

Molecular Breeding of Allergy Vaccines and Antiallergic Cytokines

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Abstract

Molecular breeding, also called DNA shuffling, is a technology that enables the generation of large libraries of novel genes and vectors, from which improved variants can be selected based on functional properties. In a common format, it involves recursive recombination and mutation, performed by random fragmentation of related DNA sequences, followed by reassembly of the fragments in a self-priming polymerase chain reaction. As in natural evolution, the technique takes advantage of crossovers, deletions, insertions, inversions and point mutations of genes to generate large pools of related sequences. Molecular breeding can be used to generate improved variants of proteins used as therapeutics, such as vaccine antigens, growth factors and immunomodulatory molecules. Moreover, the technology can be applied to evolve entire viruses or vectors, including DNA vaccines. Cytokines downregulating allergic immune responses and allergens are attractive targets for evolution by molecular breeding. This review describes approaches to generate chimeric allergens with T cell epitopes from multiple allergen homologues, while reduc-

ing the recognition by preexisting IgE. In addition, the results and applications of molecular breeding in the evolution of improved antiallergic cytokines are discussed.

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The Principle of Molecular Breeding

Molecular breeding, also called DNA shuffling, is a group of in vitro and in vivo methods based on recursive recombination and mutation of DNA sequences, followed by selection of the best clones [1, 2]. The technology enables the generation of very large libraries of novel chimeric genes, vectors or genomes. In one format, the initial step is random fragmentation of two or more homologous genes, typically performed by DNase I digestion. The starting genes can be obtained for example from a single gene by random mutagenesis [1, 2], or they can be naturally existing homologues of a given gene [3]. The DNase I fragments are subsequently reassembled into full-length genes in a self-priming polymerase chain reaction (PCR). Because of the sequence similarity of the starting genes, a fragment derived from one gene can hybridize with a fragment from another gene, thereby creating chimeras that have components from multiple members of a gene family (fig. 1). In other words, the

