

Cloning allergens via phage display

Claudio Rhyner,^a Michael Weichel,^a Sabine Flückiger,^b Stefanie Hemmann,^c
Tamara Kleber-Janke,^c and Reto Cramer^{a,b,*}

^a Swiss Institute of Allergy and Asthma Research (SIAR), Obere Strasse 22, CH-7270 Davos, Switzerland

^b BioVisioN Schweiz AG, Promenade 132A, CH-7260 Davos-Dorf, Switzerland

^c BioVisioN AG, Feodor-Lynen Strasse 5, D-30625 Hannover, Germany

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Abstract

Although an impressive list of allergenic structures has been elucidated during the last decade by classical cloning methods, the size of the repertoire of molecular structures able to elicit allergic reactions is still unknown. Selective enrichment of cDNA libraries displayed on phage surface with serum IgE from allergic individuals combined with robotic-based high-throughput screening technology has proved to be extremely successful for the rapid isolation of allergens. The basic concept of linking the phenotype, expressed as gene product displayed on the phage coat, to its genetic information integrated into the phage genome, creates fusion proteins covalently associated with the infectious particle itself. Therefore, cDNA libraries displayed on phage surface can be screened for the presence of specific clones using the discriminative power of affinity purification. The selection of IgE-binding clones involves the enrichment of phage binding to serum IgE immobilised to a solid phase during consecutive rounds of affinity selection. As a consequence of the physical linkage between genotype and phenotype, sequencing of the DNA of the integrated section of the phage genome can readily elucidate the amino acid sequence of the surface-displayed allergen. In spite of some biological limitations imposed by *Escherichia coli* as expression host, phage surface display technology has strongly contributed to the rapid isolation of a vast variety of IgE-binding structures.

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1. Introduction

A common hallmark of allergic diseases is the production of allergen-specific IgE raised against normally innocuous environmental antigens [1,2]. In sensitised atopic individuals, allergen exposure induces cross-linking of high-affinity FcεRI receptor-bound IgE on effector cells and, thus, immediate release of anaphylactogenic mediators [2]. Although the mechanisms leading to allergic reactions are quite well understood [3,4], our knowledge about the repertoire of molecular structures involved in the pathogenesis of allergic reactions is still fragmentary. This is mainly due to the complexity of most of the allergenic sources [5] and to the ability of the immune system of atopic individuals to mount IgE immune responses to a wide variety of different molecular

structures. Prominent examples of extremely complex allergenic sources are *Aspergillus fumigatus* (*A. fumigatus*) and *Dermatophagoides pteronyssinus* (house dust mite) reported to contain 81 and 16 IgE-binding proteins, respectively [6,7]. Although the incidence of sensitisation against a given allergenic structure can vary considerably, also allergens with a low prevalence of sensitisation might be clinically relevant as cross-reactive structures. This is best exemplified by profilin, a minor allergen first cloned from birch [8] and later demonstrated to be an important allergen in many sources [9,10], and ribosomal P₂ protein of *A. fumigatus* shown to cross-react with the corresponding human protein [11]. After the first report of a cloned allergen in 1988 [12], many allergens have been cloned, characterised, and produced as recombinant proteins. Sequence information and references can be accessed through a database inventoried by the Allergen Nomenclature Sub-Committee of the International Union of Immunological

* Corresponding author. Fax: +41-81-410-08-40.

E-mail address: cramerir@siar.unizh.ch (R. Cramer).

Societies (www.allergen.org). To maintain the integrity of the nomenclature system, only allergens that meet one of the two following criteria are included in this list: (I) A demonstrated prevalence of IgE reactivity above 5% or (II) A minimum of five patients showing IgE reactivity, regardless of the number of patients investigated [13]. However, a distinction between an allergen as defined molecular structure and allergen extracts, though often called allergens in clinical practice, should be made. The term allergen should only be used for single, pure proteins which are able to bind IgE *in vitro* and to elicit immediate type skin reactions *in vivo* [14].

