New Strategies for Vaccine Development

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Abstract
Introduction
Reverse vaccinology approach
Virus-like particles
DNA vaccines
Improving immunogenicity through formulation
Improving immunogenicity by including immune modulatory adjuvants
Improving immunogenicity by using next-generation delivery strategies
Viral vectors
Prime-boost strategy
Conclusions
References

Abstract

Vaccination remains the most successful strategy for preventing and controlling infectious diseases. In many cases, conventional vaccines, in particular live attenuated vaccines, inactivated microorganisms and subunit vaccines have been successful at inducing protective immunity, mainly humoral immunity, to disease-causing pathogens. However, it has become apparent that certain highly dangerous pathogens, that resist the humoral immunity, are not readily controlled
by conventional vaccination approaches. Recent knowledge of host-pathogen interactions, received from fundamental research in immunology, molecular-cellular biology and vaccinology provides understanding that the induction of T-cell immunity is required for optimal treatment against pathogens causing chronic infections. Although conventional live attenuated vaccines may induce cell-mediated immunity, there are serious safety issues regarding the application of this strategy for the development of vaccines against highly dangerous pathogens, such as human immunodeficiency virus (HIV). As a result of these problems, several new approaches to vaccine development have emerged. These approaches are distinguished by having advantages over conventional vaccine strategies. This review will focus on some of these new strategies developed to promote potent cellular immunity, including (i) virus-like particles, (ii) plasmid DNA vaccines, (iii) viral vectors, and (iv) prime-boost vaccination strategy. In addition, the reverse vaccinology approach, which facilitates systematic identification of new potential antigens of a pathogen, is discussed.

**Introduction**

The concept of vaccination was demonstrated over 200 years ago when Edward Jenner showed that prior exposure to cowpox could prevent infection by smallpox. Over the last century, the development and widespread use of vaccines against a variety of infectious agents have been a great triumph of medical science. We have vaccines for nearly thirty out of the more than seventy infectious diseases of humans. Among the greatest achievement of vaccination remains the eradication of smallpox. Other achievements are: virtual disappearance of previously disabling and lethal diseases such as diphtheria, tetanus, paralytic poliomyelitis, pertussis, measles, mumps, rubella and invasive Haemophilus influenzae type B. Hepatitis A and B are also being brought under control in an increasing number of countries.

Conventional approaches, in particular attenuation of the pathogen, inactivation of the microorganism and creation of subunit preparations, have been very successful in the development of vaccines against infectious agents. However, there are some diseases for which attempts to develop vaccines using conventional approaches have failed. These include chronic infectious diseases such as acquired immunodeficiency syndrome (AIDS) and hepatitis C (HCV). Moreover, occasionally, a vaccine has to be withdrawn because of unexpected side
effects. For instance, an inactivated vaccine against measles was withdrawn because it had provoked atypical measles after natural infection (99). More recently, in October 1999, a simian-human reassortant rotavirus vaccine was withdrawn due to intussusceptions and mortality of vaccinated infants (117). Therefore, there is a constant interest in developing new approaches to improve efficacy and safety of existing vaccines. There is also a continuous need for developing new vaccines to newly emerging pathogens, such as the severe acute respiratory syndrome virus (14) and hantavirus (26), as well as to pathogens for which no vaccines currently exist. Finally, development of therapeutic vaccines and vaccines against allergic and autoimmune diseases represents another new vaccine challenge. Achieving such ambitious goals certainly requires new approaches in vaccine development.

This review explores some new approaches for vaccine development: reverse vaccinology, virus-like particles (VLPs), ‘naked’ DNA plasmids, viral vectors, and heterologous prime-boost strategy. Reverse vaccinology approach has emerged due to the need to identify potential vaccine candidates providing new solutions for those vaccines which have been difficult or impossible to develop, whereas the other approaches mentioned above have emerged because it appears that protection against infection by many agents, particularly viruses, requires generation of potent cell-mediated immunity (106). Successful vaccines are available for many viruses, but almost exclusively ones that produce acute, self-limited infection (74). The most important characteristic of these pathogens is that neutralizing antibodies protect against infection or disease. Conventional approaches (e.g., live attenuated vaccines, inactivated vaccines and subunit vaccines) have been employed successfully against these pathogens which elicit protective immune responses with a bias towards the induction of humoral immunity. In contrast, there are no successful vaccines available for viruses that cause chronic infections, such
as HIV, HCV and herpes simplex virus. It has been shown that CD8\(^+\) T cells are especially important for the control of HIV-1 and HCV infections (77, 106). In addition to CD8\(^+\) cell responses, CD4\(^+\) T cells have been found to be critical in the maintenance of adequate CD8\(^+\) T cell function and control of viremia in both HIV and HCV infection (64, 106). Live attenuated vaccines have been the most successful in induction of efficient B- and T-cell responses (59). However, there is much concern about the use of live attenuated vaccines against viruses that cause chronic infection. Besides, such vaccines are unlikely to induce protection as the immune response to the natural infection is not sufficient to eradicate the infection. Therefore, the development of vaccines against such pathogens is more challenging, since they would have to elicit more robust cytotoxic T lymphocyte (CTL) responses in addition to antibodies (71). This review will cover the approaches are being studied today which are based on eliciting strong cell-mediated immunity.

