BRAZILIAN JOURNAL
OF MEDICAL AND BIOLOGICAL RESEARCH
www.bjournal.com.br

ISSN 1414-431X
Volume 45 (12) 1102-1340 December 2012

BIOMEDICAL SCIENCES
AND
CLINICAL INVESTIGATION

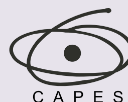
Braz J Med Biol Res, December 2012, Volume 45(12) 1102-1111

doi: 10.1590/S0100-879X2012007500142

Recombinant vaccines and the development of new vaccine strategies

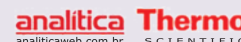
I.P. Nascimento and L.C.C. Leite

The Brazilian Journal of Medical and Biological Research is partially financed by

Ministério
da Ciência e Tecnologia


Ministério
da Educação

Institutional Sponsors

Explore High - Performance MS
Orbitrap Technology
In Proteomics & Metabolomics


All the contents of this journal, except where otherwise noted, is licensed under a Creative Commons Attribution License

Brazilian Journal of Medical and Biological Research (2012) 45: 1102-1111
ISSN 1414-431X Review

Recombinant vaccines and the development of new vaccine strategies

I.P. Nascimento and L.C.C. Leite

Centro de Biotecnologia, Instituto Butantan, São Paulo, SP, Brasil

Abstract

Vaccines were initially developed on an empirical basis, relying mostly on attenuation or inactivation of pathogens. Advances in immunology, molecular biology, biochemistry, genomics, and proteomics have added new perspectives to the vaccinology field. The use of recombinant proteins allows the targeting of immune responses focused against few protective antigens. There are a variety of expression systems with different advantages, allowing the production of large quantities of proteins depending on the required characteristics. Live recombinant bacteria or viral vectors effectively stimulate the immune system as in natural infections and have intrinsic adjuvant properties. DNA vaccines, which consist of non-replicating plasmids, can induce strong long-term cellular immune responses. Prime-boost strategies combine different antigen delivery systems to broaden the immune response. In general, all of these strategies have shown advantages and disadvantages, and their use will depend on the knowledge of the mechanisms of infection of the target pathogen and of the immune response required for protection. In this review, we discuss some of the major breakthroughs that have been achieved using recombinant vaccine technologies, as well as new approaches and strategies for vaccine development, including potential shortcomings and risks.

Key words: Human vaccines; Vectors for immunization; Safety; Recombinant vaccines

Introduction

Most current vaccines owe their success to their ability to target pathogens that have low antigenic variability and for which protection depends on antibody-mediated immunity. This is the case for polio, tetanus, diphtheria, measles, and hepatitis B, among others (Table 1) (1-3). As a consequence, vaccines capable of generating neutralizing or opsonizing antibodies against these pathogens were successful.

On the other hand, important cell-mediated immunity against intracellular pathogens (which in most cases leads to chronic infections) is much more difficult to obtain using current vaccine strategies. The live attenuated pathogen vaccines, which are capable of eliciting this type of response, although not often, may offer potential risks that cannot be overlooked, such as virulence in susceptible hosts and potential reversal of attenuation.

Recombinant vaccines rely on the capacity of one or multiple defined antigens to induce immunity against the pathogen, when administered in the presence of adjuvants or when expressed by plasmids or harmless bacterial/viral vectors. Recombinant protein vaccines permit the avoidance of several potential concerns raised by vaccines based on purified macromolecules, such as the risk of co-purification of undesired contaminants or reversal of the toxoids to their toxigenic forms, if considering diphtheria or tetanus toxoid vaccines, for example. Another fundamental issue overcome by this technology is the complexity involved in obtaining sufficient quantities of purified antigenic components.

However, one of the main challenges in the development of these new strategies of immunization consists of designing vaccines that elicit the appropriate kind of immune response to confer immunity mainly to intracellular pathogens and especially to those that establish chronic, often lifelong infections. For this, the knowledge of the biology of highly conserved antigens involved in pathogenesis and of the immune mechanisms that should be elicited for protection must be obtained to rationally design vaccine strategies that can overcome the low protective immunity naturally generated by infection (reviewed in Ref. 4).

Substantial efforts have been made towards the identification of protective antigens, which have been selected by several rational and experimental approaches (5,6). However, the use of these antigens as vaccines goes beyond their discovery. The development of efficient vaccines will require the combination of diverse strategies,

Correspondence: L.C.C. Leite, Centro de Biotecnologia, Instituto Butantan, 05503-900 São Paulo, SP, Brasil.
E-mail: lccleite@butantan.gov.br and ivanpn@butantan.gov.br

Received May 29, 2012. Accepted August 22, 2012. Available online September 7, 2012. Published December 17, 2012.

such as different delivery systems/adjuvants, to present the antigen in a manner that can elicit an adequate and efficient immune response against these antigens. The use of novel biotechnological tools has provided a new arsenal of strategies and possibilities to the field of vaccinology. Here we review some of these strategies being currently used and discuss their potential for the generation of new human vaccines, as well as the challenges that remain to be solved for their development and use (5).

Recombinant vaccine strategies

Several genes from different etiologic agents have been cloned, expressed and purified to be tested as vaccines. There are a variety of expression systems for antigenic protein components, such as bacteria, yeast, mammalian cells and insect cells, in which the DNA encoding the antigenic determinant can be inserted and expressed. However, several factors must be taken into account before selecting the system for antigen expression. The level of expression obtained using each specific expression vector and promoter, the selection marker of choice, the presence or absence of post-translational modification by the recombinant vector, among others, are essential features that interfere in the efficacy of production of recombinant antigens as vaccines. Bacterial expression systems are the most used due to the ease of handling and to their capacity for high level expression. However, for antigens in which post-translational modifications (e.g., glycosylation) are necessary, the use of mammalian or insect cells should be considered (7,8).