The progress in applying molecular biology and genetic engineering in the last decade strongly contributed to the rapid development in the field of molecular allergology. The fastest method to clone an allergen is amplification of the coding sequence from reverse transcribed mRNA by PCR [15–17]. However, direct cloning of allergens by PCR requires sequence information, at least from the N-terminal amino acid sequence, which is not available in the normal case. Therefore, classical methods for the screening based on bacteriophage lambda [18,19] have been adapted to screen cDNA expression libraries constructed with mRNA of different allergenic sources using serum IgE from sensitised individuals to detect allergen-expressing clones [20–22]. λ -based cloning strategies have been successful in generating information about the sequence of many allergens; however, efficient handling of large libraries is greatly facilitated if screening can be based on selective enrichment of clones expressing genes of interest [23–25]. The basic requirement for selective enrichment of clones consists in the physical linkage between genotype and phenotype [26,27]. This requirement is not fulfilled by λ -based cDNA libraries where genetic information, integrated into the phage genome, and expressed gene products produced exploiting the machinery of the host cell during the lytic process, are not physically linked [28]. Therefore, these kinds of libraries require immobilisation to solid phase supports for screening, hampering selective enrichment of clones by specific gene-product/ligand interaction [18,19,24]. In contrast, filamentous phage engineered to display gene products on their coats contains the DNA encoding the displayed gene product as a part of their single-stranded genome [29], providing a physical link between genotype and phenotype (Fig. 1). This

allows the survey of large libraries for the presence of specific clones using the discriminative power of affinity purification. In this contribution, we describe the application of phage display technology as an efficient method for rapid cloning of allergenic molecules.

2. Phage display technology

The basis of biological display technology relies on the physical link between the displayed entity and the gene coding for the displayed entity, thus connecting genotype and phenotype [30]. As a consequence thereof, it is possible to isolate members fulfilling specific criteria through an iterative “Darwinian” selection process from large and diverse repertoires cloned into filamentous phage [31]. Filamentous phages represent relatively simple biological systems, which are able to infect gram-negative bacteria through a specific interaction with the tip of the F pilus, and are therefore referred to as Ff phage (for a review, see [32]). The genome of Ff phage consists of a single-stranded covalently closed 6.4 kb DNA molecule encoding eleven genes. They are grouped on the genome according to their functions in the life cycle of the phage and encode five capsid proteins, three proteins responsible for the assembly of the phage, two proteins involved in DNA replication, and one protein binding single-stranded DNA [33]. The simple genomic architecture and size, together with the well-understood biology, make Ff phage ideal for the development of cloning vectors. In his pioneer work, Smith [26] realised that a fusion to phage coat proteins would create unusual proteins because they would be covalently associated with the infectious particle itself. During the first years after proposition of phage display technology, applications were limited to construction and screening of random peptide libraries, enabling the selection of small peptides able to interact with antibodies and with other target molecules [34]. Since 1990, phage display has rapidly evolved and has become a widely used technology in life sciences. The success of phage display is due to two main reasons: (a) the linkage between genotype and phenotype allowing screening of large libraries based on the power of affinity selection and (b) the possibility to construct large diverse synthetic or naïve combinatorial libraries. This is highlighted by the rapid progress obtained in the field of antibody technology recently reviewed [35]. The combinatorial strategy in antibody engineering involves random recombination of light chain and heavy chain fragments to generate Fab molecules [36] and has gained wide applications in combination with the power of filamentous phage display [30]. The construction of libraries from naïve repertoires has led to the generation of antibodies without immunisation [37] and to the first molecular analysis of a human immune response [38].

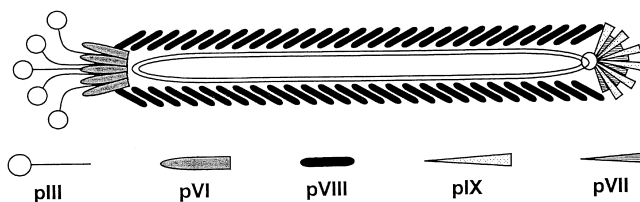


Fig. 1. Schematic representation of filamentous bacteriophage M13 showing the coat proteins usable for surface display of fusion proteins (according to [32]).

The development of semi-synthetic combinatorial and synthetic human antibody libraries, built from cloned, sequenced, and mapped human germline immunoglobulin genes, keeps great promise for rapid progress in clinical applications [39]. Besides antibodies, enzymes, enzyme inhibitors, receptors, hormones, lymphokines, and DNA-binding molecules have been target molecules displayed on phage surface [40]. Therefore, phage display technology has advanced to an industrially important technology with a great potential of applications in functional genomics [30,39,41].