**Reverse vaccinology approach:**

The first revolution in vaccinology was the use of modern recombinant DNA technology to produce subunit vaccines. This approach involves propagation of a pathogen in the laboratory conditions, followed by identification of its components that are important in pathogenesis and immunity. Then, identified protective antigens are produced in a system for large-scale recombinant protein expression (Figure 1). While successful in some cases, for example the subunit vaccines against hepatitis B (6) and Bordetella pertussis (65), this strategy is limited to pathogens with known immunodominant protective antigens. The approach is time-consuming and is amenable to only those antigens which can be purified in high quantities. However, in some cases the most abundant proteins expressed by a pathogen are not suitable vaccine
candidates and genetic tools required for identification of less abundant components may be inadequate or not available. Moreover, this strategy is not applicable to non-cultivable microorganisms.

Figure 1. Schematic representation of the conventional approach to vaccine development. This approach requires the pathogen to be grown in laboratory conditions, individual components to be identified and produced in a pure form, either directly from the microorganism or through recombinant DNA technology and then tested for their ability to induce immunity.

The second revolution in vaccine development took place at the end of 20th century as a result of the use of the genomic technology. Currently, it became possible to determine the complete genome sequence of any microorganism in a few months at a low cost. As a result, the sequences of most of the pathogens are now available. This advantage is used by reverse vaccinology approach (142) which is shown schematically in Figure 2. The strategy utilizes computer analysis of available genome sequences to predict open reading frames (ORFs) as well as localization and possible function of proteins encoded by these ORFs. Two-dimensional gel electrophoresis together with mass spectrometry, DNA microarrays, in vivo expression technology provide more information on these potential antigens. For example, microarray
analysis is used to identify genes that are turned on during invasive infection (39), and thus, the proteins encoded by these genes can be considered as potential targets for vaccine development (156). It allows choosing, in a short period of time, many vaccine candidates including those proteins which are expressed at very low levels during infection. Next step is a high throughput expression of recombinant proteins and testing them in animal models. This approach reduces the time of vaccine development to 1-2 years, whereas the development of vaccines by conventional methods could take 5 or more years. An example of one of the first applications of reverse vaccinology approach is the identification of vaccine candidates against Neisseria meningitidis serogroup B (132). Potential antigens identified by computer programs were expressed in Escherichia coli as recombinant fusion proteins, purified and used to immunize animals. The resultant sera were examined for meningococcoidal activity in the presence of complement (132). Among the proteins with bactericidal activity, a novel surface-exposed oligomeric protein Neisseria adhesin A (NadA) was found to be present in most of the meningococcus B strains. As NadA elicited production of bactericidal antibodies that correlated with protection against the pathogen in the infant rat model it was considered as a promising vaccine candidate (32). Moreover, immunization with a multicomponent vaccine containing NadA and other protective antigens discovered by this approach, gave promising results in clinical phase I and II trials (61). While the vaccine is in clinical development, research efforts are focused on the structural characterization of the main vaccine antigens. For instance, a truncated soluble variant of NadA was subjected to extensive physico-chemical characterization in order to be formulated as part of the final drug product (97).
Figure 2. The reverse vaccinology approach to vaccine development. This approach utilizes available information about genomic sequences of pathogenic microorganisms. With the use of computer software, ORFs and possible localization and functions of proteins encoded by these ORFs are predicted. This is followed by a high-throughput cloning and expression of identified ORFs. Expressed proteins undergo screening in animal models to be tested for the immunogenicity and ability to protect against challenge.

The success of the group B meningococcus paradigm facilitated the application of the reverse vaccinology strategy for the identification of vaccine candidates against other bacterial pathogens, such as Streptococcus pneumoniae (184), Porphyromonas gingivalis (150), Chlamydia pneumoniae (112), Staphylococcus aureus (46) as well as to protozoan pathogens (63). However, in order to be successful against a more complex microorganism such as Streptococcus agalactiae this approach had to evolve giving the beginning of pan-genome reverse vaccinology and comparative reverse vaccinology (157). Pan-genome reverse analysis is focused on comparing many pathogenic strains of a species in order to identify antigens that could provide maximum coverage against different serotypes resulting in the development of a universal vaccine. The identification of vaccine candidates against Streptococcus agalactiae, a group B
streptococcus, is an example of successful application of multiple genomic approaches in vaccine design (98). Comparative reverse vaccinology approach involves comparative analysis of genomes of pathogenic and non-pathogenic strains of the same species with the goal of finding pathogenicity markers (69). This strategy reduces the number of vaccine candidates to express and test in the animal model and, consequently, reduces the time for the delivery of a vaccine as genes conserved in both pathogenic and non-pathogenic strains are excluded from the selection.