Recombinant protein vaccines

Most of the vaccines under investigation today are based on highly purified recombinant proteins or subunits of pathogens (9). The classical example of recombinant protein vaccines currently in use in humans is the vaccine against hepatitis B (Table 1) (10). Hepatitis B virus (HBV) infection is a chronic liver disease occurring worldwide. HBV presents a marked tropism for human liver cells, partially due to a specific receptor that is expressed on the surface of infected cells. The current vaccines are produced by expressing the hepatitis B surface antigen (HBsAg) in yeast cells. The HBsAg assembles into virus-like particles (VLPs), which are extremely immunogenic, making the HBV vaccine a very efficacious vaccine. The yeast expression system may secrete the antigen into the culture supernatant that can facilitate its purification (11,12). Furthermore, yeast cells offer some of the eukaryotic cellular machinery responsible for the post-translational modification of proteins, being

Table 1. Licensed[a] viral and bacterial vaccines for use in humans.

	Live attenuated	Killed inactivated	Subunit
Viral	Vaccinia	Polio (IPV)	Hepatitis B (HepB-surface antigen)
	Polio (OPV)	Rabies	Human papilloma virus (HPV)
	Yellow fever	Influenza	
	Measles	Hepatitis A	
	Mumps		
	Rubella		
	Influenza		
	Rotavirus		
Bacterial	BCG (tuberculosis)	*Bordetella pertussis* (whole cell)	Tetanus (toxoid)
	Salmonella typhi (oral)	Cholera	Diphtheria (toxoid)
		Bacillus anthracis	*Neisseria meningitidis* (polysaccharide)
			Bordetella pertussis (acellular)
			Streptococcus pneumoniae, 23 valent (polysaccharide)
			Haemophilus influenzae, type b (Hib) (polysaccharide)
			Neisseria meningitidis (polysaccharide conjugate)
			Streptococcus pneumoniae, heptavalent (conjugate polysaccharides)
			Salmonella typhi Vi (capsular polysaccharide)

[a]Licensed by national regulatory agencies such as ANVISA in Brazil or FDA in the USA. OPV = oral polio vaccine; IPV = inactivated polio vaccine; BCG = bacillus Calmette-Guérin.

capable of rendering proteins glycosylated. The technology of production of the HBV vaccine has been transferred to several manufacturers and the prices have decreased due to competition, which has rendered this vaccine affordable to most developing countries.

A more recently developed example of recombinant vaccine is the vaccine against human papillomaviruses (HPVs) (13) (Table 1). HPV is one of the most common sexually transmitted diseases and this infection is associated with many types of mucocutaneous diseases in humans, including cervical, vulva, and vaginal cancers, and genital warts. There are two vaccines in use against HPV, which have both been developed based on VLPs derived from HPV-6, -11, -16, and/or -18 subtypes. These vaccines utilize the L1 recombinant proteins of each subtype, produced either in yeast or in an insect-cell system. The L1 is the major capsid protein and its expression *in vitro* results in the assembly of VLPs. The vaccines are given in a three-dose regimen, using aluminum potassium sulfate as adjuvant, which induces high titers of virus-neutralizing serum antibodies (13). These vaccines are proprietary and extremely expensive, and therefore will have limited accessibility for low-income countries for some time.

Even though vaccines based on recombinant proteins offer several advantages when compared with traditional vaccines, such as safety and production cost, most of them present weak or poor immunogenicity when given alone, and thereby require the use of adjuvants to elicit a protective and long-lasting immune response (14). The successful use of recombinant proteins as vaccines, including hepatitis B and, more recently, HPV, was possible due to the use of aluminium salt as adjuvant (9,13). Therefore, the investigation of new adjuvants is an extremely important field in vaccinology. The main difficulties for the development of new adjuvants involve understanding their molecular complexity and the mechanisms by which they operate to stimulate or induce the immune response. For example, the mechanism of action of the aluminum salts, which are the most commonly used adjuvants in human and animal vaccines worldwide, remains unknown. However, Richard Flavell's group (15) recently suggested that they would activate an intracellular innate immune response system called Nalp3 inflammosome. An alternative path for antigen presentation has been the use of live vectors, such as bacteria and viruses, in which their natural adjuvant properties are explored. Formulation and safety, among other concerns, are also important aspects to be considered (14).

Live recombinant vaccines using bacterial or viral vectors

As a result of advances in the fields of molecular biology and genetic engineering it is now possible to create live recombinant vectors capable of delivering heterologous antigens by the introduction of antigen-encoding genes. The idea behind this approach is to use the capacity of infection and the immunological properties of the live vector to elicit an immune response against its own proteins, as well as towards the heterologous protein being presented (16). A number of bacteria (such as *Salmonella typhi* (17) and bacille Calmette-Guérin (BCG) (18) and viruses (such as vaccinia and adenovirus) (19) have been investigated as live recombinant vector vaccines. In general, these approaches have advantages that are intrinsic to the pathogen itself, such as mimicry of a natural infection, their capacity of stimulating both $CD4^+$ and $CD8^+$ T-cell subsets, and, in some cases, the possibility to be administered orally.

The use of live-attenuated bacterial vaccines is not novel. However, their utilization as carriers or delivery vehicles for heterologous antigen expression represents a technology with broad applicability that may have a significant impact on vaccine development. Significant advances in molecular biology have enabled precise deletions of genes encoding important virulence factors, as well as the introduction of recombinant DNA into avirulent yet immunogenic vaccine strains. Bacterial vectors have many advantages that make them attractive systems for heterologous antigen presentation. They can elicit humoral and/or cellular immune responses and can be administered orally, thereby eliciting mucosal immunity. Most are antibiotic-sensitive strains, which allow antibiotic treatment if any adverse reaction occurs. In general, they display very favorable cost-effectiveness (9).

Several bacteria have been used as vectors, such as *Mycobacterium bovis* BCG (18), *Listeria monocytogenes* (20), *Salmonellae* spp (17) and *Shigellae* spp (21), all of which have been shown to be capable of eliciting immune responses against important viral, bacterial, protozoan, and metazoan pathogens in animal models (9). Although such bacterial vectors present similar features, they have distinct characteristics that should be considered before making a choice for any one of them. For instance, while *Listeria* elicits strong antigen-specific T helper (Th)1-driven CD8+ T cells, BCG and *Salmonella* induce immune responses with mixed Th1/Th2 patterns (9,22).