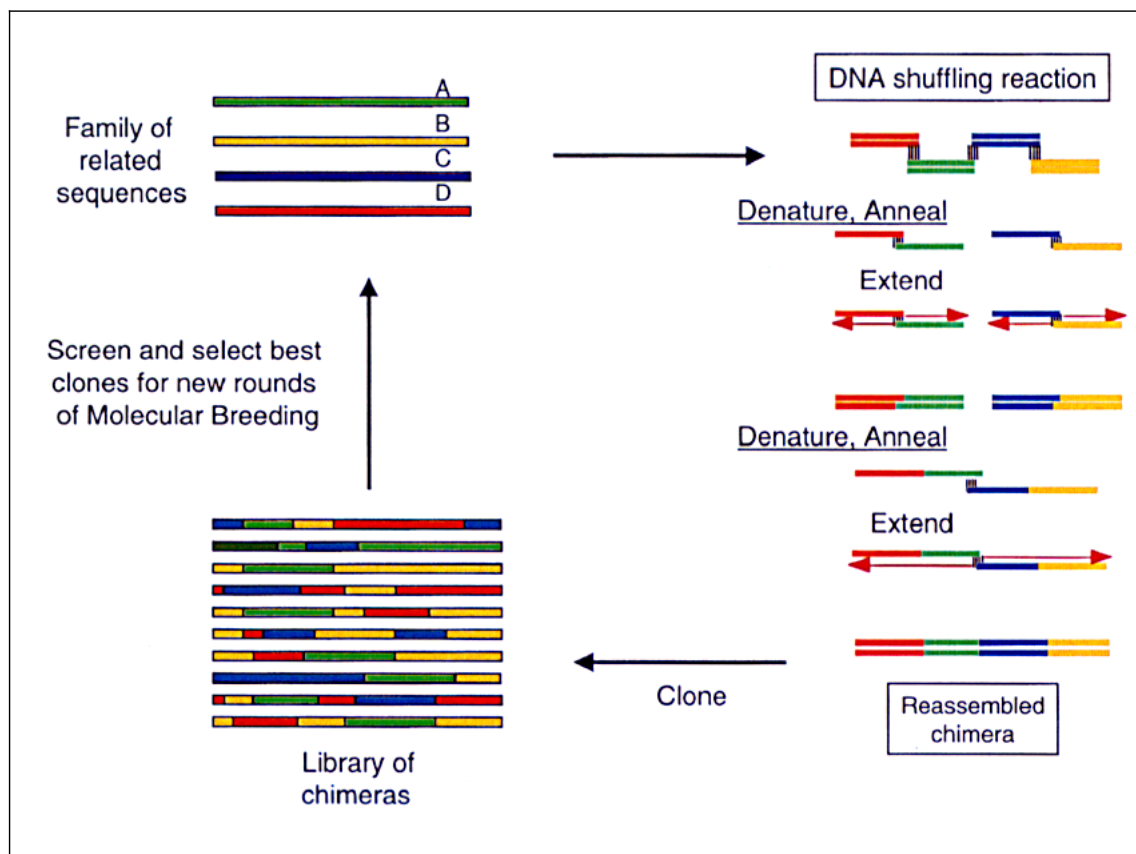


Fig. 1. An example of molecular breeding technology. A library of chimeric genes is in this example generated from four starting genes illustrated by different colors. The novel genes are subjected to a screening process to identify the best clones that are selected to new rounds of molecular breeding.

DNA fragments derived from the different starting genes act both as primers and templates, and template switching causes crossovers in areas of homologous sequences. As in natural evolution, molecular breeding takes advantage of deletions, insertions, inversions and point mutations of genes to generate large pools of related sequences. The recombination events that occur during the process enables one to combine beneficial mutations from the different starting genes.

Once the libraries of chimeric genes have been generated, they are subjected to a screening process to identify variants with the desired properties. The screening protocols need to be designed individually for each target to be evolved. However, it is typically useful to design multi-tiered screening processes, in which the first screening assay is meant to rapidly identify a group of highly active clones from the original library that typically contains a large diversity of genes, including some that are complete-

ly inactive. The second tier of screening is often more labor-intensive, but is designed to allow for more careful qualitative comparisons of the most active clones. The third tier of screening, when necessary, will confirm the levels of improvements as compared to the naturally existing genes. Efficient identification of the desired clones generated by molecular breeding is a critical component of the overall speed of the process. Carefully designed screening protocols combined with rapid library construction enables one to generate new generations of functional genes in a matter of months, rather than years, or decades, as is the case in natural evolution. The best clones from the first round of molecular breeding are typically subjected to further rounds of breeding and selection until the desired level of activity is obtained.

Natural Diversity as the Starting Gene Pool

Naturally existing gene homologues provide an excellent starting population of sequence diversity that can be recombined by molecular breeding to generate large libraries of novel functional genes. These naturally existing variants have already gone through thousands or millions of years of natural evolution, and, hence, have proven to be functionally viable genes. This increases the likelihood that the chimeric genes encode proteins that have functions similar to, and often better than, the starting genes.

The power of molecular breeding that starts with multiple naturally existing genes, also known as family DNA shuffling, was clearly demonstrated in experiments comparing molecular breeding of single cephalosporinase genes with the simultaneous breeding of four cephalosporinase genes. One cycle of single gene breeding yielded 8-fold increased activity against the antibiotic from the four separately evolved genes, versus a 270- to 540-fold increase from the four genes bred together [3]. This represents an approximately 50-fold increase in efficiency per cycle, demonstrating that trans-species recombination and breeding of natural diversity accelerates evolution.

Advantages of Molecular Breeding as Compared to Random Mutagenesis or Rational Design

Molecular breeding has several advantages as compared to random mutagenesis, performed for example by error-prone PCR. Error-prone PCR is based on amplification of genes under conditions that decrease the fidelity of DNA polymerase allowing increased frequency of mutations to be introduced into the amplified gene [4]. This technique has been successfully used to generate improved variants of several enzymes, antibodies and hormones [5–8]. However, because of the random nature of the process, error-prone PCR often generates deleterious mutations that completely destroy the capacity of the gene to encode proteins with proper structure and/or function [9]. Therefore, the beneficial mutations may often be masked by other mutations that destroy the capacity of the gene to encode functional proteins.

