Cas s 1	22	P	AAB29092
<i>Olea europea</i>	Ole e 5	16	P	P80740
	Ole e 8	21	C	AF078679
<i>Juniperus ashei</i>	Jun a 2		C	AJ404653
	Jun a 3	30	P	P81295
<i>Juniperus sabinoides</i>	Jun s 1	50	P	27
<i>Prosopis juliflora</i>		66		Unpublished data
D. Mites				
<i>Blomia tropicalis</i>	Blo t 1	39	C	AF277840
	Blo t 3	24	C	AY090091
	Blo t 11	110	C	AF525465
<i>Dermatophagoides farinae</i>	Der f 1	25	C	DQ185509
	Der f 3	30	C	D63858
	Der f 11	98	C	AF352244
<i>Dermatophagoides pteronyssinus</i>	Der p 1	25	C	DQ185508
	Der p 2	14	C	DQ185510
	Der p 6	25	P	P49277
	Der p 10	36	C	Y14906

<i>Lepidoglyphus destructor</i>	Lep d 1	15	C	X83876
---------------------------------	---------	----	---	--------

E. Fungi

<i>Alternaria alternata</i>	Alt a 1	28	C	U82633
	Alt a 6	45	C	U82437
	Alt a 13	26	C	AY514673
<i>Cladosporium herbarum</i>	Cla h 6	46	C	X78226
	Cla h 9	55	C	AY787775
<i>Aspergillus fumigatus</i>	Asp f 1	18	C	M83781,S39330
	Asp f 3	19	C	U20722
	Asp f 6	26.5	C	U53561
	Asp f 8	11	C	AJ224333
	Asp f 13	34	C	Z11580
	Asp f 18	34		28
	Asp f 22w	46	C	AF284645
<i>Penicillium chrysogenum</i>	Pen ch 13	34	C	AF321100
	Pen ch 18	32	C	AF263454
<i>Penicillium citrinum</i>	Pen c 13	33		29
	Pen c 22w	46	C	AF254643
<i>Candida albicans</i>	Cand a 3	29	C	AY136739
<i>Epicoccum purpurascens</i>	Epi p 1	33.5	P	P83340
<i>Curvularia lunata</i>	Cur l 1	31	P	20
	Enolase	48	C	AY034826
	Cytochrome c	12	C	AY034827
<i>Fusarium solani</i>		45		22
<i>Fusarium equiseti</i>		65		23

F. Insects

<i>Apis mellifera</i>	Api m 1	16	C	NM_001011614
	Api m 2	44	C	NM_001011619.
<i>Blattella germanica</i>	Blag 2	36	C	U28863
	Blag 5	22	C	U92412
<i>Periplaneta americana</i>	Per a 1	47	C	U69957
	Per a 3	72-78	C	L40819
	Per a 7	37	C	Y14854
	Serine Protease	29		Unpublished data
<i>Lepisma saccharina</i>	Lep s 1	36	C	AJ309202
<i>Polistes dominulus</i>	Pol d 4	32-34	C	AY285998
<i>Vespula maculifrons</i>	Ves m 2	44	P	30
<i>Solenopsis saebvissima</i>	Sol s 2			

G. Foods

<i>Gadus callarias</i>	Gad c 1	12	C	P02622
<i>Bos domesticus</i>	Bos d 5	18.3	C	X14712
<i>Gallus domesticus</i>	Gal d 1	28	C	P01005
<i>Penaeus aztecus</i>	Pen a 1	36	C	DQ151457
<i>Brassica juncea</i>	Brä J 1	14	C	X65972
<i>Brassica napus</i>	Bra n 1	15	P	P80208
<i>Brassica rapa</i>	Bra r 1	10,14	C	CAA46782
<i>Triticum aestivum</i>	Tri a 18			
<i>Zea mays</i>	Zea m 14	9	P	P19656
<i>Daucus carota</i>	Dau c 1/1	16	C	Z84376
<i>Corylus avellana</i>	Cor a 2	14	C	AF327622
<i>Malus domestica</i>	Mal d 1		C	X83672, Z48969
<i>Arachis hypogaea</i>	Ara h 1	63.5	C	L34402
	Ara h 2	17	C	L77197
	Ara h 3	60	C	AF093541
<i>Ricinus communis</i>	Ric c 1		C	P01089
<i>Carica papaya</i>	Car p 1	23	C	M15203

H. Latex

<i>Hevea brasiliensis</i>	Hev b 1	58	P	P15252
	Hev b 6.01	20	C	M36986
	Hev b 6.02	5	C	M36986
	Hev b 9	51	C	AJ132580
	Hev b 12	9.3	C	AY057860

screening. The screened cDNA is sequenced and cloned in a suitable vector to express recombinant protein.

Recombinant allergens are produced mainly by expression of cDNA in prokaryotic systems (*Escherichia coli*) and tested for IgE and IgG binding³¹⁻³³ and also for specific activation of T-cell³⁴⁻³⁸ and histamine release from basophils³⁹⁻⁴¹.