Among these bacterial vectors, *M. bovis* BCG and *S. typhi* are the most representative of the current status of this approach, as it can be seen by the numerous and assorted papers that have been published using both vectors (18,23). BCG offers several features that render it an attractive vaccine vector. It is safe and has been administered to over 3 billion individuals with minimum side effects, it can be administrated soon after birth, it is a potent adjuvant, and it provides the possibility of generating T cell-mediated immunity against the cloned heterologous antigen. This last feature is considered to be an essential element of a successful vaccine against intracellular pathogens. Several examples of recombinant BCG (rBCG) expressing foreign antigens from diverse pathogens have been described, such as malaria, tuberculosis (TB), HIV, leishmania, pertussis, and others. These were demonstrated to induce both hu-

moral and cellular immune responses and, in some cases, protection against challenge with the infective microorganism (18,23). Much work has been done on rBCG expressing HIV antigens, in which different antigens have been found to elicit specific antibodies, production of interferon (IFN)-γ, as well as to induce T helper cells and cytotoxic T lymphocytes (CTLs), thus demonstrating the ability of different strains of rBCG-HIV to produce both humoral and cellular immune responses against HIV antigens (18,24,25). Another example of promising results involves rBCG expressing the non-toxic subunit 1 of pertussis toxin (rBCG-S1PT). This strain was shown to induce a cellular immune response in adult and neonate mice that protected them against a lethal challenge with virulent *B. pertussis* (26). An antibiotic-free strain has been constructed by autotrophic complementation to be investigated in clinical trials (27).

Recently, many studies have focused on the use of rBCG as a means of increasing the protection against TB (28,29). Recombinant BCG expressing important *M. tuberculosis* antigens, such as Ag85A, have been shown to induce better immune responses than those elicited by standard BCG in animal models and, as a consequence, these strains are under evaluation in clinical trials (30). In fact, rBCG-Ag85A was the first rBCG vaccine to be used in a clinical trial against TB. The idea was to improve the BCG vaccine by overexpressing an immunodominant antigen that had been demonstrated to be protective. Another BCG-based vaccine that is also in clinical trials involves a more sophisticated approach in technical terms. In this case, a BCG mutant deficient in the urease gene was used to express the Listeriolysin O gene from *L. monocytogenes* (29). This rBCG::ΔureC-llo+ mutant has the advantage of being less virulent than the wild-type BCG, a characteristic that may be advantageous when considering immunocompromised individuals. In this vaccine, Listeriolysin expression could lead to disruption of the phagosome membrane, allowing BCG antigens to escape into the cytosol, thereby potentially increasing presentation to $CD8^+$ T cells and protection (31,32). Another approach has been the construction of strains of rBCG expressing cytokines involved in antimycobacterial immunity, such as IFN-γ and interleukin (IL)-2, which have been used as a means to enhance the immune response against TB (33), although concerns with their potential cytotoxicity have been raised. It is a consensus that a Th1 immune response is important for protection against TB and the production of IFN-γ by specific T cells is an important factor for protection (34), even though there is evidence of the absence of correlation between IFN-γ levels and the degree of protection (35).

TB vaccines based on the attenuation of *M. tuberculosis* have also been developed (36,37). However, this strategy has occasionally rendered *M. tuberculosis* strains less immunogenic than wild-type BCG itself, perhaps due to the deletion of important regions responsible for inducing the appropriate immune responses (29). In general, similar approaches could be applicable to recombinant *S. typhimurium*, *Vibrio cholerae*, *Listeria monocytogenes*, and *Shigella* (16,17). Another important class of presentation systems for heterologous antigens is based on viral vectors. The use of viral vectors in vaccine development has been recently reviewed (19,38,39). Vaccines based on viral vectors represent an important strategy against infectious diseases caused by intracellular pathogens, partially due to the fact that they localize in the same compartment that may mimic a natural viral infection. By delivering antigens within the host cells, processing and antigen presentation via major histocompatibility complex (MHC) class I molecules on the surface of infected cells can occur, facilitating the induction of cellular immune responses, which are known to be important in the control of intracellular infections. There are a wide variety of viral vectors under investigation as vaccine delivery vehicles. Some characteristics are desirable for a virus to be used as a delivery vehicle, such as: 1) the capacity to receive large fragments of foreign genes along with regulatory sequences, which could replace a segment of the viral genome not essential to the virus; 2) to be genetically stable; 3) to be capable of growing to higher titers and allow purification; 4) to lack persistence or genomic integration in the host, and 5) most importantly, not to induce disease or show toxicity (19,40).

Numerous viral vectors are available for vaccine development, such as vaccinia, modified vaccinia virus Ankara (MVA), adenovirus (Ad), adeno-associated virus (AAV), retrovirus/lentivirus, alphavirus, herpes virus, and many others (19,41). There are many differences between the viral vectors available. They can be classified according to the virion type (DNA or RNA), particle size, transgene capacity, and cell tropism (40,41). Viral vectors can be replicating or non-replicating viruses; the replication-defective viruses being the most tested in clinical trials, partly due to their higher safety. However, some groups are focusing on the use of replicating vectors in clinical trials as they are more likely to elicit stronger cellular and mucosal immune responses, as well as antibodies against the expressed proteins, depending on their cell tropism and sites of replication (40).

Several studies have demonstrated that recombinant viral vectors encoding genes from important pathogens, (such as malaria, HIV and TB) are able to induce both humoral and cell-mediated immune responses against their expressed antigens in immunized animals and, in some cases, may even protect the animals from lethal challenge (19,41). Co-expression of immunomodulating cytokines in viral vectors has also been used in order to enhance their immunogenicity, also with the above-cited restrictions (42).

This strategy has been used extensively in the development of vaccines against HIV. Similar to other viral vaccines or viral vector-based vaccines developed, a vaccine against HIV infection could be devised based on its attenuation. However, due to the possible risk of reversion or recombinant

events, which can lead to a pathogenic HIV phenotype, vaccines based on HIV virus attenuation have been avoided. Therefore, live recombinant viral vectors such as Ad and MVA have been proposed as safer and less concern raising approaches. Ad and MVA are among the most promising live viral vector systems and, besides having been tested as vaccines against HIV (19), are currently being used in clinical trials against other important infectious diseases such as TB (43) and malaria (41).

