The advent of recombinant allergens and allergen cloning

Wayne R. Thomas, PhD *Subiaco, Australia*

When the allergen nomenclature system was adopted in 1986, allergens were identified by their behavior on electrophoresis and chromatography and by reactivity to shared antisera. Not only was this unsatisfactory for standardization, but the processes of allergic sensitization and immunotherapy could not be studied in the framework of antigen processing and B- and T-cell epitopes. Recombinant technologies developed in the 1980s for cloning cDNA from low-abundance mRNA permitted the cloning of allergens, beginning with the major house dust mite allergen Der p 1 and hornet allergen Dol m 5. After this, a wave of cloning with IgE immunoscreening resulted in the cloning of Der p 2, Der p 5, Bet v 1, Bet v 2, and Dac g 2 along with Fel d 1 cloned after amino acid sequencing. Recombinant allergens have now been used to define the important allergens for a wide range of allergies and to develop new types of immunotherapy, some of which have shown efficacy in human trials. The clonally pure allergens have been used to solve the tertiary structures of allergens and from this how allergens might activate innate immunity. Proprietary recombinant allergens are now being used in improved diagnostic tests. (J Allergy Clin Immunol 2011;127:855-9.)

Key words: *Allergen, cloning, recombinant, history,* Dermatophagoides *species, allergen structure*

The immunochemical characterization of allergens was begun at a time when it was inconceivable that practical quantities of most common allergens could be purified to homogeneity. Indeed, it was a concern that the low doses of allergen required to elicit hypersensitivity reactions were less than those that could be monitored for purity. However, it was established that more than 1 allergen from a particular source was responsible for hypersensitivity and that each source had major and minor allergens. The importance of proteins was identified, which was an important question at the time, and it was established that major allergens, such as Amb a 1 from ragweed, could be a heterogeneous family of proteins.[1]

Amb a 1 was the vanguard of the immunochemical characterization of inhalant allergens, especially from the studies of Te Piao King.[1] Its biochemical identity and amino acid sequence, however, remained unknown because its blocked N-terminal precluded amino acid sequencing. The characterization of the codfish food allergen conducted at the same time was more informative. Its amino acid sequence was determined, and it was identified as the muscle calcium regulator parvalbumin. Parvalbumins remain the most important allergens known for fish.[2] Some other major allergens were biochemically characterized before their allergenicity was appreciated. Honey bee phospholipase A had been sequenced in toxicology studies, and the amino acid sequences of ovalbumin and ovomucoid had been determined before they were identified as the major egg allergens, as had the sequences of casein and β-lactoglobulin from milk. Although enormous advances were made in the 1960s and 1970s in the techniques of protein purification, the characterization of proteins required large amounts of purified material, and a period of stagnation in protein research occurred.[3] Amino acid sequences of the minor Amb a 3 and Amb a 5 allergens were solved,[1] but few other accomplishments were made before cDNA cloning was introduced.

IMMUNOLOGIC PUSH FOR MOLECULAR CLONING

Solid-phase synthesis of peptides made it routine to synthesize polypeptides of up to 50 amino acids, and synthetic peptides representing sequences of antigens were being used to immunize animals to induce antibodies that reacted with the native antigen. Similarly, even before the principles of antigen processing and presentation were elucidated, it was known that 10- to 12-mer synthetic peptides taken from a linear sequence of antigens could act as T-cell epitopes and immunize animals[4] or, as shown in tissue culture, inhibit the activation of human T cells.[5] Clearly this pointed to the possibility of new types of immunotherapy, as did the demonstration that variations in the sequences of T-cell epitopes in evolutionarily related antigens markedly altered immune responsiveness. The investigations of Ronald Schwartz and his colleagues showing that T-cell responses of mice to cytochrome c could be modified with sequence variants taken from different species were particularly influential for the initiation of allergen cloning by the author.[6] The use of urea-denatured allergens as a method of targeting T-cell responses had already been investigated in murine experiments with ragweed,[1] and site-directed mutagenesis, which is required to produce genetically engineered antigens, had been established in 1978.[7]

The quest to understand the link between the extraordinary polymorphism of the genes of the MHC and the immunologic responsiveness to allergens was also a major impetus. Experiments with mice had shown that the amino acid sequences of the epitopes determined which antigens could be presented by the MHC molecules of different alleles, and therefore it was thought that similar phenomena would be uncovered for allergy. The propensity of people with certain MHC alleles to produce

From the Centre for Child Health Research, University of Western Australia, Telethon Institute for Child Health Research.