Thus, the reverse vaccinology approach is aimed at the identification of potential vaccine candidates without the need for cultivating the pathogen, reducing the time and cost required for the identification of new vaccine candidates. Although this approach has been predominantly used for the development of vaccines against complex microorganisms, such as bacteria, vaccines against viral pathogens (hepatitis C and HIV) are currently under development with use of this approach (142). However, some limiting steps in reverse vaccinology include lack of algorithms that can be used to make a good correlation between antigens and their likely protective immune responses, and inability to identify non-protein antigens including polysaccharides or CD1-restricted antigens such as glycolipids (142).

**Virus-like particles:**

Virus-like particles represent another promising and viable strategy to the production of vaccines against many diseases. VLPs are a highly effective type of subunit vaccines that possess characteristics that make them a safe and effective vaccine platform. “Virus-like particles are highly organized spheres that self-assemble from virus-derived structural antigens” (93). VLPs
preparations are all based on the observation that expression of the capsid proteins of many viruses leads to the spontaneous assembly of particles that are structurally similar to authentic virus (4, 76, 123). Immunological features of viruses, such as repetitive surfaces, particulate structures and induction of innate immunity through activation of pathogen-associated molecular-pattern recognition receptors, make VLPs very effective in inducing potent B- and T-cell responses. The critical role of innate immune response induced by virus in directing the subsequent adaptive immune response of appropriate magnitude, quality and specificity has been highlighted by Gaucher and coauthors (59). Besides, the authors demonstrated that the sum of all immune arms is required for the long-lasting protection induced by yellow fever vaccine and integrated immune response constitutes the correlates of protection. Moreover, unique features of protective immune responses were identified, which can now be used as a basis for the development of novel vaccines.

In practical terms, the fact that VLPs mimic the structure of virus particles usually means that VLPs elicit strong humoral response as it allows the adaptive immune system rapidly detect and respond to these repeated and ordered structures found on viral surfaces (11, 135). Moreover, the dense, repetitive nature of VLPs makes them particularly effective in inducing antibody responses, often without adjuvants (70). In addition, due to their structure and size VLPs are capable of diffusing to lymph nodes from the site of injection where they can interact directly with B-cells to trigger antibody responses (192).

In addition to B-cell responses, VLPs also induce strong CD4 proliferative and CTL responses (116), which are the aim of current T-cell vaccine strategies. Features of VLPs, such as preferential targeting of dendritic cells (DCs), efficient delivery to both MHC class I and II molecules and the ability to directly activate the DC maturation, make them strong inducers of
CTL responses. Due to their particulate structure exogenous VLPs are readily taken up by antigen presenting cells (APCs), in particular by DCs, and presented effectively in the context of MHC class II molecules as well as they can be cross-presented on MHC class I molecules, thereby inducing a CTL response, which is essential for the clearance of intracellular pathogens such as viruses (115). For example, it has been reported that human papillomavirus (HPV) VLPs directly activate DCs leading to the expression of co-stimulatory molecules, cytokines necessary for activation of CTLs (85). Furthermore, some VLPs that retain receptor binding regions of cognate viruses can enter cells through their normal receptor and can be taken by DCs as exogenous antigens for class I presentation (13). Importantly, DC maturation and migration, essential for activating the innate and adaptive immune responses, are promoted by uptake of VLPs (54). It was shown that the stimulation of DCs does not require the virus replication but it requires an intact envelope of either an inactivated virus (50) or that of a VLP (13), or an intact non-enveloped VLP (187). This feature gives advantages to VLPs over the cognate live viruses for immune activation, because several viruses have an ability to interfere with the DC activation (125). Moreover, the self-adjuvanting effect of VLPs to target DCs is an important advantage over soluble antigens, which are less immunogenic and require the co-administration with adjuvants in several booster injections. However, not all VLPs can skew the immune response towards Th1, and the addition of innate immunity stimulators is required. This drawback can be easily overcome as VLPs have the ability to encompass immune stimulating adjuvants, such as Toll-like receptor (TLR) ligands, within their lumens. For instance, vaccination with CpG-loaded VLPs derived from the hepatitis B core antigen or the bacteriophage Qbeta was able to induce high frequencies of peptide-specific CD8+ T cells (164). TLR9 ligands have an adjuvant activity that directly affects B-cells to undergo isotype switching to Th1 (79). Similarly, single-stranded
RNA packaged within some VLPs can activate TLR7 present in B-cells and enhance B-cell responses and class switching to Th1 (79).

Importantly, in addition to priming T-cell responses, CpG-loaded VLPs are capable of boosting them without a change in carrier in contrast to heterologous prime-boost strategy (155). Finally, in most of the cases VLPs lack the DNA or RNA viral genome, but have the authentic conformation of viral capsid proteins seen with attenuated virus vaccines, thus they represent a safer alternative to attenuated viruses. Therefore, all characteristics of VLPs described above have made them attractive vaccine candidates for many viral diseases as well as carrier molecules for epitopes for other pathogens (66) (Figure 3).