Adenoviruses are non-enveloped icosahedral viruses containing a linear double-stranded DNA in their genome, which can infect and replicate in different locations in the body, such as the respiratory tract and the urinary bladder. There are over 50 subtypes of human Ad, with Ad serotype 5 (Ad5) being the best characterized and most used in several vaccination trials. Ad5 is a stable, non-replicating virus, characteristics that contribute to its safe application. This virus allows the insertion of large segments of foreign DNA (~8 kb) into its genome and, in addition, it can be obtained in high titers and easily purified. Replication-competent adenovirus vectors are also under development as vaccine carriers for HIV. The advantages of this type of adenoviruses vector are the lower doses necessary for inducing immune responses and longer persistence in the host, which may be associated with a more prolonged immune response. However, contrary to the non-replicating type, replication-competent adenovirus vectors present lower cloning capacity, limited to ~3-4 kb. Noteworthy, both systems elicit a potent and long-lasting immune response carrying the same gene inserts (44).

Antigens from HIV such as gag, pol, env, and nef have been expressed in adenovirus vectors, particularly Ad5, showing promising results in diverse animal models and in phase I trials (45). Ad5 expressing the simian immunodeficiency virus (SIV) gag protein was able to attenuate the viral infection in monkeys after a challenge with a pathogenic HIV-SIV hybrid virus (SHIV) (46). However, the same results were not observed in phase II trials in humans (47). The candidate MRK Ad5 HIV-1 clade B gag/pol/nef vaccine from Merck & Co., Inc. (USA) has been considered to be the most promising vaccine against HIV-1 to date; however, the clinical trial of this vaccine was interrupted after it was demonstrated not to be protective against HIV infection. Moreover, an increase was observed in the rate of HIV infection in vaccinees that had pre-existing immunity to Ad5 (48). New immunization strategies have been developed to overcome this problem, including the use of other adenovirus serotypes and heterologous vector prime-boost regimens (reviewed in Ref. 45).

Similar to Ad5 and other viral vaccine vectors, MVA has also been used as a vaccine platform in the development of HIV and TB vaccines, as well as for other infections. MVA is an attenuated strain derived from vaccinia virus, which was obtained after 570 passages in chicken embryo fibroblasts. This process resulted in several deletions, making the MVA strain unable to replicate in mammalian cells and inefficient in evading the immune system of the host. In addition, this process modified the host range of the virus. Studies using MVA-based recombinant vaccines in animal models have shown them to be immunogenic and protective against several infectious agents, including HIV, SIV, TB, and malaria (40,49).

Even though promising results have been obtained using vaccines based on viral vectors in recent clinical trials, the use of this technique alone has not been shown to be sufficient to induce a protective immune response. Other approaches are therefore under investigation to be used in combination with this technique. A promising technique that will be discussed in more detail below is called heterologous prime boost. It combines the use of two methods of immunization sequentially, for example, first an immunization with viral vectors, followed by a recombinant protein or live bacterial vaccines.

DNA vaccines

The direct injection of a naked DNA plasmid into muscle as a vaccine system with the ability to induce an immune response and protection after challenge is now well established, since this approach has been used to express numerous antigens from different pathogens with promising results (50-52). A DNA vaccine (or genetic vaccine as it is also called) consists of a plasmid containing: 1) one origin of replication of *Escherichia coli*, for the amplification of the plasmid; 2) a strong promoter, generally from cytomegalovirus; 3) multiple cloning sites, in which one can insert the gene to be expressed, and 4) an antibiotic as selection marker (50,51). The idea behind the DNA vaccine system is that the antigen can be expressed directly by the cells of the host in a way similar to that occurring during viral infection. As a result, the antigens can be processed as proteins synthesized in the cytoplasm, and the fragmented peptides presented to the immune system by class I MHC molecules. In addition, if the protein is exported or secreted, it can be processed by class II MHC molecules and, as a result, mount a specific antibody response (50-52).

Initially, DNA vaccines were administrated either by intramuscular (*im*) injection or using a DNA particle delivery system called Gene Gun (53). Unlike *im* injection, which requires micrograms of plasmid DNA and several doses, the Gene Gun system requires nanogram levels of plasmid DNA to induce the same level of immune response. However, the type of immune response induced in response to the same antigen by the two systems was shown to be distinct. While *im* injection raised predominantly a Th1 response, Gene Gun immunization induced a mixed Th1/Th2 or a Th2 shifted profile. These findings are particularly important in vaccine design, as it is desirable to establish previously the kind of immune response required for protection against a specific pathogen (54). DNA vaccines have several properties that could represent advantages

over other immunization procedures: there is no risk of infection, contrary to attenuated vaccines; they elicit both humoral and cell-mediated immunity, and they are capable of inducing long-lived immune responses and increased cytotoxic T-cell responses. In addition, DNA vaccines avoid problems associated with producing recombinant protein vaccines, such as inadequate folding of target molecules or high purification cost of recombinant proteins. Although DNA vaccines present many advantages, some concerns regarding suitability and capability should be investigated, such as the possibility of production of anti-DNA antibodies, integration of DNA plasmid into the cell genome (now considered a remote possibility), and low efficiency of transfection of the cells *in vivo* (55).

DNA vaccines have been used to express antigens from many different pathogens, such as influenza, HIV, malaria, TB, and leishmaniasis, leading to the induction of immune responses against these etiologic agents in several animal models, and in some cases to protection (54,56). However, DNA vaccines have been shown to be less immunogenic in non-human primates and humans, even though they have been demonstrated to be safe and well tolerated (55).