Molecular breeding has the capacity to combine beneficial mutations from multiple naturally existing variants of a given gene [10–13]. Because molecular breeding takes advantage of naturally existing functional genes, the process allows for the generation of libraries with a large diversity of functional mutants, some of which may be

very distant from the parental genes. For example, when cephalosporinase genes from four different organisms were bred, the most active variant contained sequence information from three different parents and shared less than 50% sequence identity with any of its three parents [3]. Such large changes in protein structure would hardly be accessible by any random approach. The fact that molecular breeding generates libraries with a high percentage of functional genes also significantly reduces the cost and time associated with identifying improved variants. Furthermore, complex biological screening assays are feasible, because relatively few gene products must be analyzed to detect an improvement in the starting gene activity.

When the regions of proteins that are the most critical for the function are known, site-directed mutagenesis can be an efficient means to generate the desired phenotype. For example, a potent antagonist of IL-4 was generated by introducing mutations to the receptor-binding sites of IL-4. The antagonistic IL-4 mutant protein was generated by replacing the tyrosine residue at position 124 by an aspartic acid using site-directed mutagenesis (hIL-4.Y124D) [14, 15]. hIL-4.Y124D inhibited IL-4- and IL-13-induced IgE synthesis in a dose-dependent manner, and more than 90% inhibition of IgE synthesis was observed when hIL-4.Y124D was added at 20- to 50-fold excess as compared to IL-4 or IL-13 [16, 17]. hIL-4.Y124D strongly inhibited IgE synthesis in SCID-hu BM/T mice both in the presence and absence of exogenous IL-4 [17–19], and an analogous mouse mutant IL-4 protein also prevented IgE-mediated allergic reactions in normal mice [20]. Thus, because the receptor-binding sites of IL-4 could be predicted, site-directed mutagenesis based on rational design proved to be an efficient means to generate potent antagonists of IL-4.

As hIL-4.Y124D illustrated, consistently successful rational design using site-directed mutagenesis requires a high level of understanding of structural and functional characteristics of the target molecules. In addition, the generation of large numbers of individual mutants is very labor-intensive and time-consuming. In contrast, molecular breeding does not require information of the structure-function relationships of the target molecules, and even the full-length DNA sequence does not need to be known. Moreover, molecular breeding often results in solutions that could not have been predicted based on knowledge-based approaches, because it does not rely on assumptions regarding the sites that are the most critical for the phenotype. For example, molecular breeding of green fluorescent protein (GFP) resulted in a variant that demon-

Table 1. Examples of improvements achieved by molecular breeding

Target	Level of improvement	Ref. No.
β-Lactamase	32,000 ×	1
Antibody binding	>440 ×	22
GFP	45 ×	21
Fucosidase activity	66 ×	23
Arsenate degradation	40 ×	24
Cephalosporinase	270–540 ×	3
Thymidine kinase	32 ×	11
Subtilisin	3 ×	12
IFN-α	185–285,000 ×	13
MLV	New activity	25

strated a 45-fold improvement in fluorescence [21]. Subsequent sequence analysis of the most active GFP variant identified mutations that changed hydrophobic residues into hydrophilic residues, suggesting that the fluorescence increased through improved solubility of the molecule [21]. Even if it could have been predicted that the fluorescence can be enhanced by improving the solubility of the protein, it would have been very difficult and time-consuming to rationally design mutants with improved solubility. These data further indicated that molecular breeding is an efficient and rapid means to find solutions to complex problems, even when little information is available regarding the mechanisms that limit the function of the target. Molecular breeding has been successfully used to generate improved variants of proteins, antibodies, enzymes and viruses, such as GFP, thymidine kinase, subtilisin, interferon (IFN)-α and murine leukemia virus (MLV) [1, 3, 11–13, 21–25] (table 1). As described in more detail below, we are currently conducting research towards the generation of improved variants of recombinant allergens, DNA vaccines, growth factors and immunomodulatory molecules by molecular breeding.