Although application of phage display to screen cDNA libraries has been suggested from the beginning as a significant application of the technology, this potentially interesting area of research has lagged behind the impressive progress achieved in other fields. Among the over 2000 papers describing the use of phage display available to date from the literature, only a few deal with selection of cDNAs for the reasons discussed below.

3. Description of methods

The most common approach used to link phenotype and genotype has been to fuse the displayed molecule as an N-terminal extension of the viral pIII coat protein [34]. Although phage display has been used extensively for selection of peptides and antibodies, it has its limitations when applied to the expression of unknown sequences from cDNA libraries. As many of these sequences contain stop codons in their 3' untranslated regions, it is not feasible to directly fuse these sequences to the N-terminus of the pIII or pVIII coat proteins. To overcome these problems, a cloning and expression system which allows the display of functional cDNA expression products on the surface of filamentous bacteriophage has been developed [23,24,42].

3.1. Vector construction

As a backbone for the construction of phagemid pJuFo, the pComb 3 phagemid [43] which contains two independent lacZ promoters was used. In a first step, a modified Jun leucine zipper was fused with the N-terminal domain of the truncated form of pIII present in pComb 3. At the N- and C-termini of the Jun leucine zipper, cysteine residues were added via a diglycine spacer and the Jun-pIII fusion was placed under the control of a lac promoter/operator element preceded by the pelB signal peptide to direct the produced fusion protein to the periplasmic space of *Escherichia coli*. During helper phage superinfection, the Jun-pIII fusion becomes incorporated into the virion in competition with wild type pIII resulting in packaged phagemid particles which contain both, the wild type pIII product necessary for infection and the modified Jun-pIII fusion

protein [23,43]. cDNAs to be captured on the outer phage surface were linked to the C-terminus of a modified Fos leucine zipper flanked by cysteine residues added via GlyGly spacers at the N- and C-termini. The cDNAs to be expressed with Fos were placed under the control of a separate lac promoter/operator element and directed to the periplasmic space of the *E. coli* host by the pel B signal sequence. Due to the high affinity between Jun and Fos [44] and to the favourable chemical conditions in the periplasmic space, phagemid particles decorated with the Jun leucine zipper become associated with the Fos-decorated cDNA products to form heterodimers stabilised by the formation of disulphide bridges within the pairs of engineered cysteine residues [23,45]. In this way, cDNA products become displayed on the surface of the phagemid particles and become available for affinity interactions with solid phase immobilised ligands [24,45–48]. The original pJuFo expression system has been modified further to allow direct subcloning of commercially available cDNA libraries constructed into λ -Zap II vectors [42,49]. To allow cDNA cloning using *EcoRI* and *XhoI* as restriction enzymes, the pJuFo vector was re-modelled. The internal *EcoRI* site of the vector upstream of the pelB-leader of the Fos-controlling promoter was deleted with a standard Klenow fill-in reaction [28]. The first internal *XhoI* site inside the coding region of Jun was eliminated by conservative in vitro mutagenesis of the first triplet of the *XhoI* site from CTC to CTT. The second internal *XhoI* site upstream of the pelB-leader controlling the Jun-unit was deleted by digesting pJuFo with *XhoI* and *SalI* followed by re-ligation of the phagemid. In a last step, the internal cloning sites *XbaI* and *KpnI* were changed to *EcoRI* and *XhoI*, respectively, to generate the pJuFo II vector [42,50].

3.2. Construction of libraries displayed on phage surface

cDNAs to be co-expressed with Fos can be cloned as *BglII/XbaI*, *BglII/KpnI* or *XbaI/KpnI* fragments into the pJuFo vector [23]. The first cDNA phage surface display library constructed in pJuFo was generated by ligation of cDNA inserts prepared from an *A. fumigatus* cDNA library previously constructed in λ -Zap II by *XbaI/KpnI* restriction purified by agarose gel electrophoresis [24]. The same procedure was used to construct cDNA libraries from peanut [53], *Coprinus comatus* [54], and human lung [55]. Inserts from λ -Zap II libraries are easy to prepare due to the possibility to excise the p-Bluescript phagemid in vivo [49]. However, inserts prepared by this procedure contain a 5'-overhang encoding the amino acids SerArgThrSerGlySerProGlyLeuGlnGluPheGlyThr(Arg/Ser) derived from the multiple cloning site of p-Bluescript and from the *EcoRI* adaptor. To avoid this extension, an *XbaI* adaptor was directly ligated to cDNAs prepared from *Malassezia furfur* [56]