The journey of recombinant allergens started in 1988 when first cDNA for a major allergen from *Dermatophagoides pteronyssinus* was sequenced. The sequence of events occurred in progress of recombinant allergens are presented in figure 1.

RECOMBINANT PURIFIED ALLERGENS FOR ALLERGY DIAGNOSIS

Allergen extracts from natural sources are heterogeneous mixtures containing proteins and other macromolecules which vary in allergen composition and contents. The extracts are contaminated sometimes with allergens from other sources and therefore give false positive reactions. The extracts contain certain proteolytic enzymes which can deteriorate the quality of product. Some of the natural allergens may contain denatured proteins during their extraction and/or storage (eg. Apple) and lack requisite potency to be used for allergy diagnosis. In

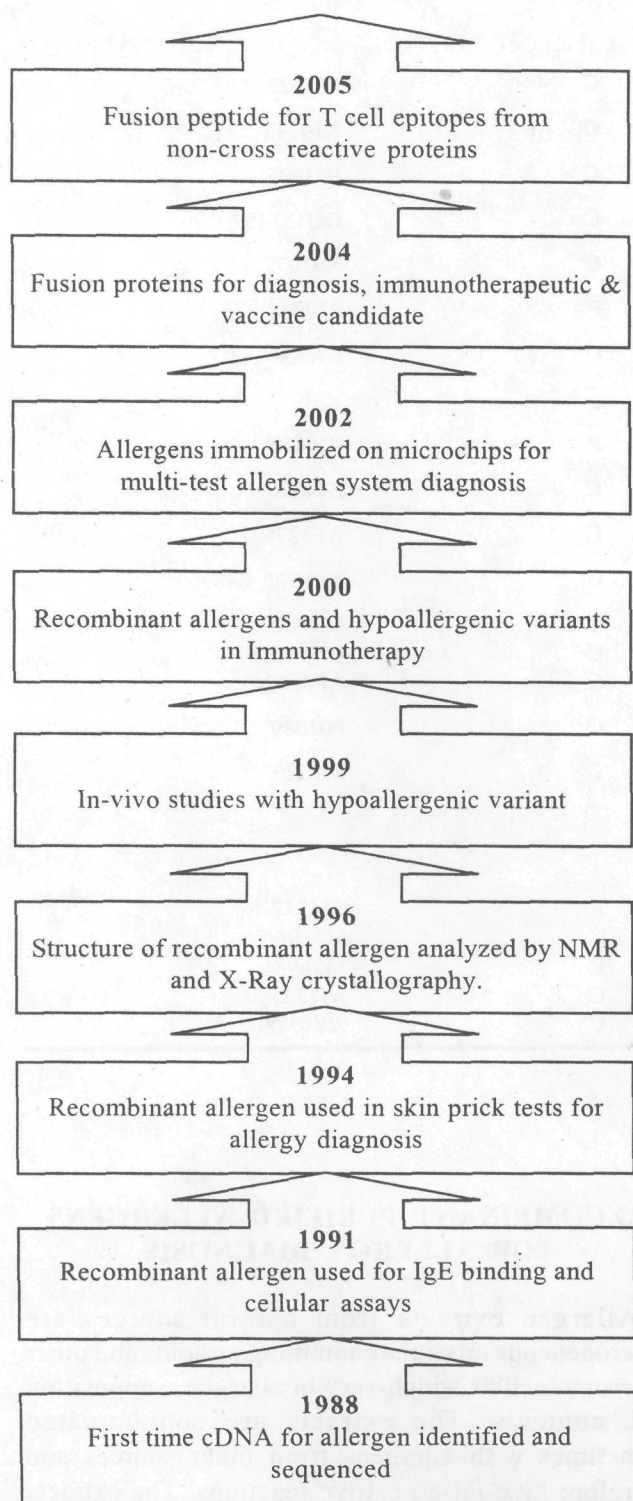


Fig 1. Sequence of events in the Journey of recombinant allergens^{38,42,43}

contrast recombinant allergens are homogenous preparations and give better results in allergy diagnosis.

The use of recombinant allergens identifies sensitization against specific allergen molecule in the extract instead of the source. Furthermore, the use of single recombinant allergen may function as effective marker of atopy and provide a useful clue to characterize a patient's sensitivity⁴⁴. Skin prick test with recombinant allergens was started during 1994-1996 with rAsp f 1/a, rBet v1 and rBet v 2^{38,45,46}. The skin testing with these allergens^{47,48} confirmed the previously conducted serological tests and the in vivo reactivity was comparable to natural allergen. The accuracy of diagnostic tests with recombinant allergens has improved significantly as revealed by IgE binding assay, skin prick test and basophil degranulation⁴⁶.

In near future, microarray technology with purified proteins / peptides will be used effectively for allergy diagnosis. Here the purified allergen molecules (mainly recombinant protein) are microarrayed on glass slides to obtain allergen microchips that allow determination of patient's IgE reactivity profile using very small amount of serum. The microarrays with purified allergens will provide an effective multi allergen test system and may substitute painful skin tests and save time required in current serological tests⁴⁹⁻⁵¹.