DNA Vaccines as Allergy Therapeutics and Applications of Molecular Breeding

DNA vaccines are attractive targets for molecular breeding and a promising approach to the development of next-generation allergy vaccines. DNA vaccines are stable and easy to manufacture, and preclinical and early-stage clinical studies suggest an excellent safety profile. DNA vaccines are also an attractive vaccine modality given the

feasibility of rapidly generating novel vaccine candidates by combining antigen sequences with various immunomodulatory molecules expressed by the same vectors, or codelivered in separate vectors.

Allergic immune responses are generally mediated by T helper 2 (Th2) cells that produce high levels of IL-4 and IL-13, cytokines which induce IgE isotype switching and differentiation of B cells into IgE-secreting cells [26–28]. Cytokines directing or enhancing T helper 1 (Th1) responses generally inhibit IgE synthesis in vitro and in vivo. For example, IL-12, which is the most potent cytokine directing Th1 differentiation, inhibits IL-4- and IL-13-induced T cell-dependent IgE synthesis in vitro [29, 30]. It also downregulates the primary IgE responses to *Nippostrongylus brasiliensis* as a result of general reduction in the Th2 response [31]. In addition, IL-18 (IFN-γ-inducing factor) synergizes with IL-12 in inducing Th1 differentiation [32], blocking IgE upregulation, airway hyperresponsiveness, and eosinophilic infiltration in the bronchoalveolar lavage after allergen challenge [33]. In addition, effective immunotherapy of allergic diseases is associated with an increased IFN-γ:IL-4 production ratio by allergen-specific T cells [34–37], further suggesting that an improved means to bias allergen-specific immune responses towards the Th1 phenotype will be beneficial in the treatment of allergic and asthmatic diseases.

Several studies suggest that DNA vaccines may efficiently direct allergen-specific Th2 responses towards the Th1 phenotype. In addition, DNA itself can act as an adjuvant. DNA extracted from *Mycobacterium bovis* BCG had potent immunostimulatory activities and protected mice against tumors, prevented metastasis and prolonged survival [38, 39]. It was then demonstrated that DNA derived from BCG augmented NK cell activity, activated macrophages and induced production of IFN-α/β and IFN-γ [40]. The effect was not specific for DNA derived from BCG, because DNA from 5 different bacterial strains and two viruses induced IFN-γ production by mouse spleen cells [41]. Moreover, these results were confirmed using synthetic CpG-containing oligonucleotides, indicating that the responses were not mediated by possible contaminants in bacterial DNA preparations [42, 43]. CpG-containing oligonucleotides were also subsequently shown to induce proliferation and differentiation of mouse B cells [44], and phosphorothioate oligodeoxynucleotides induced proliferation and Ig production by human B cells [45]. CpG-containing oligonucleotides, and DNA vaccines in general, favor Th1 responses in vitro and in vivo. CpG-containing phosphorothioate oligode-

oxynucleotides have been shown to skew differentiation of human *Dermatophagoides pteronyssinus* group 1 (Der p 1)-specific CD4⁺ T cells towards the Th1 phenotype in vitro [46]. DNA vaccines preferentially induce Th1 responses even when the same antigen induces potent IgE responses when delivered as purified protein [47–49], further suggesting that DNA vaccines will be particularly useful as allergy vaccines.

When DNA vaccine encoding the Hevea latex allergen Hev b 5 was administered subcutaneously into mice, the transcripts were simultaneously detected in multiple tissues [50]. Therefore, it may be beneficial to initiate genetic allergy vaccination with low doses, as is done in conventional immunotherapy. However, simply reducing the amount of DNA injected may not be sufficient, because only few cells expressing the antigen may be sufficient to induce anaphylactic reactions. Therefore, promoters with a range of activities are likely to be useful in the dose escalation of genetic allergy vaccines. We have generated libraries of promoter sequences by molecular breeding and a very broad range of promoter activities has been observed. Such libraries are likely to be beneficial in the development of vectors that induce different levels of antigen expression in vivo. Moreover, molecular breeding can also be applied to the entire vectors or vector components, such as the allergen sequence, cytokines or other immunomodulatory molecules, to generate more efficient DNA vaccines for allergy and asthma.

