Ann Microbiol (2010) 60:13–19
DOI 10.1007/s13213-009-0014-7

REVIEW ARTICLE

Phage display and its application in vaccine design

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Received: 8 October 2009 / Accepted: 17 December 2009 / Published online: 22 January 2010

Abstract This review focuses on phage display and its application in vaccine design. Four kinds of phage display systems and their characteristics are highlighted. Whole phage particles can be used to deliver vaccines by fusing immunogenic peptides to modified coat proteins (phage-display vaccination), or by incorporating a eukaryotic promoter-driven vaccine gene within the phage genome (phage DNA vaccination). Hybrid phage vaccination results from a combination of phage-display and phage DNA vaccination strategies, indicating the potential for evolution of phage vaccines. Phage vaccines could provide a key to unlock new approaches in combating bacterial and viral pathogens, and cancer diseases. New phage display systems will certainly emerge because of the global abundance of phage and our increasing ability to exploit them. The scope of phage display applications will continue to expand. Over the ensuing period, research should be directed toward a better understanding of the immunization mechanisms involved in phage-mediated immunization, and the development of hybrid phage vaccination.

Keywords Bacteriophage · Phage display · Phage-display vaccination · Phage DNA vaccination · Hybrid phage vaccination

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Introduction

Bacteriophages (phages) are viruses of bacteria consisting of a DNA or RNA genome contained within a protein coat. Their growth and proliferation require a suitable prokaryotic host. They either incorporate viral DNA into the host genome, replicating as part of the host (lysogenic), or propagate inside the host cell before releasing phage particles either by extruding from the membrane (as with filamentous phages) or by lysing the cell (lytic phages; Clark and March 2006; Petty et al. 2007). Phages do not replicate in eukaryotic hosts and act as inert particulate antigens metabolically. Being particulate antigens, bacteriophages are processed by antigen-presenting cells (APC), cleared from the circulation and targeted to the spleen and liver Kuppfer cells. Nowadays, the applied use of bacteriophages is clearly visible in techniques such as phage therapy, phage display, DNA vaccine delivery, therapeutic gene delivery, bacterial typing, and so on (Clark and March 2006). Recently, whole bacteriophage particles have been described as highly efficient DNA vaccine delivery vehicles (Clark and March 2004a; Jepson and March 2004; March et al. 2004). This review will focus on phage display and its application in vaccine design.

Phage display

In phage display, a foreign peptide or protein is expressed on the phage surface through transcriptional fusion with a protein coat gene. The principle underlying phage display technology is the physical linkage of the phenotype of a polypeptide to its corresponding genotype. The first report of phages displaying foreign peptides on their surface was published in 1985 (Smith 1985). The first use

of whole phage particles to elicit an immune response against displayed foreign peptides was in 1988 (de la Cruz et al. 1988). Screening phage libraries has emerged as a powerful technology for selecting polypeptides with desired biological and physicochemical properties. (Paschke 2006). Somers et al. (2002) constructed a cell-line based cDNA library for a colorectal tumor. Serological selection based on human IgG binding revealed 13 different antigens, which may be candidates for tumor vaccination or useful prognostic markers. In parallel, other studies (Hansen et al. 2001; Minenkova et al. 2003; Pavoni et al. 2004, 2006) identified a panel of tumor-associated antigens using phage-displayed libraries of breast cancer. Several selected autoantigens have potential in breast cancer diagnosis.

In addition to tumor-derived antigens, peptide mimics are also suitable candidates for the generation of epitope-specific cancer vaccines. Riemer et al. (2005) screened a linear 9mer phage display peptide library using the anti-high molecular-weight melanoma-associated antigen (HMW-MAA) monoclonal antibody (mAb) 225.28 S. Fifteen peptides were selected in the biopanning procedure. Biopanning is an affinity selection technique that selects for peptides binding to a given target (Ehrlich et al. 2000). It involves four major steps: (1) preparation of a phage display library, (2) conjugating the library to the target, (3) washing away unbound phages, and (4) elution of bound phages. In Riemer′s study, all 15 of the above-mentioned peptides showed partial homology to the amino sequence of the HMW-MAA core protein. One mimotope fused to an immunogenic carrier induced epitope-specific anti-melanoma immune responses. Up to 10^9 different peptide mimics can be screened at the same time using phage displayed random peptide libraries. Furthermore, the optimal epitope can be selected rationally by the choice of the screening antibody. This is an important feature because antibodies targeting different epitopes on the same molecule can have opposite effects on cell growth (Ferrone and Wang 2001).