**Figure 3.** Features of virus-like particles. The ability of VLPs to self-assemble allows incorporation of activators of innate immunity as well as plasmid DNA within their lumen and makes them a promising vehicle for delivering immune stimulating
adjuvants and genetic material into target cells. Due to their particulate structure VLPs are readily taken up by APCs and presented effectively in the context of MHC class I molecules, thereby inducing a CTL response. The dense, repetitive nature of VLPs makes them particularly effective in inducing antibody responses. Proteinaceous composition of VLPs permits chemical conjugation or genetic fusion, thereby allowing them to serve as carrier molecules for other pathogens. Figure created by using reference (129).

The most straightforward application of VLPs is to use them for vaccination against the corresponding virus from which they are derived. Examples of such VLPs used for vaccines and vaccine development are hepatitis B virus, HPV, hepatitis E virus, influenza, hepatitis C virus, poliovirus, HIV, Ebola virus, Marburg virus, Norwalk virus, Rotavirus, SARS coronavirus VLPs (66). Recently, VLP-based vaccines for HBV and HPV have been licensed commercially. Recombivax (Merck & Co., Inc.) (104) and Engerix (GlaxoSmithKline (GSK)) (7), both were approved in the USA in 1980s. Gardasil (Merck) was approved in 2006 (55). In addition, VLPs can also be used to present foreign epitopes to the immune system. This can be achieved by genetic fusion or chemical conjugation. Genetic fusion of the protein transduction domain of HIV-1 Tat protein with the core gene of HBV provide an example of this approach (27). Alternatively, foreign vaccine proteins may be chemically conjugated to pre-formed VLPs. For example, this approach has been used in the production of HBV core antigen VLPs chemically conjugated with M2 peptide of influenza A virus (51). Chemical conjugation also allows non-protein targets, such as glycans or other small haptens to be attached to VLPs. One recent example is the VLP-based vaccine against nicotine dependence. This vaccine was developed by covalently coupling nicotine to the surface of VLPs (103). Therefore, the ability of VLPs to serve as carriers of epitopes derived from either the parental virus or foreign sources has enhanced and broadened their potential as prophylactic and therapeutic vaccines.
This technological innovation, especially genetic fusion and chemical conjugation, has given the beginning of numerous chimeric VLP-based vaccines. Chimeric VLPs can act as carriers of immunologic epitopes derived from viral, microbial pathogens as well as they can be used to induce autoantibodies to disease-associated self-molecules involved in chronic diseases. For instance, VLPs derived from both double- and single-stranded DNA and RNA viruses encompassing 14 different families have been used for the production of chimeras (134). Among clinically tested vaccines based on VLPs carrying microbial epitopes are two anti-malaria vaccines, Malarivax (Apovia) (118) and RTS,S (GSK) (67). VLP display of self-antigens is used successfully to target molecules that are involved in the pathogenesis of a variety of chronic diseases, including Alzheimer’s disease (190), hypertension (5, 172), obesity (53) and certain cancers, specifically, epithelial cancers (58). Clinical proof of concept has been achieved with a VLP-based anti-angiotensin II vaccine against hypertension. This vaccine known as AngQb has been shown to reduce blood pressure in preclinical models of disease and in humans in a phase IIa clinical trial (5, 172). Therefore, ability of VLPs to carry heterologous epitopes made it possible to target antigens that were previously refractory to vaccine-based approaches. Recently, VLPs have been explored for their ability to serve as delivery vehicles for plasmid DNA (pDNA). The efficient packing of pDNA in VLPs derived from several viruses, such as papillomavirus (PV) (173), polyomavirus (30) and hepatitis E virus (165) was demonstrated. Touze and Coursaget showed higher gene transfer in diverse eukaryotic cell lines using papillomavirus like particles for DNA delivery compared with DNA alone or delivered with liposomes (173). Takamura et al. showed that VLPs derived from orally transmitted viruses, such as hepatitis E, were especially suitable for the oral application of DNA vaccines (165). In a mouse model, orally applied PV VLPs packed with pDNA encoding the HIV-1 Gag protein
showed the ability to induce Gag-specific memory CTLs and the generation of Gag-specific serum immunoglobulin G (IgG) and mucosal immunoglobulin A (IgA) antibodies (191). Thus, virus-like particles are a very promising vehicle for delivering genetic material into target cells.

VLP technology has the potential for therapeutic vaccination as an economic and effective treatment option for chronic diseases. However, one of the drawbacks that may delay the application of this strategy is a low compositional and architectural consistency of VLPs produced using existing manufacturing methods. This problem creates a demand for the development of new large-scale bioprocesses having high yield, quick process time and reduced total cost. Future directions in manufacturing may include an in vitro chemical self-assembly of VLPs based on capsid components (66).