Evolution of Allergens by Molecular Breeding

The specific immunotherapy of allergy, allergy vaccination, is performed by subcutaneous injections of increasing amounts of the specific allergens. This approach has been shown to be highly effective in a selected group of patients with IgE-mediated allergies, such as respiratory allergies and bee venom allergy [37, 51, 52]. The therapy significantly suppresses airway hyperresponsiveness, and eosinophilic infiltration in the bronchoalveolar lavage and reduces the levels of circulating allergen-specific IgE antibodies, resulting in sustained reduction in frequency and severity of allergic symptoms [37, 51, 52].

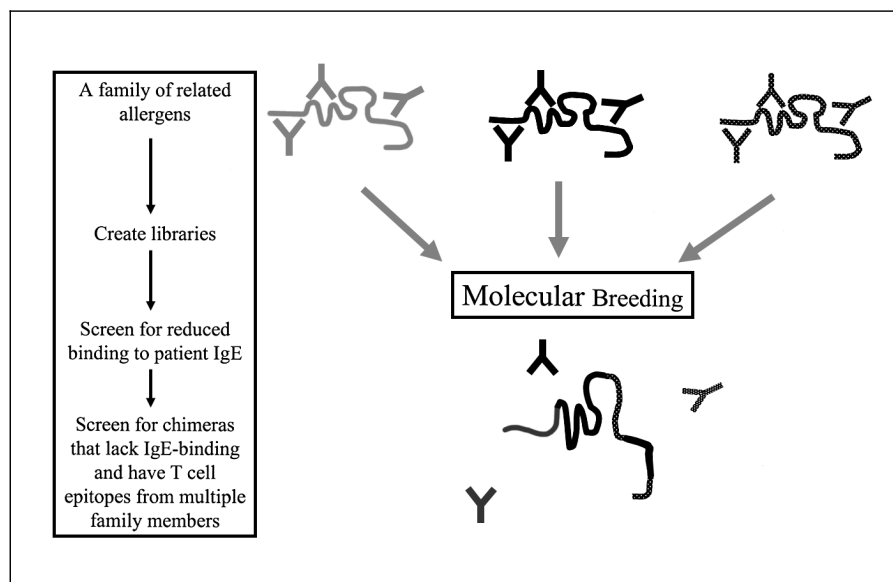
The exact mechanisms that mediate successful immunotherapy of allergy are still somewhat unclear, but it is widely accepted that the beneficial effects are at least partially mediated by modulation of T cell function. Immunotherapy has been shown to skew the phenotype of allergen-specific T cells towards Th1 [34, 35], and the treatment may also induce anergy of the specific T cells [52],

for example, through induction of IL-10 production by the T cells, as was shown to be the case in bee venom immunotherapy [53]. In addition, because CD8⁺ T cells can either induce or inhibit IL-4- or IL-13-dependent IgE synthesis depending on the ratios of IL-4, IL-13 and IFN- γ produced [54–57], modulation of the function of CD8⁺ T cells may also contribute to the efficacy of allergy vaccinations. Nevertheless, the fact that the mechanisms are mediated through modulation of T cell responses suggests that approaches to improve these therapies should focus on improving T cell recognition of the relevant immunogenic peptides and directing the subsequent T cell response towards the nonallergic phenotype. Molecular breeding has the potential to solve several of the problems associated with inadequate T cell response following allergy vaccinations.