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Reprint requests: Wayne R. Thomas, PhD, Telethon Institute for Child Health Research, 100 Roberts Rd, Subiaco, Western Australia 6008, Australia. E-mail: wayne@ichr.uwa.edu.au.

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antibodies to the minor ragweed allergens had been described by Marsh et al,[8] especially the association of antibodies to Amb a 5 with the class II allele HLA-Dw2. However, Amb a 5 is a 45-amino-acid peptide and not a strong allergen, so studies to discover MHC restrictions of responses to major allergens and how they varied with allergen sequences, such as isoforms of grass allergens, were high on the research agenda. It is now known that immune responses to major allergens typically do not show consistent associations with particular MHC alleles, but these investigations were a major stimulus for sequencing allergens.

FROM IMMUNOCHEMISTRY TO MOLECULAR CLONING

Molecular cloning resulted from the realization that DNA containing a gene of one organism can be transferred and made to function in a genetically different organism. The most frequent incarnation of this is to transfer a gene encoding a protein of interest to the bacterium *Escherichia coli*, where each *E coli* produces 10 to 100 copies of the gene. The cloning results from the ability to transfer 1 gene to each *E coli* and to propagate it to produce limitless progeny. The cloning of the DNA allows it to be isolated for analysis and genetic engineering, and the bacterial hosts of the DNA can be made to produce the product of the gene. In practice, the DNA used for cloning the "genes" that code for proteins from eukaryotes is made as a DNA copy of the mRNA that codes for the protein (cDNA). The cloning of DNA transferring antibiotic resistance between bacteria, accomplished in 1973, was one of the most clear-cut conceptual and practical advances made in biology. Even so, many milestones needed to be passed before the ability to confer antibiotic resistance to bacteria became a technology for the characterization and production of recombinant proteins.[7] Because of the abundance of its mRNA in the hen oviduct, the ovalbumin allergen Gal d 2 was a trailblazer in molecular cloning along with rabbit globin. Its nucleotide sequence was solved during the development of sequencing methodology. The expression of recombinant ovalbumin in *E coli* reported in 1978 was made at the same time as the more exalted expression of growth hormone from cDNA constructed from the mRNA of a pituitary tumor.[7]

The use of cDNA cloning for proteins produced from low-abundance mRNA required substantial technical advances in library construction and screening methodologies. The production of large cDNA libraries suitable for high-density screening was achieved by cloning with the λ gt10 and gt11 bacteriophages. Their high-density lawns of plaques could be screened by using DNA hybridization, and importantly for IgE antibody based screening, high-throughput immunoscreening methods for bacterial colonies and plaques were devised.[9]

Another important development was the microsequencing of proteins immobilized on polyvinylidene difluoride membranes.[3] This was not only able to provide 20 to 30 N-terminal residues from submicrogram amounts of protein, but sequencing could be directly performed from bands electroblotting onto the membranes after SDS-PAGE.

CLONING OF THE FIRST ALLERGENS

The feasibility of cDNA cloning of house dust mite allergens was demonstrated by the detection of Der p 1 and other IgE-binding polypeptides among the *in vitro* translation products of mRNA made from the bodies of *Dermatophagoides pteronyssinus* mites. Libraries were then constructed with λ gt10 and λ gt11 vectors and screened by using a plaque immunoassay with anti–Der p 1 antisera and by using DNA hybridization with oligonucleotides synthesized from the sequences of the N-terminal and peptide fragments from trypsin digests.[10] Clones that encoded Der p 1 were identified by their translated amino acid sequences, which also had high identity to the N-terminal sequence published for Der f 1. Publication was delayed so that IgE binding could be demonstrated but was eventually made with the knowledge that IgE binding to Der p 1 was very sensitive to denaturation and might need considerable work to achieve. The reports of Der p 1 cloning only just preceded the report of the cloning of Dol m 5 from white-faced hornet venom. This was accomplished by using an identical strategy, except that the mRNA was extracted from a specific organ, the acid venom gland.[11] IgE binding was also not demonstrated.