DNA vaccines:
Plasmid DNA vaccination was discovered over 10 years ago as a potent method to elicit humoral and cellular immune responses to foreign antigens (169). The simplicity and attractiveness of DNA vaccines were obvious, as they were represented by plasmid DNA which was easy to produce in laboratory bacterial strains at comparatively low cost, as well as easy to store and transport since plasmid DNA is chemically and biologically stable. Conventional plasmids used for vaccination are double-stranded DNA molecules, which can be subdivided into two functional units: an eukaryotic expression cassette (transcriptional unit) and a plasmid backbone (68) (Figure 4). The transcription unit consists of regulatory elements: eukaryotic promoter, enhancer and a polyadenylation signal required for the appropriate expression of the gene of interest in the target cell. An additional expression cassette for genes of immunomodulatory proteins may be inserted in order to enhance the immune response. The plasmid backbone
includes elements, such as antibiotic resistance genes and an origin of replication required for the production of plasmid DNA in bacterial cells. In addition, unmethylated CpG motifs, which stimulate the immune response in the host, can be incorporated into this functional unit (Figure 4).

**Figure 4.** Schematic drawing of a plasmid DNA vector. The plasmid DNA can be divided into a transcriptional unit and a bacterial backbone. The transcriptional unit includes a promoter, which can drive high level of protein expression in eukaryotic cells, an ORF for a vaccine antigen, and transcription/termination sequences (Poly A). The bacterial backbone consists of a bacterial origin of replication and an antibiotic resistance gene. Unmethylated CpG motifs can be incorporated into plasmid DNA to stimulate immune responses in the host cell.
Figure 5. Mechanisms of antigen presentation after DNA immunization. Application of a plasmid DNA vaccine leads to: (A) Direct transfection of professional APCs resulting in antigen presentation to T cells; (B) Cross-priming, when antigens...
generated by somatic cells can be taken up by professional APCs to prime T-cell responses. Figure created by using reference (68).

The principle of DNA vaccination is based on the production of the antigen(s) of interest \textit{in vivo} by cells of the vaccinated person or animal, whereby pathogen-derived genetic information combined with eukaryotic expression system is applied. It means that the \textit{in vivo} synthesized antigens retain their native structures, including post-translational modifications, accurately mimicking their ‘natural’ counterpart. There are two main mechanisms by which the antigen encoded by plasmid DNA is processed and presented to elicit an immune response: direct transfection of professional APCs and cross-priming (68) (Figure 5A). When the gene of interest is transfected directly into professional APCs and expressed after transfer into the nucleus, the resulting protein is directed into the MHC class I pathway of antigen presentation. On the other hand, if the antigen is first secreted or released following expression in myocytes and then taken up by APCs, the protein is targeted to the MHC class II pathway of antigen presentation, although such antigens may also be able to ‘cross-over’ into the MHC class I pathway following internalization into APCs (cross-priming) (Figure 5B). In contrast to DNA vaccines, exogenous delivery of killed or subunit vaccines leads preferentially to the Th2 immune response, since the antigens are taken up by APCs and targeted into the MHC class II molecules. Therefore, the ability to present antigens through both MHC classes makes DNA vaccines able to stimulate both arms of the immune system simultaneously (24).

Besides the intrinsic immunological advantages of DNA vaccines, there are other factors making these vaccines attractive. As DNA vaccine plasmids are non-live, non-replicating and non-spreading, there is little risk of either reversion to a disease-causing form or secondary infection. Moreover, DNA vaccines are highly flexible, encoding several types of genes
including viral or bacterial antigens, and immunological and biological proteins. In addition, the use of DNA approach avoids the risks linked to the manufacture of killed vaccines, as exemplified by the tainting of a polio vaccine with live polio virus owing to a production error (122).

Due to their simplicity and versatility DNA vaccines became a promising strategy to develop immunity against several infectious diseases including HIV, tuberculosis, malaria, influenza and the newly emerging Ebola and SARS viruses (33, 42, 124, 141, 158). DNA immunization was used in a wide variety of model systems (72, 177, 178). As a result, four veterinary DNA vaccines have been approved for commercial sale by the respective regulatory agencies in the USA and Canada. One against West Nile virus in horses (37), one against infectious hematopoietic necrosis virus in schooled salmon (56), one for treatment of melanoma in dogs (17) and the most recent licensure, growth hormone releasing hormone product for foetal loss in swine (171). However, a much lower immunogenicity of DNA vaccines has been observed in higher primates and clinical trials in humans and high doses and/or multiple immunizations are required to induce protective immune responses (80, 90), which hampers the practical application of DNA vaccines.