Phage display systems

Four kinds of display system have been developed so far, namely, filamentous phage, phage lambda, T4 phage and T7 phage. A list of phages and their genome sizes, display sites, and advantages is given in Table 1.

In M13 filamentous phage display, several coat proteins have been used as targets for the fusion of foreign peptides. The first and most commonly used is the p3 coat protein (Smith 1985; Marks et al. 1992), which is present as three to five copies in M13. The major coat protein p8 (2,670 copies per phage particle) is also used (Banhar 2001). Fusions to the p3 protein seem to have few size restrictions. The p3 display system is convenient for expression of large proteins of about 100 kDa, but only six to eight amino acids can be fused to the p8 protein, due to the large number of copies present.

Phage lambda has been used to display proteins fused to the D head protein or pV tail protein (Maruyama et al. 1994; Cicchini et al. 2002). Each phage lambda particle contains 405 copies of D head protein and six copies of pV protein. Of the two lambda coat proteins, the display of peptides as D head protein fusions seems to be more promising, because pV tail fusions express low levels of fusion proteins, and low yields of phage, presumably due to disruption of formation of the tail during assembly. Compared with filamentous phage display, phage lambda display has a number of potential advantages. First, translocation through the *Escherichia coli* membrane is not required and thus a wider variety of proteins can be displayed. Second, the ability to display multiple copies of the same protein on the surface of a single phage lambda particle results in a much more effective immune response.

Phage T4 capsid is an elongated icosahedron decorated with two non-essential outer capsid proteins (Sathaliyawala et al. 2006; Li et al. 2007), the highly antigenic outer capsid protein (HOC, 39 kDa, 155 copies per capsid) and the small outer capsid protein (SOC, 10 kDa, 810 copies per capsid). Either a single SOC or HOC site can be used to display large foreign molecular immunogens. Furthermore, the main advantages of the phage T4 system over other display technologies have been substantiated by using phage T4 SOC/HOC dual sites to display antigens (Wu et al. 2007). The phage T4 HOC/SOC bipartite display system is attractive for the expression of cDNA, and the display of peptides or proteins at high copy number on the phage capsid surface.

T7 phage particles assembled in the cytoplasm of *E. coli* cells and progeny phage are released by cell lysis, hence displayed peptides do not need to be capable of secretion through the periplasm and the cell membrane, as required in filamentous bacteriophage (Russel 1991). The T7 capsid protein is normally produced in two forms: 10A (344 aa) and 10B (397 aa). The 10B form is produced by a translational frameshift at amino acid 341 of 10A, and normally makes up about 10% of the capsid protein (Condron et al. 1991). However, functional capsids can be composed entirely of either 10A or 10B, or of various ratios of these two proteins. T7 phage has the ability to display small peptides in high copy number and large peptides or proteins in low- or mid-copy number. The T7 phage particle is extremely robust, and is stable to harsh conditions that inactivate other phage. This phage display system has been widely used (Kang et al. 2004; McKenzie et al. 2004; Videlock et al. 2004; Takakusagi et al. 2005a, 2005b; Tan et

Table 1 Features of phage display systems. *SOC* Small outer caspid protein, *HOC* highly antigenic outer caspid protein

Phage	Genome (size)	Display sites	Advantages
Filamentous phage	Single-stranded looped DNA (6.4 kb)	N-terminal of p3 C-terminal of p3-JUN/Fos C-terminal of p6 N-terminal of p8	P3 can be used to screen high affinity ligands by univalent display. P8 has the capacity to display proteins in high copy number
Lambda phage	Double-stranded linear DNA (50 kb)	N- or C-terminal of pD, C terminal of pV	Capable of displaying complicated, high molecular weight proteins
T4 phage	Double-stranded linear DNA (160 kb)	C-terminal of SOC N-terminal of HOC	Capable of displaying larger proteins in high copy number; displaying inserts with stop codon
T7 phage	Double-stranded linear DNA (39 kb)	C-terminal of cp10	Displaying small peptides in high copy number and larger peptides or proteins in low or mid-copy number; displaying inserts with stop codon

al. 2005; Ishi and Sugawara 2008; Jestin 2008). However, like other phage display systems, T7 also has its limitations. One limitation is that the displayed peptide will not be post-translationally modified as in eukaryotic systems, another is that only peptides less than 50 residues can be expressed in high copy number.