To increase the effectiveness of these vaccines some approaches have been designed that constitute a second generation of DNA vaccines: plasmid alterations that augment the gene expression, as well as systems that co-express cytokines or other molecules capable of enhancing the immune responses, are some of these new strategies. Among the molecules used for co-expression are genes that induce apoptosis and genes encoding ligands for Toll-like receptors (TLRs) (55,57). Other important approaches that have been developed consist of the formulation of DNA in ways that can protect the DNA from degradation or facilitate its uptake into cells. One good example is the DNA encapsulation into microparticles or the use of live vectors such as viruses or bacteria to protect and facilitate delivery of DNA into specific cells (55,57). The uptake of DNA into cells can also be improved using *in vivo* electroporation, a technique by which small amounts of electric current applied *in vivo* are used to cause the localized formation of pores in cells, which allow more DNA to enter the target cells (58). However, widespread use of electroporation in vaccination campaigns is difficult to envisage. Despite the relative success in improving the immunogenicity induced by DNA vaccines, the precise cellular mechanisms by which a DNA vaccine works in the body are still not totally elucidated. Again, since DNA vaccines alone have been shown not to be sufficient to induce a strong immune response, strategies such as prime boost have been used to improve the immune response for the development of efficient vaccines against a variety of infectious diseases.

The prime-boost approach

Current vaccination traditionally known to be effective requires immunization of an individual with two or more doses and this consists of a “prime-boost regime”. As the vaccines used in the prime and boost consist of the same formulation, such regime is called homologous prime-boost. On the other hand, an immunization regime involving different formulations used sequentially in more than one administration will be called heterologous prime-boost. Research results accumulated over the past decade have shown that heterologous immunization can be more effective than homologous immunization, especially against intracellular pathogens, the infectious agents of higher complexity that are currently considered to be more challenging for vaccine development (59).

The heterologous prime-boost or simply “prime-boost” immunization, as it is commonly called, is a strategy, which involves the administration of the same antigens but formulated in different ways, either as purified antigens or recombinant protein in the presence of appropriate adjuvants, as live recombinant viral or bacterial vectors or DNA vaccines. This approach has opened new venues for vaccine development, and appears to be able to induce a more adequate and efficient immune response against intracellular pathogens. The idea behind the heterologous prime-boost immunization is to combine both humoral and cellular immunity, potentially elicited by each delivery system individually, in an attempt to enhance and modify the immune response induced against a specific antigen. For example, subunit vaccines will usually induce a predominant humoral immune response, while recombinant live vector vaccines and DNA vaccines are effective delivery systems for eliciting cell-mediated immunity (CMI) (59).

The great potential of this strategy has been well demonstrated in the context of HIV vaccine development. Monkeys (*Macaca fascicularis*) primed with the recombinant vaccinia virus expressing SIVmne gp160 antigen and boosted with the recombinant gp160 protein were protected against an intravenous challenge with SIVmne virus. These results were considered among the most promising obtained in the early effort of HIV vaccine development (60). On the other hand, the combination of DNA vaccines with other immunization approaches has also proven to induce greatly increased immunogenicity. Mice primed with a DNA vaccine encoding the hemagglutinin gene of influenza and boosted two weeks later with a recombinant viral vector Fowl poxvirus (FPV) expressing the same antigen were able to produce high levels of anti-hemagglutinin serum antibodies, predominantly of the IgG2a isotype, unlike animals immunized with each vector alone (61).

Since these seminal investigations, several groups have obtained good results using either similar combinations or alternative protocols (62). Many different combinations of heterologous prime-boost will be possible: DNA vaccine-recombinant protein; live recombinant bacteria/virus-recombinant protein; live recombinant bacterial/virus-DNA vaccine (and vice versa). However, in spite of some positive results, in general prime-boost immunization

protocols initiating with recombinant vectors followed by recombinant protein have produced disappointing results (63). Interestingly, the order of the prime and boost has been shown to alter the immune response obtained. In a prime-boost strategy of immunization against malaria, mice immunized with consecutive DNA and MVA vectors encoding antigens from *Plasmodium berghei* have been shown to be protected against challenge with *P. berghei* sporozoites, and such protection was associated with high levels of peptide-specific IFN-γ-secreting $CD8^+$ T cells. However, reversal of the order of the immunization or substitution of the viral vector resulted in failure of protection (64). This result showed the importance of using DNA as a priming vehicle and attenuated virus as a booster.

Prime-boost strategies have been applied for the development of vaccines against important infectious diseases such as HIV, TB, and malaria, demonstrating promising results even in clinical trials. In the last HIV clinical trial using a combination of two earlier vaccines that had previously failed, researchers found that the prime-boost combo reduced by 31% the risk of contracting HIV (65). Unfortunately, they have also shown that the observed protection was limited to 1 year. In spite of this short-lived protection, the authors believe this result is encouraging and that a new and safer HIV vaccine will soon be available. Presently, clinical trials are ongoing to further assess this line of research (66).

The exact mechanism underlying the efficacy of the heterologous prime-boost vaccination is still poorly understood, being likely that several distinct mechanisms participate in the success of this approach. One mechanism proposed suggests that the different characteristics of the vectors are important. A second advantage of a heterologous prime-boost is the fact that the use of different immunization strategies results in reduced induction of anti-vector immunity. A third, and possibly the most relevant mechanism, is due to immunodominance. During priming immunization, T cells will be induced against the most immunodominant epitopes of the antigen. Upon heterologous boosting, which shares only the relevant antigen with the prime immunization, the immune response will focus preferentially on the expansion of immunodominant T cells induced by priming (67,68); live recombinant vectors, such as MVA and adenovirus, seem to be especially efficient in boosting pre-existing memory immune responses, especially primed T-cell responses (65,66,69).

A number of studies have shown that at least one plasmid vector (consisting of DNA vaccine) or a recombinant viral vector should be included as a component of the prime-boost vaccination in order to elicit a potent cell-mediated immunity (59,64,70). Although DNA vaccines so far have shown low immunogenicity when used alone, they have also proven to act as strong priming vehicles, while viral vectors seem to be much more effective when used as boosters. As a consequence, DNA prime-viral vector boost regimes have become the main scheme of choice to induce T cell-mediated immune responses (59,64,70).