Allergy vaccine development is hampered by the multitude of allergens and by the antigenic heterogeneity of these proteins. In addition, because it has been shown that the amino acid composition of immunogenic peptides can affect the cytokine synthesis profiles of the specific T cells [58], it is possible that allergens have selected peptides that favor Th2 differentiation. Therefore, many of the naturally existing allergens may have an intrinsic tendency to direct specific T cells towards the Th2 phenotype reducing their efficacy as allergy vaccines. Molecular breeding is an efficient means to generate chimeras that have peptides from multiple family members. For example, when the envelope genes of six related murine leukemia viruses (MLV) were shuffled, the chimeras that provided altered cell tropism, namely infectivity of CHO K1 hamster cells, contained epitopes from three different parental viruses. In the case of MLV envelope proteins, the chimeric antigens were also functional in that the viruses were able to infect host cells [25, 59]. However, in the case of allergy vaccines, the proteins do not need to retain their natural properties suggesting that the frequency of useful chimeras may be even higher.

Although relatively little is known about the properties that affect the immunogenicity of allergens and antigens in general, and how individual amino acids affect the function of the specific T cells, antigens are likely to contain regions that both negatively or positively affect antigen presentation. Moreover, the amino acid composition and the length of the immunogenic peptides can dramatically affect their affinity to the transporters associated with antigen presentation, which are required for the transport of antigenic peptides to the MHC class I molecules [60, 61]. Importantly, because the factors that affect the immunogenicity and peptide presentation of antigens

Fig. 2. An example of evolution of allergens by molecular breeding. Libraries of chimeric genes are generated and negative selection is used to delete chimeras that are recognized by patient IgE. The remaining variants are screened for their capacity to modulate T cell proliferation and cytokine synthesis.



are incompletely understood, rational design is unlikely to have a broad impact on the generation of more immunogenic antigen variants. Molecular breeding does not rely on a priori assumptions of the mechanisms that limit the processes, and therefore, provides an approach that has the potential to simultaneously combine epitopes from multiple family members of allergens and improve the immunogenicity of the antigens through enhanced presentation of the relevant peptides. Analysis of immunogenic properties of allergen variants can be performed using human peripheral blood T cells, because preexisting immunity is the target of the therapy (fig. 2). The optimal allergen variants, based on in vitro studies using patient T cells, can be further tested in vivo by studying delayed type hypersensitivity after injections to the skin of allergic patients.

Molecular Breeding of Allergens with Reduced Risk of Anaphylaxis

Anaphylactic reactions are a major risk factor in the immunotherapy of allergic diseases. Mast cells, basophils and eosinophils are key cells in inducing IgE-dependent allergic symptoms, including anaphylactic reactions. Recognition of specific antigen by IgE bound to high-affinity IgE receptors on mast cells, basophils or eosinophils results in cross-linking of the receptors leading to degranulation of the cells and rapid release of mediator mole-

cules, such as histamine, prostaglandins and leukotrienes, causing allergic symptoms. Although immunotherapy of allergy is generally initiated using very low allergen concentrations, significant quantities of the antigens may reach mast cells and basophils potentially binding to the IgE present on these cells.

Molecular breeding can be used, not only to generate allergen chimeras/variants that have T cell epitopes derived from several family members as described in the previous section, but also to reduce the likelihood that antigens are recognized by the preexisting IgE antibodies of the patients. Shuffled allergen variants that are not recognized by IgE antibodies can be selected using patient sera and negative selection (fig. 2). Previous studies have demonstrated the feasibility of engineering of allergen variants with reduced binding to patient IgE by introducing deletions and mutations outside T cell epitopes [62–64]. Subsequent analysis using T cell assays in vitro enabled the identification of variants that efficiently activate specific T cells [62–64]. Allergens with reduced IgE binding were also shown to be efficacious in mouse models of immunotherapy [65]. Molecular breeding can generate a large diversity of such allergen variants, from which the optimal chimeras can be selected based on functional assays (fig. 2). These second-generation allergy vaccines may allow for treatments with improved efficacy and therapeutic index with reduced risk of anaphylactic reactions.