RECOMBINANT PREPARATIONS FOR SPECIFIC IMMUNOTHERAPY

Immunotherapy does immunomodulation or the induction of robust specific immunity against disease-causing antigens. Presently immunotherapy involves repeated injection of increasing doses of whole mass allergen extracts and it exhibits variable efficacy⁵². Allergen extracts also contain irrelevant proteins, which may develop additional sensitivities in patients during therapy. The fungal extracts cause severe systemic reactions, therefore it is difficult to achieve optimal maintenance dose. The purified recombinant allergens are likely to overcome these problems associated with natural extracts. Attempts have been made to develop modified recombinant forms viz. allergen fragments⁵³⁻⁵⁹, deletion or substitution

mutants⁶⁰⁻⁷⁶, hypoallergenic isoforms⁷⁷, and defined allergen oligomers⁷⁸⁻⁸⁰.

Some hypoallergenic variants may exhibit low immunogenicity, but fusion of different antigens in the form of hybrid molecules strongly increases their immunogenicity⁸¹. Hybrid allergens consisting of different allergen molecules have been produced⁸². For example hybrid of yellow jacket and paper wasp venom allergen (antigen 5s) which shows reduced allergenicity but retains immunogenicity of natural allergen.

Further T-cell epitope duplication and disulfide bond disruption approaches have been used to reduce IgE reactivity and basophil degranulation but increased T-cell proliferation leading towards tolerance against allergen¹³. There are reports of synthesizing fusion proteins of more than one allergenic protein by PCR and using those in diagnosis and therapy^{83,84}. Recently, a rice-based edible vaccine expressing multiple T cell epitopes showed oral tolerance in mice model⁸⁵.

Clinical trials with DNA vaccines produced by genetic engineering could inhibit infiltration of inflammatory cells⁸⁶⁻⁸⁸. Injection of plasmid DNA encoding clinically relevant allergens induced immune responses with T_H1 bias and promoted formation of IFN- γ producing CD4+ T cells⁸⁹. These IFN- γ secreting T_H1 cells could stimulate B cells to synthesize IgG2a antibodies and inhibited IgE production⁹⁰. Further DNA vaccines conjugated with immunostimulatory DNA to the principal allergenic moiety of allergen are also made and tested^{79,91}.

There is opportunity to construct live vaccines using recombinant DNA technology. cDNA encoding for allergens can be inserted into non-pathogenic 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 T_H1 like response. Recently a novel approach has been used for specific allergy treatment. Here a fusion protein CrSbSC-Bet v1 containing bacterial cell surface protein (S-layer) and major birch pollen allergen Bet v1 was produced, that showed reduced allergenicity with immunomodulating capacity⁹².

CURRENT STATUS AND LIMITATION OF RECOMBINANT ALLERGENS

Structural and immunological similarities between recombinant and natural allergens indicate that a panel of recombinant allergens will be sufficient for effective diagnosis and therapy of allergic diseases. The recombinant allergens enable characterization of IgE binding sites of allergens, T-cell epitopes etc. This opens the possibility of using recombinant allergens in various immunotherapeutic approaches based on synthetic peptides, allergen fragments, T-cell epitope multiplication, allergen isoforms, hypoallergenic variants and DNA vaccines. In countries like Austria, Brazil, Canada, France, Singapore, Spain, Sweden, Switzerland, Taiwan and Venezuela recombinant allergens are already in clinical use⁴⁸. In spite of benefits recombinant allergens are not much popular due to some shortcomings associated with them. For example, identification and cloning of all relevant allergens from complex allergen source can be very time and resource consuming. There is a chance of contamination with DNA or endotoxin depending on host where recombinant allergens are produced. Products of genes expressed in foreign hosts may deviate structurally, biologically or immunologically from their natural counterparts⁴⁸.

REFERENCES

1. Valenta R, Vrtala S, Focke-Tejkl M, Bugajska-Schretter, Ball T, Twardosz A, Spitzauer S, Gronlund H, Kraft D. Genetically engineered and synthetic allergen derivatives: candidates for vaccination against type I allergy. *Biol Chem.* 1999; **380**: 815-24.
2. Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. *J Allergy Clin Immunol.* 1998; **102**: 558-62.
3. Chapman MD, Smith AM, Vailes LD, Arruda LK, Dhanaraj V, Pomes A. Recombinant allergens for diagnosis and therapy of allergic disease. *J Allergy Clin Immunol.* 2000; **106**: 409-18.
4. Nelson HS, Ikle D, Buchmeier A. Studies of allergen extract stability: the effects of dilution and mixing. *J Allergy Clin Immunol.* 1996; **98**: 382-88.
5. Trivedi B, Valerio C, Slater JE. Endotoxin content of standardized allergen vaccines. *J Allergy Clin Immunol.* 2003; **111**: 777-83.