One possible mechanism to explain the success of these prime-boost regimes relies on the induction of high-avidity T cells. Mice immunized with DNA prime/live vector boost protocols expressed high frequencies of high-avidity T cells and were capable of eliminating target cells expressing 10- to 100-fold less immunogenic peptide than mice vaccinated with either vector alone (70). Other features characteristic of the vaccine vectors used in prime-boost immunization may as well be essential for their ability to induce increased CMI (Table 2). The presence of cytosine-phosphodiester bond-guanine (CpG) motifs in the plasmid of the DNA vector has also been shown to strongly stimulate the production of IL-12, the main inducer cytokine of Th1 cells. The use of non-replicating DNA vaccines followed by live vectors may result in an immune response focused almost exclusively on the encoded antigen. The efficient presentation of the encoded antigen by MHC class I and class II molecules will result in efficient induction of $CD4^+$ T and $CD8^+$ T cells (70). The types of antigens and the types of vectors used, the order of vector administration, the routes and interval between priming and boosting vaccinations, among other factors, should be taken into account to determine the effectiveness of the prime-boost strategies (Table 1). Further investigation of the mechanism of action of this promising strategy will allow its optimization, and eventually lead to improved vaccines.

Final considerations

We have seen here that the prevention of important infectious diseases such as HIV, TB and malaria, among others, continues to be a challenge for the vaccinology field in the 21st century. Furthermore, it is most likely that vaccines for such pathogens will not become available by following the classical approaches of successful traditional vaccines.

Nonetheless, considerable advances in the fields of immunology, molecular biology, recombinant DNA, microbiology, genomics, bioinformatics, and related areas have provided novel insights to help elucidate important pathogenic mechanisms involved in these infectious diseases and in pathogen interaction with the host. Altogether, these advances have led to the development of several new vaccine strategies with promising results. It seems now clear that an integrated approach will be necessary to foster continued progress in the immunology field, which probably constitutes the limiting factor for the development of new vaccines.

It is also important to realize that the challenges of vaccine development are not limited to the discovery of safe and effective antigens, adjuvants and delivery systems. The balance between cost, benefits and risk should certainly be evaluated before translating a vaccine candidate to the clinic.

Millions of children worldwide die from infectious diseases, despite currently available vaccines. Thus, social, political and economic policies are not less important issues and cannot be overlooked.

Table 2. Properties of vaccine vectors that contribute to the efficacy of heterologous prime/boost vaccination strategies.

Vector	Properties	Immune consequence	Most used vaccination		References
			Prime	Boost	
DNA vaccine	Encoded antigens delivered to MHC class I and class II processing pathways	$CD4^+$ Th1 and $CD8^+$ T cells	DNA	Viral	59,61,63, 64,70
	Low level and constant expression of protein	Prolonged immune stimulation and induction of high-affinity T cells		BCG	59,63,64
	Presence of CpG motifs	Adjuvant for CMI		RP/Adj	59,63,64
	Expresses only vaccine antigen	Focused response on antigen			
Viral	Efficient delivery to MHC class I and class II process pathways	Expansion of T-cell responses induced by DNA vaccination	Viral	RP/Adj	59,60,63, 64
	Higher levels of encoded antigen	Expansion of high-affinity T cells primed by DNA vaccine			
	Presence of CpG motifs and other TLR agonists	Adjuvant for CMI and strong production of pro-inflammatory cytokines			
	Non-productive replication in mammalian cells	Immune response largely focused on encoded antigen and safe for human use			
Bacterial (BCG)	Encoded antigens delivered to MHC class II processing pathways	Induction of $CD4^+$ Th1/Th2 cells	Bacterial	Viral	59,63,64,70
Recombinant protein	Requires adjuvant and multiple immunizations	CD4 T cell and humoral responses	RP/Adj	RP/Adj	59,63,64
	Requires strong adjuvant	Poor induction of cellular responses, particularly of $CD8^+$ T cells			

BCG = bacillus Calmette-Guérin; MHC = major histocompatibility complex; CpG = cytosine-phosphodiester bond-guanine; TLR = Toll-like receptor; Th1/Th2 = T helper cells 1 or 2; CMI = cell-mediated immunity; RP/Adj = recombinant protein/adjuvant.

References

1. Plotkin SA. Immunologic correlates of protection induced by vaccination. *Pediatr Infect Dis J* 2001; 20: 63-75.
2. Ada G. Overview of vaccines and vaccination. *Mol Biotechnol* 2005; 29: 255-272.
3. Robinson HL, Amara RR. T cell vaccines for microbial infections. *Nat Med* 2005; 11: S25-S32.
4. Lemaire D, Barbosa T, Rihet P. Coping with genetic diversity: the contribution of pathogen and human genomics to modern vaccinology. *Braz J Med Biol Res* 2012; 45: 376-385.
5. Sette A, Rappuoli R. Reverse vaccinology: developing vaccines in the era of genomics. *Immunity* 2010; 33: 530-541.
6. Cardoso FC, Pacifico RN, Mortara RA, Oliveira SC. Human antibody responses of patients living in endemic areas for schistosomiasis to the tegumental protein Sm29 identified through genomic studies. *Clin Exp Immunol* 2006; 144: 382-391.
7. Hansson M, Nygren PA, Stahl S. Design and production of recombinant subunit vaccines. *Biotechnol Appl Biochem* 2000; 32 (Part 2): 95-107.
8. Clark TG, Cassidy-Hanley D. Recombinant subunit vaccines: potentials and constraints. *Dev Biol* 2005; 121: 153-163.
9. Perrie Y, Mohammed AR, Kirby DJ, McNeil SE, Bramwell VW. Vaccine adjuvant systems: enhancing the efficacy of sub-unit protein antigens. *Int J Pharm* 2008; 364: 272-280.
10. Michel ML, Tiollais P. Hepatitis B vaccines: protective efficacy and therapeutic potential. *Pathol Biol* 2010; 58: 288-295.
11. Dertzbaugh MT. Genetically engineered vaccines: an overview. *Plasmid* 1998; 39: 100-113.
12. Adkins JC, Wagstaff AJ. Recombinant hepatitis B vaccine: a review of its immunogenicity and protective efficacy against hepatitis B. *BioDrugs* 1998; 10: 137-158.
13. Govan VA. A novel vaccine for cervical cancer: quadrivalent