As a result of the limited immunogenicity of DNA vaccines, considerable efforts are underway to solve this problem. There are several ways in which antigen expression and immunogenicity can be improved for DNA vaccine platform: optimization of the transcriptional elements in the plasmid backbone with the aim to improve antigen expression levels; strategies to improve protein expression of the gene of interest, inclusion of adjuvants in the formulation or as immune modulators, and the development of new delivery systems for specific targeting and/or better DNA uptake (84). The latter two approaches influence the magnitude, the type of
immune response as well as they can facilitate antigen entry into DCs. Approaches that are aimed at targeting selected antigens to APCs, especially to DCs, have shown potential in vaccinating against disease in animal models and humans as targeting vaccines to DC appears to be essential for the induction and modulation of immune responses.

**Improving immunogenicity through formulation**

One current trend in DNA vaccine formulation is the use of biodegradable polymeric microparticles and liposomes (121). Polymeric delivery systems and liposomal delivery systems represent novel DNA delivery platforms that protect DNA from degradation and facilitate targeting to specific tissues, thereby increasing the transfection efficacy of target cells. It has been shown that microparticle constructs, such as poly (D,L-lactic-co-glycolic acid) copolymers (PLGA), can be easily phagocytosed by DCs in vitro or in vivo (120, 179) and this can stimulate further DC maturation in the lymph node (139). The immune responses of DNA-loaded microparticles were investigated in mice, non-human primates and humans (82, 111, 126, 127, 181). It was shown that the parenteral administration of PLGA encapsulated DNA induces a significant CTL response (105). In addition, the oral administration of encapsulated DNA in PLGA microparticles elicited systemic and mucosal immune responses upon challenge (81). Moreover, polymeric matrices can be designed by the incorporation of ligands, such as folates, transferrin, antibodies and sugars, to enhance tissue targeting (108). Similar to polymers, liposomes are taken up by APCs and mediate MHC class I antigen presentation, and have been shown to induce cellular and humoral immunity (180, 182). Furthermore, immunogenicity of DNA vaccines can be improved by directly targeting the encoded vaccine protein to DCs in vivo. Nchinda et al. demonstrated that the incorporation of antigens into an antibody against the DC
endocytic receptor, DEC205, enhanced the antigen presentation by DCs in lymphoid tissues, thereby improving the efficacy of DNA vaccines (119).

**Improving immunogenicity by including immune modulatory adjuvants**

The DNA itself has adjuvant properties through CpG motifs. For instance, synthetic oligodeoxynucleotides containing unmethylated CpG motifs that are DNA specific sequences recognized by TLR9 can act as immune adjuvants in mice, as they boost the humoral and cellular responses to co-administered antigens (73). Moreover, the addition of many CpG motifs into a plasmid backbone has improved the immunogenicity of DNA vaccines inducing Th1-biased immune response (133). However, it has been recently shown that DNA vaccine immunogenicity has been attributed to its double-stranded structure, which activates TANK-binding kinase 1-dependent innate immune signaling pathway in the absence of TLRs (31).

In addition to self-adjuvanting effect of DNA, co-administration of genetic adjuvants such as cytokines, chemokines or co-stimulatory molecules is a separate approach in which genes are co-delivered in order to enhance the magnitude and type of desired immune responses to DNA vaccines. For example, the employment of Th1-associated cytokines such as interleukin (IL)-12 and IL-15 in non-human primates induced antigen specific cellular immune responses (29, 151). DNA vaccines co-injected with plasmids encoding Th2-inducing cytokines such as IL-4 augmented antigen-specific humoral immune responses (87). Furthermore, proinflammatory cytokines have the ability to modulate immune responses to DNA vaccines. For instance, the granulocyte-macrophage colony-stimulating factor (GM-CSF) is capable of enhancing humoral and cellular immune responses (168). However, the timing of GM-CSF administration influences the Th1/Th2 immune response (83). Chemokines can also modulate the immune response and
protective immunity. Sin and colleagues observed that co-injection with IL-8 and RANTES plasmid DNAs dramatically enhanced antigen-specific Th1 type cellular immune responses and protection from lethal herpes simplex virus-2 challenge (159). In addition, co-delivery of the costimulatory molecules CD86 during DNA vaccination enhanced both helper and cytotoxic T-cell responses (2).

Improving immunogenicity by using next-generation delivery strategies

The direct injection of plasmid DNA into muscle or skin is still the most widely used. However, the biggest drawback of this delivery method is the low efficacy achieved in larger animals and humans. Therefore, development of physical delivery methods to increase the transfection efficiency of target cells has been a primary focus of research in more recent years. Physical methods for pDNA transfection comprise electroporation, ballistic needle-free delivery systems and microporation.

Microporation involves use of hundreds of microneedles that enable topical immunization with naked plasmid DNA as they can bypass the stratum corneum, thereby delivering plasmid DNA to APCs of the skin. This induces stronger and less variable immune responses than via needle-based injections (109).