Phages for vaccine delivery

Whole bacteriophage particles have recently been described as highly efficient DNA vaccine delivery vehicles. In phage-display vaccination, phages can be reconstructed to display a specific protective antigen on their surface. The protective antigen was transcriptionally fused with a coat protein. Alternatively, proteins can also be artificially conjugated to the surface of phages particles. Many studies (di Marzo Veronese et al. 1994; Bastien et al. 1997; Irving et al. 2001; Wang and Yu 2004) have now indicated that immunization with whole phage particles displaying antigenic proteins in animal models can induce specific antibody responses. In this context, the interesting research done by van Houten and colleagues is noteworthy (van Houten et al. 2006). In their study, the f1.K phage was engineered to have an additional Lys residue near the N-terminus of the major coat protein, p8, to allow efficient conjugation to the antigen. The f1.K phage was derived from phage f1 by site-directed mutagenesis. The dimeric synthetic peptide, B2.1, was conjugated to f1.K (f1.K/B2.1) in high copy number and compared as an immunogen to B2.1 conjugated to ovalbumin (OVA/B2.1) and to phage-displayed recombinant B2.1 peptide in a murine model. All immunogens elicited anti-peptide antibody titers, with those elicited by OVA/B2.1 exceeding those by f1.K/B2.1; both titers were greater than that elicited by recombinant B2.1 phage. However, higher peptide to carrier antibody ratios were elicited by the f1.K/B2.1 conjugate, indicating that phage appears better at focusing the antibody response against peptide than the traditional carrier OVA. A weak anti-phage response was elicited due to restricted B cell epitopes on p3 and the low copy number of the outer immunogenic domains of p3. A study in our laboratory has demonstrated that T7 bacteriophage particles displaying the latent membrane protein 1 of Epstein-Barr virus is highly immunogenic in Wistar rats (unpublished data).

In DNA vaccination, a vaccine gene is cloned into a eukaryotic cassette. The host is inoculated with plasmid (naked DNA) produces an immune response to vaccine protein synthesized in vivo (Dietrich et al. 1999). Phage DNA vaccination is a novel strategy for DNA vaccine administration. Rather than directly vaccinating with phages carrying vaccine antigens on their surface, in phage DNA vaccination, phages have been used to deliver a DNA vaccine expression cassette (Clark and March 2004b). In this system, the gene encoding the vaccine antigen, under the control of a suitable eukaryotic promoter, is cloned into the phage genome (Fig. 1). Because it is a virus-like particle, it can target the vaccine to APC. Research has confirmed that such phage DNA vaccination should indeed improve immune efficacies compared with naked DNA vaccination. Long-lasting and significantly higher antibody responses have been observed in mice (Clark and March 2004a), and rabbits (March et al. 2004), following phage delivery of DNA vaccines, in some cases comparable with those produced after vaccination with recombinant protein. In a recent study (Hashemi et al. 2009), BALB/c mice were inoculated with filamentous phage particles containing an expression cassette for Herpes simplex virus 1(HSV-1) glycoprotein D. A dose-response relationship was observed in both humoral and cellular immune responses induced by recombinant filamentous phage inoculation. The results were similar to those from DNA vaccination.