The amino acid amino sequence of Der p 1 was immediately of considerable interest.[12] Der p 1 was revealed to be a cysteine protease similar to papain, and therefore the potent allergenicity might be linked with an adjuvant effect of protease activity. The overall tertiary structure was also immediately revealed because the structures of papain and the related actinidin enzymes had been solved. Although not perfectly folded, high IgE-binding recombinant allergens were soon produced, and the publication in 1992 of rDer p 1 made in *Saccharomyces cerevisiae* with near-natural and high-frequency IgE binding formally demonstrated the cloning of a major allergen.[13] Because, like Der p 1, IgE binding of the Dol m 5 had been well established, the main reason for cloning was to complete the characterization of the venom allergens and to explore the molecular basis for allergenic cross-reactivity between vespid species. The sequence showed identity to plant pathogenesis-related proteins, now designated as the V5/Tpx-1–related family. The family members are remarkably conserved across fungi, plants, animals, and parasites, but their precise functions are unclear.

CLONING BY MEANS OF IGE SCREENING

The first reports of IgE-binding recombinant allergens resulted from the use of IgE immunoassays used to screen cDNA expression libraries. Der p 2[14,15] and Der p 5[16] from the house dust mite, Bet v 1 from birch pollen,[17] and Dac g 2 from orchard grass[18] were cloned this way. The cloning of Bet v 1 was a watershed. It unveiled the allergenicity of PR10 pathogenesis-related proteins, which are now known to induce strong IgE responses in a diverse range of pollen and food hypersensitivities and to be a cause of the oral allergy syndrome. It was also an ideal allergen for pioneering studies of recombinant allergens for immunotherapy.[19] Most birch pollen–sensitive patients direct 80% of their anti-birch IgE to this allergen, and immunotherapy to treat birch pollen–induced allergic rhinitis is commonly practiced. Bet v 1 was also the first recombinant allergen used to determine NMR and X-ray crystallographic structures.[2] Recombinant Der p 2 and Der f 2 have also been frequently used for structural studies[2] and were the first members of ML (MD2-like) domain proteins, which is an inappropriate name because MD2 was modeled on Der p 2.

MAJOR ALLERGENS FROM MAJOR SOURCES

The cloning of major allergens from other important sources quickly followed with the cloning of the group 1 and group 5 grass

pollen allergens,[20,21] Fel d 1 from cat,[22] and Amb a 1 from ragweed,[23] as reported in 1991. Birch profilin (Bet v 2) was also cloned at this time.[24] It is not a major allergen of birch pollen, but plant profilins from many different species are pollen, food, and contactant allergens and cross-react. From this perspective, they are important allergens and are very important in diagnosis. The first molecular characterization of a fungal allergen resulted from the discovery that Asp f 1, the major allergen of *Aspergillus fumigatus*, was the mitogillin cytotoxin.[25] The primary structure had already been determined by means of amino acid sequencing. Molecular cloning of allergens from the German[26] and American[27] cockroaches were important later undertakings, and the cloning of Ara h 1 and Ara h 2 from peanut[28,29] were milestones for food allergy.

The cloning of Blo t 5 from *Blomia tropicalis* is especially noteworthy because here cDNA cloning revealed the nature of a major allergen that had not previously been recognized by using immunochemical methods and showed that it was unexpectedly different from the major allergens of *Dermatophagoides* species.[30] Its high IgE binding and lack of cross-reactivity with *Dermatophagoides* species allergens provided the means to define the prevalence of an allergy that is widespread in the highly populous countries of Southeast Asia and South America. Even today, only small quantities of natural Blo t 5 have been isolated. Molecular cloning has very recently discovered the related high-IgE-binding Blo t 21 allergen, making this an important area of contemporary research.

PRODUCTION OF RECOMBINANT ALLERGENS

Molecular cloning was expected to lead to the production of unlimited supplies of clonally pure allergens and genetic engineering to study epitopes and produce novel allergens for improved immunotherapy. The allergens first cloned by using an IgE immunoassay were easily expressed in *E coli* as proteins with indistinguishable allergenicity to their natural counterparts, as shown for rDer f 2 based on skin test reactions and basophil degranulation.[31] Expression of rBet v 1 and rBet v 2 similar to the natural allergen was also readily accomplished in *E coli*.[19] The major grass allergens, as shown for Phl p 5 and Phl p 1, were expressed as recombinant proteins with high IgE-binding activity and, combined with rPhl p 2 and profilin, were shown to be able to absorb much of the IgE activity of allergic sera to pollen extract.[19]

The production of rFel d 1 is a noteworthy success story. Early studies that separately expressed the 2 polypeptide chains of this allergen only accomplished the production of low-IgE-binding polypeptides. It was subsequently found that direct juxtapositioning of the DNA encoding the 2 chains produced a correctly folded molecule that has been used for X-ray crystallography and extensive immunologic studies.[32] Fel d 1 is a dimer of a heterodimer, showing that a multichain structure is not a barrier for the production of recombinant allergens.