6. Frick OL, Teuber SS, Buchanan BB, Morigasaki S, Umetsu DT. Allergen immunotherapy with heat-killed *Listeria monocytogenes* alleviates peanut and food-induced anaphylaxis in dogs. *Allergy* 2005; **60**: 243-50.
7. van der Veen MJ, Mulder M, Witteman AM, van Ree R, Aalberse RC, Jansen HM, van der Zee JS. False-positive skin prick test responses to commercially available dog dander extracts caused by contamination with house dust mite (*Dermatophagoides pteronyssinus*) allergens. *J Allergy Clin Immunol.* 1996; **98**: 1028-34.
8. Scheiner O, Kraft D. Basic and practical aspects of recombinant allergens. *Allergy* 1995; **50**: 384-91.
9. Gajhede M, Osmark P, Poulsen FM, Ipsen H, Larsen JN, Joost van Neerven RJ, Schou C, Lowenstein H, Spangfort MD. X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. *Nat Struct Biol.* 1996; **3**: 1040-45.
10. Fedorov AA, Ball T, Mahoney NM, Valenta R, Almo SC. The molecular basis for allergen cross-reactivity: crystal structure and IgE-epitope mapping of birch pollen profilin. *Structure* 1997; **5**: 33-45.
11. Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Rapp BA, Wheeler DL. GenBank. *Nucleic Acids Res.* 2002; **30**: 17-20.
12. Valenta R, Lidholm J, Niederberger V, Hayek B, Kraft D, Gronlund H. The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). *Clin Exp Allergy* 1999; **29**: 896-904.
13. Saarne T, Kaiser L, Gronlund H, Rasool O, Gafvelin G, van Hage-Hamsten M. Rational design of hypoallergens applied to the major cat allergen Fel d 1. *Clin Exp Allergy* 2005; **35**: 657-63.
14. Topham CM, Srinivasan N, Thorpe CJ, Overington JP, Kalsheker NA. Comparative modelling of major house dust mite allergen Der p 1: structure validation using an extended environmental amino acid propensity table. *Protein Eng.* 1994; **7**: 869-94.
15. Mueller GA, Benjamin DC, Rule GS. Tertiary structure of the major house dust mite allergen Der p 2: sequential and structural homologies. *Biochemistry* 1998; **37**: 12707-14.
16. Rouvinen J, Rautiainen J, Virtanen T, Zeiler T, Kauppinen J, Taivainen A, Mantylarvi R. Probing the molecular basis of allergy. three-dimensional structure of the bovine lipocalin allergen Bos d 2. *J Biol Chem.* 1999; **274**: 2337-43.
17. Lascombe MB, Gregoire C, Poncet P, Tavares GA, Rosinski-Chupin I, Rabillon J, Goubran-Botros H, Mazie JC, David B, Alzari PM. Crystal structure of the allergen Equ c 1. A dimeric lipocalin with restricted IgE-reactive epitopes. *J Biol Chem.* 2000; **275**: 21572-77.
18. Maleki SJ, Kopper RA, Shin DS, Park CW, Compadre CM, Sampson H, Burks AW, Bannon GA. Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation. *J Immunol.* 2000; **164**: 5844-49.
19. Fahlbusch B, Koch A, Douwes J, Bischof W, Gehring U, Richter K, Wichmann HE, Heinrich J. The effect of storage on allergen and microbial agent levels in frozen house dust. *Allergy* 2003; **58**: 150-3.
20. Gupta R, Sharma V, Sridhara S, Singh BP, Arora N. Identification of serine protease as a major allergen of *Curvularia lunata*. *Allergy.* 2004 Apr; **59**(4): 421-7.
21. Bisht V, Arora N, Singh BP, Pasha S, Gaur SN, Sridhara S. Epi p 1, an allergenic glycoprotein of *Epicoccum purpurascens* is a serine protease. *FEMS Immunol Med Microbiol.* 2004; **42**: 205-11.
22. Verma J, Singh BP, Sridhara S, Gaur SN, Arora N. Purification and characterization of a cross-reactive 45-kD major allergen of *Fusarium solani*. *Int Arch Allergy Immunol.* 2003r; **130**: 193-99.
23. Verma J, Sridhara S, Rai D, Gangal SV. Isolation and immunobiochemical characterization of a major allergen (65 kDa) from *Fusarium equiseti*. *Allergy.* 1998; **53**: 311-15.
24. Verma J, Singh BP, Gangal SV, Arora N, Sridhara S. Purification and partial characterization of a 67-kD cross-reactive allergen from *Imperata cylindrica* pollen extract. *Int Arch Allergy Immunol.* 2000; **122**: 251-56.
25. Shankar J, Gupta PD, Sridhara S, Singh BP, Gaur SN, Arora N. Immunobiochemical analysis of cross-reactive glutathione-S-transferase allergen from different fungal sources. *Immunol Invest.* 2005; **34**: 37-51.
26. Kumari D, Kumar R, Sridhara S, Arora N, Gaur SN, Singh BP. Sensitization to blackgram in patients with bronchial asthma and rhinitis: clinical evaluation and characterization of allergens. *Allergy* 2006; **61**: 104-10.
27. Gross GN, Zimburean JM, Capra JD. Isolation and partial characterization of the allergen in mountain cedar pollen. *Scand J Immunol* 1978; **8**: 437-41.
28. Shen HD, Lin WL, Tam MF, Chou H, Wang CW, Tsai JJ, Wang SR, Han SH. Identification of vacuolar serine proteinase as a major allergen of *Aspergillus fumigatus* by immunoblotting and N-terminal amino acid sequence analysis. *Clin Exp Allergy* 2001; **31**: 295-302.
29. Shen HD, Lin WL, Tsai JJ, Liaw SF, Han SH. Allergenic components in three different species of *Penicillium*: crossreactivity among major allergens. *Clin Exp Allergy* 1996; **26**: 444-51.
30. Jacobson RS, Hoffman DR, Kemeny DM. The cross-reactivity between bee and vespid hyaluronidases has a structural basis (abst). *J. Allergy Clin. Immunol.* 1992; **89**: 292.
31. Valenta R, Duchene M, Vrtala S, Birkner T, Ebner C, Hirschehr R, Breitenbach M, Rumpold H, Scheiner O, Kraft D. Recombinant allergens for immunoblot diagnosis