and *Lepidoglyphus destructor* [57] using standard methods [28], allowing direct subcloning of the cDNAs into *XbaI/XhoI*-restricted pJuFo. The new pJuFo II phagemid allows rapid shuffling of all inserts present in commercial λ -Zap II libraries by directional cloning into *EcoRI/XhoI*-restricted vector [42,49] as well as construction of any cDNA library generated with the λ -Zap II cloning kit (Stratagene, La Jolla, CA) and has been used to construct phage surface-displayed cDNA libraries from *Alternaria alternata* [58], *Cladosporium herbarum* [59,60], wheat germ [61], mouse B cells [50], and many other sources. In all cases, pJuFo/cDNA ligation mixtures were transformed into *E. coli* XL1-Blue cells (Stratagene) by electroporation to generate phage surface display libraries by helper phage superinfection. All methods used are standard methods used in molecular biology and do not require any special equipment [42,62].

3.3. Screening of phage surface-displayed libraries

The Fos/cDNA fusions, linked to the outside of the phage capsid through Jun/Fos heterodimerisation, become solvent exposed. Therefore, phage displaying any given gene product can be affinity purified from non-binding phage by iterative panning cycles against immobilised ligands [24,29,48]. The screening procedure is, in contrast to the screening of λ -based libraries, fast and easy to perform. The most important consideration of phage screening is that the ligand used to screen the phage library is tagged or immobilised in a way allowing to retain its biological function [45]. In general, the known or expected characteristics of the ligand will dictate the immobilisation procedure used. To clone IgE-binding molecules, the panning procedure was optimised to enrich phage displaying allergens on their surface [23,24]. Briefly, monoclonal mouse anti-human IgE is coated onto polystyrene microtitre wells and used to capture IgE from serum of individuals suffering from allergy. This procedure allows the generation of a specific IgE-ligand surface depleted from interacting and cross-reacting antibodies of other isotypes present in the polyclonal sera [24]. To enrich for IgE-binding phage, approximately 10^{11} colony-forming units of the library is added to a single well and incubated for 2 h at 37 °C to allow binding. After removal of phage and extensive washing, binding phages are eluted from the well by different strategies including acidic buffer, competitive elution or a cleaving enzyme specific to a site cloned in the pIII molecule [52]. To prepare phage for a further round of affinity enrichment, eluted phages from the former round are simply used to infect exponentially growing *E. coli* XL1-Blue cells to amplify specific phage. Several rounds of affinity enrichment are required to select specific phagemids from background binding. Enrichment depends on many factors including the

quality of the library, the affinity of the ligand for the target, and the number of specific ligands available for a specific target. Therefore, enrichment of a phage surface display library using complex ligands like human serum will depend on the percentage of specific ligands (e.g., antibody) present in the serum used. It is almost impossible to select specific phagemids from background if binding of a wanted gene product to its specific ligand is limiting compared to the unspecific binding [52]. In spite of all the limitations intrinsic to the system, phage surface-displayed cDNA libraries have been successfully used to isolate many genes. Although most of the cDNA libraries constructed and screened using pJuFo vectors were devoted to the isolation of IgE-binding molecules from complex allergenic sources [53–61], many other applications have been reported [29,40,46,50,63–66] demonstrating the versatile applicability of the pJuFo cloning system.