Allergens that did not initially fold correctly in *E coli* were eventually produced in other hosts. The hornet allergen Dol m 5 is produced as a well-folded allergen when expressed in the yeast *Pichia pastoris*.[33] The work of a number of research groups resulted in the production of correctly folded group 1 house dust mite allergens.[34] Constructs of cDNA that contain the complete 80-residue proenzyme sequence can either direct the synthesis of the mature allergen in *P pastoris* or produce a proenzyme that can be matured in an acidic environment. Less efficient production can be achieved in *E coli*.

Although many allergens can be produced as recombinant proteins, there are some difficult or neglected areas. Although Amb a 1 was cloned in 1991, little attention has been paid to the production of the recombinant allergen. It can be produced in high yield in *E coli* as a polypeptide but with little IgE-binding activity.[35] Because Amb a 1 is constituted by a gene family of proteins with quite disparate amino acid sequences (30%), a standardized reagent might circumvent variations in the family members found in extracts. Further technology and knowledge are also required to recapitulate the structures of some natural allergens because there are posttranslational modifications and complexes formed with nonproteinaceous ligands. Taking house dust mite as an example, the extensive O-glycosylation of the chitinase allergen is important.[34]

cDNA FOR DEFINING THE SPECTRUM OF ALLERGENS

cDNA cloning provided an alternative to studying extracts for analyzing the spectrum of allergens produced from different sources. House dust mite allergy provides a good example. Purification of many of the allergens from extracts to the homogeneity and yield required for analysis is not only difficult, but also the representation of allergens in the extracts might be quantitatively and qualitatively different to the allergens in the environment. It is not known how the allergens are disseminated from the mites to inhaled air, and there is abundant evidence of degradation in extracts and the varying production of allergens with different culture conditions. Analysis with cloned allergens has simplified the understanding of the important allergenic specificities.[34] Although some areas of uncertainty remain, analyses of *D pteronyssinus* have shown that Der p 1 and Der p 2 account for 50% to 60% of the IgE binding and most of rest can be accounted for by binding to Der p 4, 5, 7 and 21. Similar results have been obtained for skin tests. Der p 5, 7, and 21 have almost only been studied as recombinant allergens but are not necessarily produced in small quantities, as shown by means of proteomics.

REALIZATION OF THE GOALS

Widespread application of recombinant allergens in clinical practice and research has yet to realized. However, this is beginning and will add to the substantial pool of knowledge made possible by molecular cloning and the forging of new diagnostic and therapeutic opportunities (Table I). With the recent introduction of proprietary recombinant allergens, laboratories worldwide can now experiment with recombinant allergens and thereby conduct investigations using known and reproducible quantities of allergens. A major target is to use component-resolved diagnosis to differentiate the source of allergic sensitization from cross-reactivity and to identify patients who might have problems with cross-sensitization, such as to food and pollen allergens.[36] A noteworthy success is the demonstration that patients whose reactions to latex extract are only mediated by IgE antibody to the latex profilin Heb v 8 can undergo surgical treatment without latex-avoidance procedures. The availability of rBlo t 5 to study mite allergy in tropical and subtropical countries will be central to analyzing a major allergy in developing countries, where the prevalence of allergic diseases is rapidly increasing.

TABLE I. Outcomes from the molecular cloning of allergens

Definition of allergens: allergen nomenclature based on sequence
Allergen discovery
Hierarchy of allergens produced by allergen sources
Understanding and recognizing cross-reactivities
Elucidation of gene families of major allergens
Allergens to solve 3-dimensional structures
Geographic variation in allergen sequences
Sequences for peptide immunotherapy
Recombinant proteins for diagnosis and immunotherapy
Recombinant proteins for investigations with defined and reproducible reagents
Sequences for peptides to study T-cell responses
Species-specific allergens for epidemiologic studies
Genetic engineering of hypoallergens
Genetic engineering to enhance immunotherapy

Immunotherapy with peptides designed to contain T-cell epitopes was quickly pursued by using cat allergy and Fel d 1 as the prototype. A short course of peptide injections achieved an efficacy similar to that produced by standard cat dander extract treatment, but the investigations were curtailed by the occurrence of interesting but unwanted side effects.[37] Trials of a new strategy of peptide immunotherapy are ongoing.