- of tree-pollen allergy. *J Allergy Clin Immunol.* 1991; **88**: 889-94.
32. Valenta R, Vrtala S, Ebner C, Kraft D, Scheiner O. Diagnosis of grass pollen allergy with recombinant timothy grass (*Phleum pratense*) pollen allergens. *Int Arch Allergy Immunol.* 1992; **97**: 287-94.
33. Rupa P, Mine Y. Immunological comparison of native and recombinant egg allergen, ovalbumin, expressed in *Escherichia coli*. *Biotechnol Lett.* 2003; **25**: 1917-24.
34. Rogers BL, Morgenstern JP, Griffith IJ, Yu XB, Counsell CM, Brauer AW, King TP, Garman RD, Kuo MC. Complete sequence of the allergen Amb alpha II. Recombinant expression and reactivity with T cells from ragweed allergic patients. *J Immunol.* 1991; **147**: 2547-52.
35. Yssel H, Johnson KE, Schneider PV, Wideman J, Terr A, Kastelein R, De Vries JE. T cell activation-inducing epitopes of the house dust mite allergen Der p 1. Proliferation and lymphokine production patterns by Der p 1-specific CD4+ T cell clones. *J Immunol.* 1992; **148**: 738-45.
36. Ebner C, Szepefalusi Z, Ferreira F, Jilek A, Valenta R, Parronchi P, Maggi E, Romagnani S, Scheiner O, Kraft D. Identification of multiple T cell epitopes on Bet v 1, the major birch pollen allergen, using specific T cell clones and overlapping peptides. *J Immunol.* 1993; **150**: 1047-54.
37. Rogers BL, Morgenstern JP, Garman RD, Bond JF, Kuo MC. Recombinant Fel d 1: Expression, purification, IgE binding and reaction with cat-allergic human T cells. *Mol Immunol.* 1993; **30**: 559-68.
38. Moser M, Crameri R, Brust E, Suter M, Menz G. Diagnostic value of recombinant *Aspergillus fumigatus* allergen I/a for skin testing and serology. *J Allergy Clin Immunol.* 1994; **93**: 1-11.
39. Valenta R, Duchene M, Pettenburger K, Sillaber C, Valent P, Bettelheim P, Breitenbach M, Rumpold H, Kraft D, Scheiner O. Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. *Science* 1991; **253**: 557-60.
40. Valenta R, Duchene M, Ebner C, Valent P, Sillaber C, Deviller P, Ferreira F, Tejkl M, Edelmann H, Kraft D. Profilins constitute a novel family of functional plant pan-allergens. *J Exp Med.* 1992; **175**: 377-85.
41. Valenta R, Sperr WR, Ferreira F, Valent P, Sillaber C, Tejkl M, Duchene M, Ebner C, Lechner K, Kraft D. Induction of specific histamine release from basophils with purified natural and recombinant birch pollen allergens. *J Allergy Clin Immunol.* 1993; **91**: 88-97.
42. Valenta R, Kraft D. From allergen structure to new forms of allergen-specific immunotherapy. *Curr Opin Immunol.* 2002; **14**: 718-27.
43. Hufnagl K, Winkler B, Focke M, Valenta R, Scheiner O, Renz H, Wiedermann U. Intranasal tolerance induction with polypeptides derived from 3 noncross-reactive major aeroallergens prevents allergic polysensitization in mice. *J Allergy Clin Immunol.* 2005; **116**: 370-76.