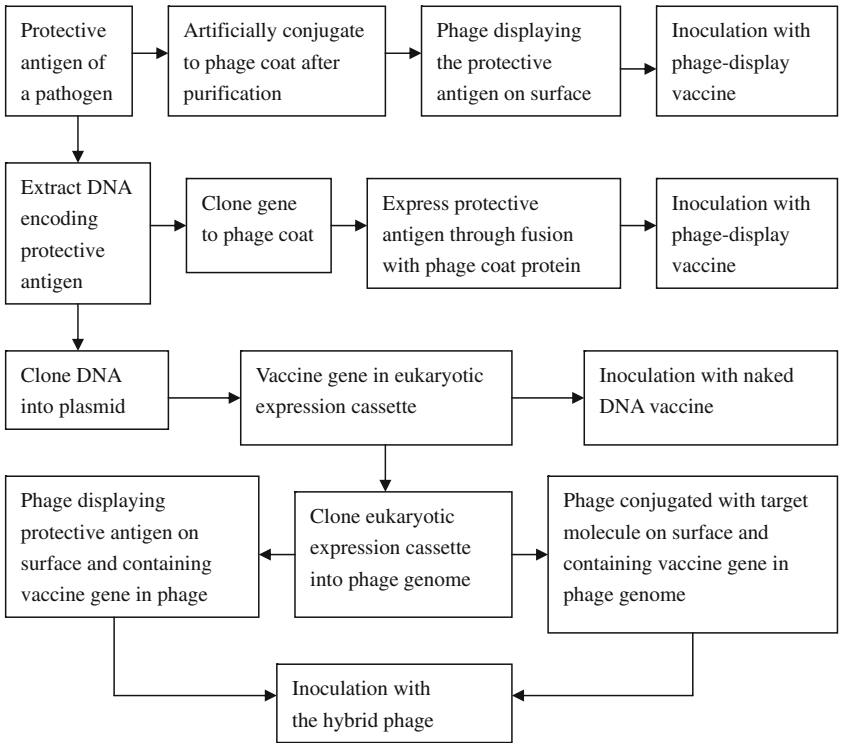

Fig. 1 Strategies of vaccine design based on phage display techniques

More recently, the concept of producing a 'hybrid phage vaccine' has been proposed by some researchers. The hybrid phage delivers both protein and DNA vaccine in one construct (Clark and March 2004b). A eukaryotic promoter-driven DNA encoding the protective antigen is cloned into the bacteriophage genome, and a variant of the same antigen is present on the phage surface. Thus, hybrid phage vaccination results from a combination of phage-display and phage DNA vaccination strategies, indicating the potential evolution of phage vaccines. To date, this method of vaccine delivery remains hypothetical, but it is expected that such a vaccine will effectively induce both the humoral and cellular immune responses. In addition, the displayed protein in a hybrid phage could be a targeting molecule, aimed to increase uptake of the phage by specific cell types. For instance, Molenaar et al. (2002) reported that ^{35}S-radiolabeled filamentous bacteriophage M13 conjugated with galactose stimulates uptake of the phage by galactose-recognizing hepatic receptors. Dickerson et al. (2005) reported that engineered filamentous bacteriophage displaying cocaine-sequestering antibodies delivered into the central nervous system are capable of treating cocaine addiction. Receptor-targeted phage might have important implications for the use of phage as a DNA vaccine or therapeutic gene delivery vehicle directed to specific cell types (Barry et al. 1996; Dunn 1996; Larocca et al. 1998, 1999; Piersanti et al. 2004).

Advantages of phage vaccines

Whole phage particles possess many intrinsic characteristics that make them ideal as vaccine delivery vehicles, both for phage-display and DNA vaccines. Phage vaccines are cheap, and can be produced easily on a large scale. Phages are highly stable. Diluting lambda phage in water results in only a marginal loss in titer over a 2-week period. Phages are even stable within the pH range 3–11 over a 24 h period, thus successful oral administration of phage vaccines might be possible (Jepson and March 2004). Because the displayed protein is fused with a coat protein, it is not susceptible to nuclease degradation under the protective protein matrix. Unlike other virus vectors, phage cannot replicate in a eukaryotic host. Phage particles are naturally immunostimulatory (Kleinschmidt et al. 1970). They carry sufficient CD4+ T cell epitopes to elicit immune responses (Meola et al. 1995). Vaccination with phage particles also induces a highly immunogenic signal against phage coat protein, which provides an easily detectable marker to confirm the vaccination effect in animals (March et al. 2006). Furthermore, high phage antibody titers do not interfere with the immune response against the expressed DNA vaccine antigen, and, if anything, are more likely to efficiently target the phage to APC (March et al. 2004). A previous study had demonstrated that phage particles recruit bystander T cells to induce a mimotope-specific humoral response to a natural antigen (Schöll et al. 2002). Phages

can be used to result in much better immune responses than equivalent standard plasmid DNA vaccination at much lower doses (Clark and March 2004a).