3.4. Towards high-throughput screening technology

Enrichment of phage surface-displayed cDNA libraries for IgE-binding phage allows rapid isolation of phage populations containing multiply represented clones [24,39]. The absolute number of clones displaying the same gene product among billions of clones obtained after amplification will depend on many factors including the number of clones displaying the product present in the original library and the number of allergen-specific IgE molecules present in the serum used for screening. Therefore, identification of clones encoding allergens derived from rare mRNA species or from allergens eliciting weak IgE responses and thus resulting in low amounts of allergen-specific IgE in serum remains a major problem [29,40]. To exploit the full potential of molecular libraries, new high-throughput screening formats are required. A strategy based on a combination of phage display and high-density arrays for fast identification of all different clones present in selectively enriched phage surface-displayed cDNA libraries has been developed [6]. We adapted robotics-based automatic devices for picking and high-density spotting of clones onto filter membranes, originally developed for automated library handling in genomics [29], to screen cDNA libraries displayed on phage surface [46–48]. Briefly, enriched phagemid libraries were amplified in bacteria, plated, and arrayed into 384-well microtitre plates by robotic colony picking [6,29]. cDNA inserts of arrayed libraries were amplified by high-throughput PCR and gridded in duplicate onto high-density filter membranes (Fig. 2). Filters were iteratively probed with digoxigenin-labelled, randomly sequenced inserts until all clones were identified. As an example, high-throughput screening of 13,440 clones from an enriched *A. fumigatus* cDNA library allowed us to assign 99.4% of the clones to a discrete sequence after three rounds of

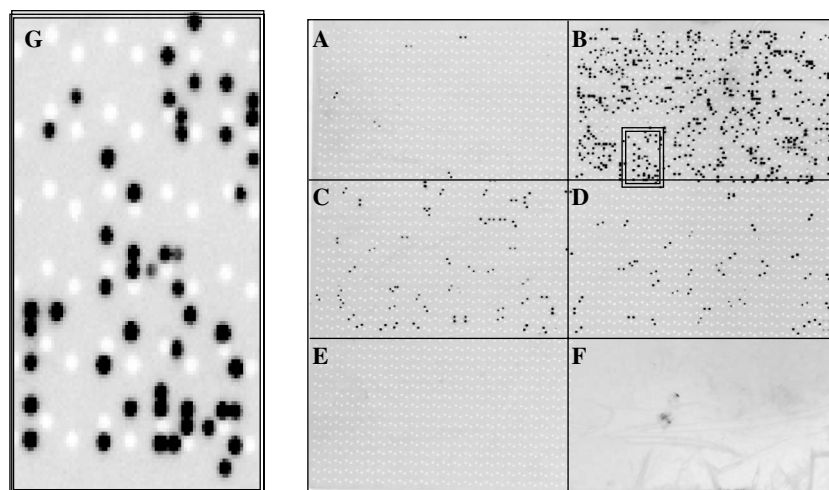


Fig. 2. Hybridisation of high-density arrays of spotted clones enriched from a cDNA library of *A. fumigatus* expressed on phage surface with a digoxigenin-labelled probe derived from the Asp f 1 allergen. (A–E) Arrays of 2688 clones spotted in duplicate obtained from enrichment of the library with sera of five individual patients. (F) Hybridisation background of the filter in the absence of spotted clones. (G) Magnification of the segment from B, showing positive clones spotted in duplicate around the matrix dots (white spots).

hybridisation and 153 sequencing reactions [6]. Eighty-one different sequences encoding IgE-binding proteins likely to cover the main allergen repertoire of the mould were found. The technology, based on phage display of cDNA libraries and robot-assisted handling of enriched phagemids, permitted a fast identification of virtually all different clones present in the enriched library, drastically reducing the manual work involved. The consequent application of functional enrichment with serum IgE of allergic patients and robotic-based screening will allow us to rapidly generate information about the whole repertoire of different molecular structures able to elicit allergic reactions. This will be especially important because recent work indicates that the epitope complexity of natural allergen extracts can be re-created using recombinant allergens [67], demonstrated to be more specific and sensitive than extracts for the diagnosis of allergic conditions in clinical practice [68].

4. Performance and limitation of phage display

The power of the phage display technology relies on the linkage of the functional display of molecular libraries with the ability of each member in the library to self-replicate and, at the same time, to encode the nucleotide sequence of the displayed polypeptide in its genome. The physical link between phenotype and genotype allows the fast isolation of desired clones through consecutive rounds of affinity enrichment [23,42,43]. The selection format is usually based on biopanning of the libraries on solid phase surfaces carrying immobilised ligands [34], affinity chromatography [45] or magnetic bead selection [51]. During selection, those clones able to bind to the target are preferentially