44. van der Veen MJ, Mulder M, Witterman AM, van Ree R, Aalberse RC, Jansen HM, van der Zee JS. False-positive skin prick test responses to commercially available dog dander extracts caused by contamination with house dust mite (*Dermatophagoides pteronyssinus*) allergens. *J Allergy Clin Immunol.* 1996; **98**: 1028-34.
45. Menz G, Dolecek C, Schonheit-Kenn U, Ferreira F, Moser M, Schneider T, Suter M, Boltz-Nitulescu G, Ebner C, Kraft D, Valenta R. Serologic and skin test diagnosis of birch pollen allergy with recombinant Bet v 1, the chief allergen of birch. *Pneumologie* 1996; **50**: 632-40.
46. Pauli G, Oster JP, Deviller P, Heiss S, Bessot JC, Susani M, Ferreira F, Kraft D, Valenta R. Skin testing with recombinant allergens rBet v 1 and birch profilin, rBet v 2: diagnostic value for birch pollen and associated allergies. *J Allergy Clin Immunol.* 1996; **97**: 1100-09.
47. Heiss S, Mahler V, Steiner R, Spitzauer S, Schweiger C, Kraft D, Valenta R. Component-resolved diagnosis (CRD) of type I allergy with recombinant grass and tree pollen allergens by skin testing. *J Invest Dermatol.* 1999; **113**: 830-37.
48. Schmid-Grendelmeier P, Crameri R. Recombinant allergens for skin testing. *Int Arch Allergy Immunol.* 2001; **125**: 96-111.
49. Bacarese-Hamilton T, Gray J, Ardizzoni A, Crisanti A. Allergen microarrays. *Methods Mol Med.* 2005; **114** : 195-207.
50. Harwanegg C, Laffer S, Hiller R, Mueller MW, Kraft D, Spitzauer S, Valenta R. Microarrayed recombinant allergens for diagnosis of allergy. *Clin Exp Allergy* 2003; **33** : 7-13.
51. Deinhofer K, Sevcik H, Balic N, Harwanegg C, Hiller R, Rumpold H, Mueller MW, Spitzauer S. Microarrayed allergens for IgE profiling. *Methods* 2004; **32**: 249-54.
52. Portnoy JM. Immunotherapy for asthma: unfavorable studies. *Ann Allergy Asthma Immunol.* 200; **87**: 28-32.
53. Zeiler T, Taivainen A, Rytönen M, Rautiainen J, Karjalainen H, Mantylä R, Tuomisto L, Virtanen T. Recombinant allergen fragments as candidate preparations for allergen immunotherapy. *J Allergy Clin Immunol.* 1997; **100**: 721-27.
54. Hayek B, Vangelista L, Pastore A, Sperr WR, Valent P, Vrtala S, Niederberger V, Twardosz A, Kraft D, Valenta R. Molecular and immunologic characterization of a highly cross-reactive two EF-hand calcium-binding alder pollen allergen, Aln g 4: structural basis for calcium-modulated IgE recognition. *J Immunol.* 1998; **161**: 7031-39.
55. Ball T, Fuchs T, Sperr WR, Valent P, Vangelista L, Kraft D, Valenta R. B cell epitopes of the major timothy grass pollen allergen, phl p 1, revealed by gene fragmentation as