Improved Antiallergic Cytokines by Molecular Breeding

Cytokines that either modulate Th differentiation or directly block IgE switch process in B cells provide an attractive approach to the treatment of allergic diseases [66]. IL-12 and IL-18 strongly enhance Th1 cell differentiation and IFN- γ production, which was shown to correlate with reduced IgE synthesis in vitro and in vivo [29, 30, 33, 67]. IFN- α , IFN- γ and TGF- β are the only cytokines that directly inhibit the ϵ switching process by modulating the level of germline ϵ transcription [68–70], and therefore, they provide an alternative approach to prevention of allergic immune responses. IL-10 inhibits IgE synthesis by human peripheral blood mononuclear cells by suppressing the accessory-cell function of monocytes [71], and IL-10 production by allergen-specific T cells is upregulated after successive immunotherapy [53], suggesting that IL-10 may also have therapeutic potential in the treatment of allergic diseases. Other cytokines that have shown antiallergic effects in vitro or in animal models include IL-8 [72], vasoactive intestinal peptide [73, 74] and glycosylation-inhibiting factor [75, 76]. Furthermore, antagonists of IL-4, IL-5 and IL-13 have been shown to inhibit IgE synthesis and allergic immune responses in vitro and in vivo [17–19, 77–79], and therefore these molecules also are attractive targets for generation of improved antiallergic treatments.

We chose IFN- α as the first target cytokine to study the feasibility of applying molecular breeding towards developing improved variants of cytokines. IFN- α is a promising antiallergic cytokine, because it acts both by supporting Th1 differentiation [80, 81] and by directly inhibiting IL-4- and IL-13-induced germline ϵ transcription and IgE switching in B cells [27, 70, 82, 83]. Moreover, IFN- α has been shown to have beneficial effects in some patients with hyper-IgE syndrome [84]. However, it is likely that the properties of IFN- α need to be further improved in order to obtain consistently beneficial clinical effects.

Molecular breeding of human IFN- α genes was used to generate libraries of IFN- α variants. The human genome contains a family of over 20 tandemly duplicated genes encoding IFN- α molecules that have 85–98% sequence identity at the amino acid level, providing an excellent starting population for further improvement by molecular breeding. Natural homologues of IFN- α were directly isolated from human genomic DNA by PCR, and the libraries of chimeras were generated by molecular breeding [13]. Natural human IFN- α has very weak activity on mouse cells, which is consistent with the fact that the

mouse IFN- α receptor is only 49% identical with that in humans [85]. However, already after a single round of molecular breeding, human IFN- α variants that had potent activity on mouse cells could be identified. Importantly, because the clones were pooled prior to being screened, only a total of 68 murine antiviral assays were required to screen a library of 1,672 clones [13]. The clones with the highest antiviral activity from the first round of breeding were selected for a second round, and new libraries of chimeras were generated. The second round of breeding was anticipated to allow for the combination of beneficial mutations from different variants. This indeed appeared to be the case, as further improvements in antiviral activity were achieved after the second round of breeding [13]. The best-shuffled chimeras after only two rounds of molecular breeding had a 185-fold improved activity for the murine receptor as compared to the most potent wild-type IFN- α , and they were 285,000-fold improved relative to IFN- α 2a, which is clinically the most widely used IFN- α type [13]. These data indicate that molecular breeding can be successfully used to modulate the properties of cytokines, and suggest that the technology will also be useful in generating more potent variants of antiallergic therapies.

Concluding Remarks

Although drug treatments to relieve the symptoms of allergic patients have become more and more efficient during the recent years, improved means to treat allergic diseases are highly desired. Since specific immunotherapy, allergy vaccination, can permanently reduce the frequency of symptoms in allergic individuals [37], it is a very attractive means to treat allergy. DNA vaccines may provide an improved method to direct Th1 responses, and therefore, may further boost the efficacy of allergy vaccinations. Molecular breeding has the potential to significantly contribute in many ways to the development of more efficacious and safer forms of immunotherapy. We are conducting studies directed towards the generation of improved antiallergic cytokines, DNA vaccine vectors and chimeric allergens by molecular breeding. These second-generation cytokines and allergens may provide improved efficacy and reduced risk of anaphylactic reactions following allergy vaccinations.

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