Another physical method widely employed in DNA vaccination is particle-mediated epidermal delivery (PMED) also called the “gene gun”. In this procedure, DNA-coated microparticles composed of gold are accelerated to high velocity to penetrate cell membranes in the epidermis where a variety of cells, including the Langerhans cells, the APCs of the skin, can be directly transfected (130). As a result, the gene gun immunization have 10-100-fold more expression of the DNA-encoded protein than intramuscular vaccinations and 100-fold less DNA
is required for the same level of expression (12). The gene gun technique has been used to immunize nonhuman primates against a variety of diseases, including HIV, Ebola, Japanese encephalitis, hepatitis E and B, influenza, smallpox etc (52). Thus, PMED can induce higher antibody and/or CD8+ T cell responses in mice and monkeys with substantially lower doses of DNA in comparison to needle-based approaches.

An alternative approach based on the use of electric pulses to transiently permeabilize cell membranes, thus permitting cellular uptake of plasmid DNA, is electroporation. It has been extensively studied in large animal species such as dogs, pigs, cattle and non-human primates to deliver DNA vaccines (10, 75, 148, 176). The potential of electroporation for DNA vaccination has been demonstrated by the increased protein expression and a robust stimulation of the immune response. Electroporation might allow for less frequent immunizations with the DNA vaccines, and can improve both cellular and humoral responses (149).

Currently, the DNA platform represents almost one quarter of all gene therapy vector systems under clinical evaluation (84). The interest to further development of this vaccination strategy is strengthened by recent licenses in the area of animal health and by the improvements in immune potency reported in the non-human primate model systems.

**Viral Vectors:**

The idea of using viruses as gene-delivery systems to combat diseases stems from a documented immunogenicity and safety profile of the majority of existing live-attenuated vaccines. Attenuated live-virus vaccines appear to be the most effective immunogens that mimic natural infection. Another important attribute of these vaccines is high cost efficiency for mass
vaccination as they are optimal for large-scale production. Recent developments in genetic engineering coupled with the accumulated data on the nucleotide structure of viral genomes and functions of viral genes allowed precise manipulations of viral genomes cloned as bacterial plasmids (167) or bacterial artificial chromosomes (36). This set of diverse technologies is applicable to the generation of recombinant DNA and RNA viruses and is based on reverse genetics, which is the most powerful tool in modern virology and has the potential for a variety of applications, from the basic understanding of virus biology to the development of genetically engineered viral vaccines and therapeutic gene delivery systems. Reverse genetics approach can be applied for the construction of genetically attenuated vaccine strains (100) or development of live viral vectors that are engineered to induce immunity to an array of proteins encoded by genes inserted into the vector (22). One of the first licensed recombinant virus vaccines was represented by vaccinia virus carrying the gene encoding rabies virus glycoproteins. This vaccine reduced the spread of rabies virus in wildlife populations and also reduced the spill-over of virus infection from wildlife to domestic animals and humans (23, 96).

Reverse genetics approach led to the development of a wide range of different DNA (1, 163) and RNA (20, 131, 186) viruses as vectors for efficient delivery of vaccine antigens. This strategy is especially attractive for the development of vaccines against highly dangerous pathogens, such as HIV, for which even a short course of replication of an attenuated live virus in the host is not acceptable due to the possibility of integration of vaccine genes or reversion of the vaccine strain to wild-type. The appropriate balance between safety and immunogenicity is a critical issue for the construction of any live attenuated vaccine. Inadequate safety is the main reason for retaining of many replication-competent viral vectors from entry into human clinical trials. In order to increase the safety of viral delivery vectors, the development of replication-
defective viruses produced in complementing cell systems was introduced (43). However, the abrogation of the ability to replicate in the vaccinated host is commonly associated with lower immune responses induced by replication-incompetent viral vectors. In this connection, replication-competent viral vectors that originate from commercial live-attenuated vaccines seem to be a promising perspective for the development of novel safe and immunogenic vaccines (20, 161).

Major concerns associated with viral vector vaccines include the potential risk of changes in viral tropism induced by introduced modifications, and the presence of pre-existing antivector host immunity. The latter drawback can be managed by certain approaches including engineering of vaccine vectors from viral strains that have not circulated widely in host populations (101) or by mutating viral surface proteins in order to evade host neutralizing antibodies (147). Additional approach to circumvent the problem of pre-existing antivector immunity is to exploit the asymmetry in induction of systemic and mucosal immune responses. Specifically, if the mucosal immune system remains naïve after systemic immunization against certain virus, it might still be amenable to immunization with recombinant viral vector based on the same or related virus. For instance, mucosal administration of measles and measles-rubella vaccines was shown to be more efficient than subcutaneous administration in pre-immunized humans (16, 41). On the contrary, in the case of active mucosal immunity to a vector, subcutaneous vaccination may be an efficient way to overcome pre-existing immunity (48). However, the pre-existing immunity is not detrimental for some viral vectors, thus measles virus (MV) is able to induce cellular and humoral immunity after re-vaccination (92).
Currently, a variety of recombinant viral vectors are either under preclinical or early clinical investigation (89). Among the most promising viral vectors are adenovirus vectors (183, 189), adeno-associated virus (AAV) vectors (91), poxvirus vectors (62), alphavirus vectors (113, 145), MV (88), vesicular stomatitis virus (VSV) (78), poliovirus (35), and herpes virus (44). Each of these vectors has unique properties that should be considered for the selection of the optimal vector for the vaccination strategy of choice.