human papillomavirus (types 6, 11, 16 and 18) recombinant vaccine (Gardasil). *Ther Clin Risk Manag* 2008; 4: 65-70.
14. Perez O, Batista-Duharte A, Gonzalez E, Zayas C, Balboa J, Cuello M, et al. Human prophylactic vaccine adjuvants and their determinant role in new vaccine formulations. *Braz J Med Biol Res* 2012; 45: 681-692.
15. Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* 2008; 453: 1122-1126.
16. Shata MT, Stevceva L, Agwale S, Lewis GK, Hone DM. Recent advances with recombinant bacterial vaccine vectors. *Mol Med Today* 2000; 6: 66-71.
17. Curtiss R III, Xin W, Li Y, Kong W, Wanda SY, Gunn B, et al. New technologies in using recombinant attenuated *Salmonella* vaccine vectors. *Crit Rev Immunol* 2010; 30: 255-270.
18. Bastos RG, Borsuk S, Seixas FK, Dellagostin OA. Recombinant *Mycobacterium bovis* BCG. *Vaccine* 2009; 27: 6495-6503.
19. Rollier CS, Reyes-Sandoval A, Cottingham MG, Ewer K, Hill AV. Viral vectors as vaccine platforms: deployment in sight. *Curr Opin Immunol* 2011; 23: 377-382.
20. Bruhn KW, Craft N, Miller JF. *Listeria* as a vaccine vector. *Microbes Infect* 2007; 9: 1226-1235.
21. Roland KL, Tinge SA, Killeen KP, Kochi SK. Recent advances in the development of live, attenuated bacterial vectors. *Curr Opin Mol Ther* 2005; 7: 62-72.
22. Detmer A, Glenting J. Live bacterial vaccines - a review and identification of potential hazards. *Microb Cell Fact* 2006; 5: 23.
23. Galen JE, Pasetti MF, Tennant S, Ruiz-Olvera P, Sztein MB, Levine MM. *Salmonella enterica* serovar Typhi live vector vaccines finally come of age. *Immunol Cell Biol* 2009; 87: 400-412.
24. Stover CK, de la Cruz V, Fuerst TR, Burlein JE, Benson LA, Bennett LT, et al. New use of BCG for recombinant vaccines. *Nature* 1991; 351: 456-460.
25. Lim EM, Lagranderie M, Le Grand R, Rauzier J, Gheorghiu M, Gicquel B, et al. Recombinant *Mycobacterium bovis* BCG producing the N-terminal half of SIVmac251 Env antigen induces neutralizing antibodies and cytotoxic T lymphocyte responses in mice and guinea pigs. *AIDS Res Hum Retroviruses* 1997; 13: 1573-1581.
26. Nascimento IP, Dias WO, Quintilio W, Hsu T, Jacobs WR Jr, Leite LC. Construction of an unmarked recombinant BCG expressing a pertussis antigen by auxotrophic complementation: protection against *Bordetella pertussis* challenge in neonates. *Vaccine* 2009; 27: 7346-7351.
27. Nascimento IP, Dias WO, Mazzantini RP, Miyaji EN, Gamberini M, Quintilio W, et al. Recombinant *Mycobacterium bovis* BCG expressing pertussis toxin subunit S1 induces protection against an intracerebral challenge with live *Bordetella pertussis* in mice. *Infect Immun* 2000; 68: 4877-4883.
28. Triccas JA. Recombinant BCG as a vaccine vehicle to protect against tuberculosis. *Bioeng Bugs* 2010; 1: 110-115.
29. Kaufmann SH, Gengenbacher M. Recombinant live vaccine candidates against tuberculosis. Current Opinion in Biotechnology. http://www.ncbi.nlm.nih.gov/pubmed/22483201.
30. Nicol MP, Grobler LA. MVA-85A, a novel candidate booster vaccine for the prevention of tuberculosis in children and adults. *Curr Opin Mol Ther* 2010; 12: 124-134.
31. Grode L, Seiler P, Baumann S, Hess J, Brinkmann V, Nasser EA, et al. Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* bacille Calmette-Guerin mutants that secrete listeriolysin. *J Clin Invest* 2005; 115: 2472-2479.
32. Hoft DF. Tuberculosis vaccine development: goals, immunological design, and evaluation. *Lancet* 2008; 372: 164-175.
33. Murray PJ, Aldovini A, Young RA. Manipulation and potentiation of antimycobacterial immunity using recombinant bacille Calmette-Guerin strains that secrete cytokines. *Proc Natl Acad Sci U S A* 1996; 93: 934-939.
34. McShane H. Tuberculosis vaccines: beyond bacille Calmette-Guerin. *Philos Trans R Soc Lond B Biol Sci* 2011; 366: 2782-2789.
35. Kagina BMN, Abel B, Scriba TJ, Hughes EJ, Keyser A, Soares A, et al. Specific T cell frequency and cytokine expression profile do not correlate with protection against tuberculosis, following BCG vaccination of newborns. http://www.ncbi.nlm.nih.gov/pubmed/20558627.
36. Martin C, Williams A, Hernandez-Pando R, Cardona PJ, Gormley E, Bordat Y, et al. The live *Mycobacterium tuberculosis* phoP mutant strain is more attenuated than BCG and confers protective immunity against tuberculosis in mice and guinea pigs. *Vaccine* 2006; 24: 3408-3419.
37. Waters WR, Palmer MV, Nonnecke BJ, Thacker TC, Scherer CF, Estes DM, et al. Efficacy and immunogenicity of *Mycobacterium bovis* DeltaRD1 against aerosol i infection in neonatal calves. *Vaccine* 2009; 27: 1201-1209.
38. Pincha M, Sundarasetty BS, Stripecke R. Lentiviral vectors for immunization: an inflammatory field. *Expert Rev Vaccines* 2010; 9: 309-321.
39. Draper SJ, Heeney JL. Viruses as vaccine vectors for infectious diseases and cancer. *Nat Rev Microbiol* 2010; 8: 62-73.
40. Drexler I, Staib C, Sutter G. Modified vaccinia virus Ankara as antigen delivery system: how can we best use its potential? *Curr Opin Biotechnol* 2004; 15: 506-512.
41. Limbach KJ, Richie TL. Viral vectors in malaria vaccine development. *Parasite Immunol* 2009; 31: 501-519.
42. Abaitua F, Rodriguez JR, Garzon A, Rodriguez D, Esteban M. Improving recombinant MVA immune responses: potentiation of the immune responses to HIV-1 with MVA and DNA vectors expressing Env and the cytokines IL-12 and IFN-gamma. *Virus Res* 2006; 116: 11-20.
43. Kaufmann SH. Fact and fiction in tuberculosis vaccine research: 10 years later. *Lancet Infect Dis* 2011; 11: 633-640.
44. Tatsis N, Ertl HC. Adenoviruses as vaccine vectors. *Mol Ther* 2004; 10: 616-629.
45. Barouch DH. Novel adenovirus vector-based vaccines for HIV-1. *Curr Opin HIV AIDS* 2010; 5: 386-390.
46. Shiver JW, Fu TM, Chen L, Casimiro DR, Davies ME, Evans RK, et al. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* 2002; 415: 331-335.
47. Priddy FH, Brown D, Kublin J, Monahan K, Wright DP, Lalezari J, et al. Safety and immunogenicity of a replication-incompetent adenovirus type 5 HIV-1 clade B gag/pol/nef vaccine in healthy adults. *Clin Infect Dis* 2008; 46: 1769-1781.
48. Buchbinder SP, Mehrotra DV, Duerr A, Fitzgerald DW, Mogg R, Li D, et al. Efficacy assessment of a cell-mediated

immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet* 2008; 372: 1881-1893.
49. Gomez CE, Najera JL, Krupa M, Perdiguero B, Esteban M. MVA and NYVAC as vaccines against emergent infectious diseases and cancer. *Curr Gene Ther* 2011; 11: 189-217.
50. Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, et al. Direct gene transfer into mouse muscle *in vivo*. *Science* 1990; 247: 1465-1468.
51. Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dwarki VJ, et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 1993; 259: 1745-1749.
52. Oliveira SC, Rosinha GM, de-Brito CF, Fonseca CT, Afonso RR, Costa MC, et al. Immunological properties of gene vaccines delivered by different routes. *Braz J Med Biol Res* 1999; 32: 207-214.
53. Yang NS, Burkholder J, Roberts B, Martinell B, McCabe D. *In vivo* and *in vitro* gene transfer to mammalian somatic cells by particle bombardment. *Proc Natl Acad Sci U S A* 1990; 87: 9568-9572.
54. de Oliveira CI, Nascimento IP, Barral A, Soto M, Barral-Netto M. Challenges and perspectives in vaccination against leishmaniasis. *Parasitol Int* 2009; 58: 319-324.
55. Belakova J, Horynova M, Krupka M, Weigl E, Raska M. DNA vaccines: are they still just a powerful tool for the future? *Arch Immunol Ther Exp* 2007; 55: 387-398.
56. Lima KM, dos Santos SA, Santos RR, Brandao IT, Rodrigues JM Jr, Silva CL. Efficacy of DNA-hsp65 vaccination for tuberculosis varies with method of DNA introduction *in vivo*. *Vaccine* 2003; 22: 49-56.
57. Saade F, Petrovsky N. Technologies for enhanced efficacy of DNA vaccines. *Expert Rev Vaccines* 2012; 11: 189-209.
58. Chiarella P, Fazio VM, Signori E. Application of electroporation in DNA vaccination protocols. http://www.ncbi.nlm.nih.gov/pubmed/20504275.
59. Radosevic K, Rodriguez A, Lemckert A, Goudsmit J. Heterologous prime-boost vaccinations for poverty-related diseases: advantages and future prospects. *Expert Rev Vaccines* 2009; 8: 577-592.
60. Hu SL, Abrams K, Barber GN, Moran P, Zarling JM, Langlois AJ, et al. Protection of macaques against SIV infection by subunit vaccines of SIV envelope glycoprotein gp160. *Science* 1992; 255: 456-459.
61. Leong KH, Ramsay AJ, Boyle DB, Ramshaw IA. Selective induction of immune responses by cytokines coexpressed in recombinant fowlpox virus. *J Virol* 1994; 68: 8125-8130.
62. Ranasinghe C, Ramshaw IA. Genetic heterologous prime-boost vaccination strategies for improved systemic and mucosal immunity. *Expert Rev Vaccines* 2009; 8: 1171-1181.
63. Excler JL, Plotkin S. The prime-boost concept applied to HIV preventive vaccines. *AIDS* 1997; 11 (Suppl A): S127-S137.
64. Schneider J, Gilbert SC, Blanchard TJ, Hanke T, Robson KJ, Hannan CM, et al. Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. *Nat Med* 1998; 4: 397-402.
65. Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, Paris R, et al. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N Engl J Med* 2009; 361: 2209-2220.
66. Girard MP, Osmanov S, Assossou OM, Kieny MP. Human immunodeficiency virus (HIV) immunopathogenesis and vaccine development: a review. *Vaccine* 2011; 29: 6191-6218.
67. McShane H, Hill A. Prime-boost immunisation strategies for tuberculosis. *Microbes Infect* 2005; 7: 962-967.
68. Woodland DL. Jump-starting the immune system: prime-boosting comes of age. *Trends Immunol* 2004; 25: 98-104.
69. Dominguez MR, Silveira EL, de Vasconcelos JR, de Alencar BC, Machado AV, Bruna-Romero O, et al. Subdominant/cryptic CD8 T cell epitopes contribute to resistance against experimental infection with a human protozoan parasite. *PLoS One* 2011; 6: e22011.
70. Estcourt MJ, Ramsay AJ, Brooks A, Thomson SA, Medveckzy CJ, Ramshaw IA. Prime-boost immunization generates a high frequency, high-avidity CD8(+) cytotoxic T lymphocyte population. *Int Immunol* 2002; 14: 31-37.