The safety issue of phage vaccine is an important matter and should be outlined. Phages were shown to be safe by the oral route in human volunteers. No adverse events related to phage application have been reported (Bruttin and Brussow 2005). In another study, phage lambda particles displaying porcine Circovirus 2 caspid protein were shown to be immunogenic in pigs (Gamage et al. 2009). No untoward local or systemic reactions occurred following immunization.

On the other hand, a few problems worthy of note still remain regarding phage immunization for humans. It is difficult to ensure that all conformationally active epitopes will be displayed correctly on the phage surface. Since phages require a prokaryotic host for growth, eukaryotic glycosylation signals will be absent from the phage vaccine, and there is a risk of filamentous bacteriophages infecting F pilus-positive *E. coli* in the intestinal flora with application via the oral route (Riemer et al. 2005).

Representative studies on phage-based vaccination

Recently, three groups of vaccine strategies have been investigated in both animal and human clinical trials. The first is peptide-based vaccines. The immunodominant peptide was used for immunization either alone or in combination with adjuvant. The second is dendritic cell-based vaccines. Dendritic cells pulsed with MHC class I restricted peptides or with natural peptides could induce potent antitumor immunity (Banchereau and Steinman 1998). The last is recombinant viruses or nucleotide acid-based vaccines. Peptide-based vaccines and recombinant viruses or nucleotide acid-based vaccines are limited by the lack of appropriate delivery systems for the effective activation of an immune response. Production of dendritic cell-based vaccines is laborious and expensive; furthermore, it may not be optimal for migrating to tumor sites (Kalos 2003).

Since the recognition of the potential of recombinant phage as an immunological reagent and vaccine, there has been a burgeoning interest in phage-based vaccine design. Vaccines against completely different organisms can be constructed using identical procedures, which simplifies production and reduces costs. Phage vaccines could provide us with the key to unlock new approaches in combating bacterial and viral pathogens, as well as cancer diseases. Some representative studies on phage-based vaccination in the past 5 years are as shown in Table 2. Although results obtained in animal models do not necessarily represent the condition in the target species, they do demonstrate the validity of the strategy (March et al. 2006).

Conclusion

Many different phage display systems are exploited today and new ones will certainly emerge because of the global abundance of phage and our increasing ability to exploit them. To date, phage display has become a powerful biotechnique and has been used extensively in a diverse range of fields such as proteomics, cloning and sequencing of unknown genes, identification of antigen epitopes, vaccine design, etc. The scope of phage display applications will continue to expand. Over the ensuing period, research should be directed toward a better understanding of the immunization mechanisms involved in phage-mediated immunization, and the development of hybrid phage vaccination.

Table 2 Representative studies on phage-based vaccination. *CSFV* Classical swine fever virus

Phage display system	Protein or peptides displayed	Reference
Lambda	HBsAg	March et al. 2004
Lambda	Porcine Circoviurs 2 capsid protein	Gamage et al. 2009
M13 filamentous phage	Tumor-associated antigens	Fosså et al. 2004
M13 filamentous phage	HSV-1 glycoprotein D	Hashemi et al 2009
fd filamentous phage	Melanoma-associated antigens	Riemer et al. 2005
fd filamentous phage	Melanoma antigen	Fang et al. 2005
fd filamentous phage	SE-CA-HSP90	Wang et al. 2006
T7	S-HBsAg	Tan et al. 2005
T7	Ep15 peptide of West Nile virus	Herrmann et al. 2007
T7	Tumor antigens	Shadidi et al. 2008
T4	Anthrax antigen	Shivachandra et al. 2006
T4	HIV antigen	Sathaliyawala et al. 2006
T4	CSFV antigen	Wu et al. 2007
T4	Foot-and-mouth disease vaccine	Ren et al. 2008

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