Recombinant allergens have also been trialed. The most recent trials with subcutaneous injections of rBet v 1 showed that it had the same efficacy for treating allergic rhinitis as the pollen extract, as did a similar trial for grass pollen allergy.[19] Immunotherapy with subcutaneous injection of rBet v 1 modified by means of fragmentation and polymerization produced promising results but without the increased efficacy or reduced side effects that might be expected of a hypoallergen. The usefulness of hypoallergens will be more apparent when allergens genetically engineered to retain their normal folding while reducing IgE binding to surface determinants have been tested. Much of the recent immunotherapy research has been directed to sublingual immunotherapy, which can be self-administered and is less confronting than injections. The announcement of efficacy with sublingual rBet v 1 in a phase IIb/III clinical trial (VO59.08) study at the European Academy of Allergy and Clinical immunology in 2010 might mark a significant advance. Recombinant technology would not only be ideal for the production of large of amounts of allergen, but also it is likely that further experimentation with this route of delivery would be less constrained than the injection regimens.

Finally, the knowledge made available by molecular cloning enables allergy research to be conducted within the framework of mainstream immunology. Gene synthesis, which was not considered feasible when molecular cloning began, enables any laboratory to cost-effectively synthesize allergens of choice, and peptide synthesis can be used to measure T-cell responses to the epitopes of the full spectrum of allergens found in extracts.[38] The molecular structures solved through the availability of recombinant allergens have provided the lead to determine how allergens interact with the innate immune system,[34] which is increasingly being recognized as an integral step for allergic sensitization.

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Correction

With regard to the January 2011 Current Perspectives article entitled "Allergen-specific immunotherapy for respiratory allergies: From meta-analysis to registration and beyond" (J Allergy Clin Immunol 2011;127:30-38), Table V contained several inaccuracies. A corrected version of the table follows.

TABLE V. Large randomized controlled studies on grass pollen SLIT tablets for seasonal allergic rhinitis

Number of patients SLIT/placebo	Daily dose	Age (mean in yrs) SLIT	Age (mean in yrs) Placebo	Symptom scores[1] (adjusted mean) SLIT	Symptom scores[1] (adjusted mean) Placebo	Symptom scores[1] (adjusted mean) Reduction[2]	Symptom scores[1] (median) SLIT	Symptom scores[1] (median) Placebo	Symptom scores[1] (median) Reduction[2]	Medication scores[1] (adjusted mean) SLIT	Medication scores[1] (adjusted mean) Placebo	Medication scores[1] (adjusted mean) Reduction[2]	Medication scores[1] (median) SLIT	Medication scores[1] (median) Placebo	Medication scores[1] (median) Reduction[2]	References
Adults																
153/136	75000 SQ-T	36	33	2.47	2.94	-16	2.13[4]	2.53[4]	-16[4]	1.46	2.05	-28	0.35[4]	1.24[4]	-72[4]	55
316/318	75000 SQ-T	33.8	34.5	2.85[3]	4.14[3]	-31[3]	2.1	3.2	-34	1.65[3]	2.68[3]	-39[3]	0.8	1.7	-53	54
155/156	300 IR	28.7	29.1	3.58	4.93	-27[4]	2.91	4.62	-37	0.28	0.43	-35	0.10[4]	0.27[4]	-63[3]	56,59
Children																
126/127	75000 SQ-T	10.1	10.1	2.62[4]	3.61[4]	-28[4]	2.71	3.75	-28	2.68[5]	3.53[5]	-65[5]	0.64	1.83	-65	57
131/135	300 IR	10.5	11.5	3.25	4.51	-28	2.48[4]	4.08[4]	-39	0.60[4]	0.79[4]	-24[4]	0.39[4]	0.76[4]	-49	58

SQ-T: Standardized Quality-Tablet; *IR*: Index of Reactivity.
[1]The definition of pollen season varies from one study to another. Here, we show the symptoms for all studies during the pollen season defined as $>10^{55}$-30 grains/m^3 air.
[2]% compared with placebo.
[3]These figures differ from those given in the publications but have been reviewed by the regulatory authorities in the EMA registration file.
[4]These data were provided by the manufacturers.
[5]Only raw means are available and not adjusted means because parametric analysis could not be performed as assumption of normality was not confirmed.