- candidates for immunotherapy. *FASEB J.* 1999; **13**: 1277-90.
56. Vrtala S, Akdis CA, Budak F, Akdis M, Blaser K, Kraft D, Valenta R. T cell epitope-containing hypoallergenic recombinant fragments of the major birch pollen allergen, Bet v 1, induce blocking antibodies. *J Immunol.* 2000; **165**: 6653-59.
 57. van Hage-Hamsten M, Johansson E, Roquet A, Peterson C, Andersson M, Greiff L, Vrtala S, Valenta R, Gronneberg R. Nasal challenges with recombinant derivatives of the major birch pollen allergen Bet v 1 induce fewer symptoms and lower mediator release than rBet v 1 wild-type in patients with allergic rhinitis. *Clin Exp Allergy* 2002; **32**: 1448-53.
 58. Zeiler T, Taivainen A, Rytkonen M, Rautiainen J, Karjalainen H, Mantyjarvi R, Tuomisto L, Virtanen T. Recombinant allergen fragments as candidate preparations for allergen immunotherapy. *J Allergy Clin Immunol.* 1997; **100**: 721-27.
 59. Smith AM, Chapman MD. Reduction in IgE binding to allergen variants generated by site-directed mutagenesis: contribution of disulfide bonds to the antigenic structure of the major house dust mite allergen Der p 2. *Mol Immunol.* 1996; **33**: 399-405.
 60. Noguchi E, Shibasaki M, Nishiyama C, Okumura Y, Takita H. IgE responsiveness to *Dermatophagoides farinae* in young asthmatic children: IgE binding study using recombinant allergens of Der f1, Der f2 and mutant proteins of Der f2. *Int Arch Allergy Immunol.* 1996; **110**: 380-87.
 61. Engel E, Richter K, Obermeyer G, Briza P, Kungl AJ, Simon B, Auer M, Ebner C, Rheinberger HJ, Breitenbach M, Ferreira F. Immunological and biological properties of Bet v 4, a novel birch pollen allergen with two EF-hand calcium-binding domains. *J Biol Chem.* 1997; **272**: 28630-37.
 62. Takai T, Yokota T, Yasue M, Nishiyama C, Yuuki T, Mori A, Okudaira H, Okumura Y. Engineering of the major house dust mite allergen Der f 2 for allergen-specific immunotherapy. *Nat Biotechnol.* 1997; **15**: 754-58.
 63. Arquint O, Helbling A, Crameri R, Ferreira F, Breitenbach M, Pichler WJ. Reduced in vivo allergenicity of Bet v 1d isoform, a natural component of birch pollen. *J Allergy Clin Immunol.* 1999; **104**: 1239-43.
 64. Ferreira F, Ebner C, Kramer B, Casari G, Briza P, Kungl AJ, Grimm R, Jahn-Schmid B, Breiteneder H, Kraft D, Breitenbach M, Rheinberger HJ, Scheiner O. Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *FASEB J.* 1998; **12**: 231-42.
 65. Okada T, Swoboda I, Bhalla PL, Toriyama K, Singh MB. Engineering of hypoallergenic mutants of the *Brassica* pollen allergen, Bra r 1, for immunotherapy. *FEBS Lett.* 1998; **434**: 255-60.
 66. Olsson S, Whitley P. Contribution of disulphide bonds to antigenicity of Lep d 2, the major allergen of the dust mite *Lepidoglyphus destructor*. *Mol Immunol.* 1998; **35**: 1017-23.
 67. Schramm G, Kahlert H, Suck R, Weber B, Stuwe HT, Muller WD, Bufer A, Becker WM, Schlaak MW, Jager L, Cromwell O, Fiebig H. "Allergen engineering": variants of the timothy grass pollen allergen Phl p 5b with reduced IgE-binding capacity but conserved T cell reactivity. *J Immunol.* 1999; **162**: 2406-14.
 68. Korematsu S, Tanaka Y, Hosoi S, Koyanagi S, Yokota T, Mikami B, Minato N. C8/119S mutation of major mite allergen Derf-2 leads to degenerate secondary structure and molecular polymerization and induces potent and exclusive Th1 cell differentiation. *J Immunol.* 2000; **165**: 2895-902.
 69. Bonura A, Amoroso S, Locorotondo G, Di Felice G, Tinghino R, Geraci D, Colombo P. Hypoallergenic variants of the *Parietaria judaica* major allergen Par j 1: a member of the non-specific lipid transfer protein plant family. *Int Arch Allergy Immunol.* 2001; **126**: 32-40.
 70. Kronqvist M, Johansson E, Whitley P, Olsson S, Gafvelin G, Scheynius A, van Hage-Hamsten M. A hypoallergenic derivative of the major allergen of the dust mite *Lepidoglyphus destructor*, Lep d 2.6Cys, induces less IgE reactivity and cellular response in the skin than recombinant Lep d 2. *Int Arch Allergy Immunol.* 200; **126**: 41-49.
 71. Beezhold DH, Hickey VL, Sussman GL. Mutational analysis of the IgE epitopes in the latex allergen Hev b 5. *J Allergy Clin Immunol.* 2001; **107**: 1069-76.
 72. Rabjohn P, West CM, Connaughton C, Sampson HA, Helm RM, Burks AW, Bannon GA. Modification of peanut allergen Ara h 3: effects on IgE binding and T cell stimulation. *Int Arch Allergy Immunol.* 2002; **128**: 15-23.
 73. Swoboda I, De Weerd N, Bhalla PL, Niederberger V, Sperr WR, Valent P, Kahlert H, Fiebig H, Verdino P, Keller W, Ebner C, Spitzauer S, Valenta R, Singh MB. Mutants of the major ryegrass pollen allergen, Lol p 5, with reduced IgE-binding capacity: candidates for grass pollen-specific immunotherapy. *Eur J Immunol.* 2002; **32**: 270-80.
 74. Vrtala S, Fischer S, Grote M, Vangelista L, Pastore A, Sperr WR, Valent P, Reichelt R, Kraft D, Valenta R. Molecular, immunological, and structural characterization of Phl p 6, a major allergen and P-particle-associated protein from Timothy grass (*Phleum pratense*) pollen. *J Immunol.* 1999; **163**: 5489-96.
 75. Tang B, Banerjee B, Greenberger PA, Fink JN, Kelly KJ, Kurup VP. Antibody binding of deletion mutants of Asp f 2, the major *Aspergillus fumigatus* allergen. *Biochem Biophys Res Commun.* 2000; **270**: 1128-35.