enriched from the large number of unspecific clones present in the library. The relatively small number of selected clones is amplified to generate a new population of phages for further rounds of selection. These procedures suffer from several limitations and biases including non-specific binding, washing and elution efficiency, differences in the ability of the eluted phages to re-infect and propagate in *E. coli*, and the small number of clones that can be manually analysed from the large enriched populations containing multiply represented clones [52]. The last point may be the most limiting factor in surveys of cDNA libraries enriched with complex ligands such as serum IgE. Complex allergenic sources contain large numbers of different IgE-binding structures and, on the other hand, the immune system elicits polyclonal responses to each single structure. As a consequence thereof, immobilised IgE from serum as ligand is highly heterogeneous and contains unknown amounts of allergen-specific IgE raised against the allergen mixture. Therefore, single clones enriched from cDNA libraries using IgE as ligand are expected to be multiply represented in the large phagemid population obtained after repetitive rounds of selection [29,40]. The relative enrichment of each single clone will depend, among other parameters, on the frequency of a specific clone in the original library and on the number of native clone-specific IgE molecules immobilised to the solid phase. Manual selection of clones, although successfully employed [53–61], is unlikely to be efficient in detecting rare clones. To circumvent this problem, high-throughput screening technology allowing a sufficient degree of parallelisation is required [6,29,39,46–48]. The combination of selective enrichment and automated robot technology for picking and high-density spotting of clones onto filter membranes has proved to be very

efficient for the isolation of virtually all allergen-specific cDNA clones present in libraries selected with serum IgE [6,46–48]. However, phage surface display technology suffers from biological limitations inherent to the system itself. Obvious biological limitations are the restricted codon usage of *E. coli* as host, the lack of post-translational modifications [69], and the potential toxicity of some expressed gene products for the host [70]. However, further and more serious limitations of phage display derive from the characteristics of the phage life cycle and from the limited folding capacity of *E. coli*. Filamentous phages are released from the host without breaking the integrity of the cell membrane and thus, in contrast to lytic phages, heterologous fusions to phage coat proteins must cross the lipid bilayer of the inner membrane [71]. As a consequence thereof, any fusion to coat proteins with peptides or proteins unable to cross the membrane will stop transfer of the hybrid capsid protein to the periplasm and will therefore not be assembled into the phage coat. In fact, display of transmembrane proteins such as receptor molecules, cell wall proteins or other structures embedded into membrane bilayers has never been reported to be found by screening of phage surface-displayed cDNA libraries. However, strictly intracellular proteins like mitochondrial manganese dependent superoxide dismutase [72], acidic ribosomal P₂ protein [11] or nuclear transport factor 2 [58] can be selected from phage surface-displayed cDNA libraries making the technology attractive for many applications. Although an upper size limit for a protein to be displayed on phage surface has to be expected from the outer membrane “pore” size that permits the exit of assembled phage, proteins up to 86 kDa have been shown to be functionally displayed on phage surface [73].

5. Concluding remarks

Since the first description of phage display in 1985 [26], phage vectors have been used to display and select a variety of ligands, but the potential of phage as vectors for cDNA cloning was not a main focus of interest. Recently, phage vectors have been remodelled and developed for the expression of cDNA libraries fused to the gIII [23,29,40] or to the gVI genes [74] and used to clone genes of interest by affinity enrichment of phage displaying the desired gene products [50,63–65,75]. Historically cDNA libraries displayed as gIII-fusions have been successfully used to clone IgE-binding proteins [53–61]. The repertoire of allergen structures cloned by phage display covers secreted, cytoplasmic, and surface proteins with or without enzymatic activity as well as structural and storage proteins. Notably, all the IgE-binding structures previously isolated from various complex allergenic sources by classical methods have been detected, among a

vast variety of new structures, by combining phage surface display and robotic-based high-throughput screening technology [6,47,48]. In spite of many limitations related to the use of *E. coli* as heterologous host and restrictions imposed by the life cycle of filamentous phage, display of cDNA libraries on phage surface has been a very successful method for rapid isolation of allergenic molecules. Many of the recombinant allergens have been evaluated for their diagnostic performance in vitro [14,76] and in vivo [14,68]. The recombinant proteins have been shown to be superior to conventional extracts in terms of specificity and as a diagnostic tool to elucidate patient- and disease-specific sensitisation patterns [77,78]. Therefore, recombinant allergens bear a great potential for improvement of the current practice of specific immunotherapy and, perhaps, for the development of prophylactic vaccines.

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