76. Ferreira F, Ebner C, Kramer B, Casari G, Briza P, Kungl AJ, Grimm R, Jahn-Schmid B, Breiteneder H, Kraft D, Breitenbach M, Rheinberger HJ, Scheiner O. Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *FASEB J*. 1998; **12**: 231-42.
77. Ferreira F, Hirtenlehner K, Jilek A, Godnik-Cvar J, Breiteneder H, Grimm R, Hoffmann-Sommergruber K, Scheiner O, Kraft D, Breitenbach M, Rheinberger HJ, Ebner C. Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. *J Exp Med*. 1996; **183**: 599-609.
78. Vrtala S, Hirtenlehner K, Susani M, Akdis M, Kussebi F, Akdis CA, Blaser K, Hufnagl P, Binder BR, Politou A, Pastore A, Vangelista L, Sperr WR, Semper H, Valent P, Ebner C, Kraft D, Valenta R. Genetic engineering of a hypoallergenic trimer of the major birch pollen allergen Bet v 1. *FASEB J*. 2001; **15**: 2045-47.
79. Creticos PS, Lichtenstein LM. Progress in the development of new methods of immunotherapy: potential application of immunostimulatory DNA-conjugated to allergens for treatment of allergic respiratory conditions. In: Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe (Ed. Frankf A M). 2003; **94**: 304-12.
80. Spiegelberg HL, Raz E. DNA-based approaches to the treatment of allergies. *Curr Opin Mol Ther*. 2002; **4**: 64-71.
81. King TP, Jim SY, Monsalve RI, Kagey-Sobotka A, Lichtenstein LM, Spangfort MD. Recombinant allergens with reduced allergenicity but retaining immunogenicity of the natural allergens: hybrids of yellow jacket and paper wasp venom allergen antigen 5s. *J Immunol*. 2001; **166**: 6057-65.
82. Linhart B, Jahn-Schmid B, Verdino P, Keller W, Ebner C, Kraft D, Valenta R. Combination vaccines for the treatment of grass pollen allergy consisting of genetically engineered hybrid molecules with increased immunogenicity. *FASEB J*. 2002; **16**: 1301-03.
83. Linhart B, Hartl A, Jahn-Schmid B, Verdino P, Keller W, Krauth MT, Valent P, Horak F, Wiedermann U, Thalhamer J, Ebner C, Kraft D, Valenta R. A hybrid molecule resembling the epitope spectrum of grass pollen for allergy vaccination. *J Allergy Clin Immunol*. 2005; **115**: 1010-16.
84. Pittner G, Vrtala S, Thomas WR, Weghofer M, Kundi M, Horak F, Kraft D, Valenta R. Component-resolved diagnosis of house-dust mite allergy with purified natural and recombinant mite allergens. *Clin Exp Allergy* 2004; **34**: 597-603.
85. Takagi H, Hiroi T, Yang L, Tada Y, Yuki Y, Takamura K, Ishimitsu R, Kawauchi H, Kiyono H, Takaiwa F. From The Cover: A rice-based edible vaccine expressing multiple T cell epitopes induces oral tolerance for inhibition of Th2-mediated IgE responses. *PNAS* 2005; **102**: 17525-30.
86. Li GP, Liu ZG, Qiu J, Ran PX, Zhong NS. DNA vaccine encoding Der p 2 allergen generates immunologic protection in recombinant Der p 2 allergen-induced allergic airway inflammation mice model. *Chin Med J (Engl)*. 2005; **118**: 534-40.
87. Holm J, Gajhede M, Ferreras M, Henriksen A, Ipsen H, Larsen JN, Lund L, Jacobi H, Millner A, Wurtzen PA, Spangfort MD. Allergy vaccine engineering: epitope modulation of recombinant Bet v 1 reduces IgE binding but retains protein folding pattern for induction of protective blocking-antibody responses. *J Immunol*. 2004; **173**: 5258-67.
88. Hartl A, Hochreiter R, Stepanoska T, Ferreira F, Thalhamer J. Characterization of the protective and therapeutic efficiency of a DNA vaccine encoding the major birch pollen allergen Bet v 1a. *Allergy* 2004; **59**: 65-73.
89. Eyal Raz, Helen Tighe, Yukio Sato, Maripat Corr, Jean A. Dudler, Mark Roman, Susan L. Swain, Hans L. Spiegelberg, and Dennis A. Carson Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *PNAS* **93**: 5141-45
90. Simons FE, Imada M, Li Y, Watson WT, HayGlass KT. Fel d 1 peptides: effect on skin tests and cytokine synthesis in cat-allergic human subjects. *Int Immunol*. 1996; **8**: 1937-45.
91. Tighe H, Takabayashi K, Schwartz D, Van Nest G, Tuck S, Eiden JJ, Kagey-Sobotka A, Creticos PS, Lichtenstein LM, Spiegelberg HL, Raz E. Conjugation of immunostimulatory DNA to the short ragweed allergen amb a 1 enhances its immunogenicity and reduces its allergenicity. *J Allergy Clin Immunol*. 2000; **106**: 124-34.
92. Bohle B, Breitwieser A, Zwolfer B, Jahn-Schmid B, Sara M, Sleytr UB, Ebner C. A novel approach to specific allergy treatment: the recombinant fusion protein of a bacterial cell surface (S-layer) protein and the major birch pollen allergen Bet v 1 (rSbsC-Bet v 1) combines reduced allergenicity with immunomodulating capacity. *J Immunol*. 2004; **172**: 6642-48.