A high level of antigen expression and efficient targeted delivery are the main goals in the process of the development of viral vector vaccines. Using the targeted vector delivery, the antigen-coding gene can be directed to professional APCs or to a specific tissue. For example, members of alphavirus genus and poxviruses are able to transduce DCs without inhibiting the immunostimulatory function of these APCs (114, 174). The transduction of DCs may elicit direct priming of an immune response. However, in the case of poxviruses it was shown that CTL responses against modified vaccinia virus Ankara were dominated by cross-priming \textit{in vivo}, despite the ability of the virus to infect DCs (57).

Adenoviruses (9), MV (15) and poliovirus (34) infect predominantly via mucosal surfaces. Vectors based on these viruses may therefore be effective for mucosal administration and induction of mucosal immunity. In addition, it was shown that VSV recombinants expressing the hemagglutinin protein of influenza virus (146) or the MV hemagglutinin (152) were able to induce protective immune responses after intranasal immunization in rodents. However, in the case of VSV as an intranasal vaccine vector potential neurotoxicity should be taken into consideration (143). A positive characteristic of VSV as a vaccine vector is that no proteins encoded by the VSV genome have the capacity to interfere with the host interferon
response (162). It seems likely that the cytokine response activated in target cells during the VSV infection will possesses an adjuvant activity for antigen-specific responses to vaccine antigens.

Viral vectors are different in terms of their capacity for the insertion of foreign genetic material. Thus, non-enveloped viruses such as poliovirus, adenovirus, and AAV have a rigid capsid structure that does not allow incorporation of more genetic material than the size of wild-type virus genome. For example, adenoviral capsid can package ~105% of the adenovirus genome size. It means that a transgene of 3-4 kilo base pairs (kbp) can be packaged by a replication-competent E3-deleted adenovirus. Replication-defective vectors bearing additional deletions of essential E1 region in their genomes are able to accommodate up to 7.5 kbp of foreign DNA (140). Some viruses with larger genomes can tolerate larger insertions of foreign genetic material, but vector construction may in turn be more laborious.

Viral vectors that replicate in the nucleus represent potential risk of incorporation of their genes into the host genome. Thus, vectors with entirely cytoplasmic replication cycle, such as poxviruses or MV, may be preferable to reduce the risk of integration. For some applications, especially in gene replacement therapy, integrating vectors, such as retroviruses, could be beneficial.

Another important characteristic of viral vaccine vectors is that some of them can induce innate immune responses through pattern recognition receptors (PRRs), acting as adjuvants to the delivered antigens. This adjuvant effects are mediated through type I interferon production and an inflammatory response. It has been proposed that the more PRRs are activated, the more robust the immune response induced by the vaccine (136). A group of membrane-bound signaling PRRs is represented by an array of TLRs and the mannose receptor (8, 18). Each
member of the TLR family recognizes distinct pathogen-associated molecular pattern and mediates the activation of NF-kB and over signaling pathways (166). TLRs recognize several molecular patterns associated with viruses including surface glycoproteins (TLR1, 2 and 4) (19, 60) and nucleic acids (TLR3, 7, 8 and 9) (3, 40, 94, 175). TLR3 is a receptor for double-stranded (ds) RNA that transmits signals that activate NF-kB and IFN-β promoter (102). The replication cycle of many RNA viruses has a double-stranded RNA replication intermediate, which is able to activate innate immune response through the TLR3 pathway (144). TLR3 signaling may be of particular importance for the immune responses to viruses that do not directly infect DCs as it was shown to promote cross-priming of CD8+ T cells in response to dsRNA (154).

The diversity of viruses and replication strategies employed by them creates unlimited perspectives for their use as gene-delivery vehicles to combat diseases. Although many problems associated with safety and efficient manufacturing of viral vectors remain to be addressed this strategy is one of the most promising in respect of development of a cure against unconquerable diseases, such as AIDS, malaria, tuberculosis, influenza, and various cancers.

**Prime-boost strategy:**

Commonly, schemes of vaccination include repeated administrations of the same vaccine (homologous boosting) to achieve protective humoral immune responses. However, immunity developed after the primary immunization by a vaccine antigen can impair effective antigen presentation after subsequent injections. This is believed to be a reason for a low cellular immune response induced by the use of homologous boosting. As a solution to this problem, the
sequential administration of a vaccine antigen using different antigen-delivery systems (heterologous boosting) was proposed and referred to as ‘prime-boosting’. This strategy evolved after the gene-based vaccines had emerged as promising alternatives to traditional vaccine strategies. The basic prime-boost strategy involves priming the immune system to an antigen delivered by one vector and then boosting this immunity by re-administration of the antigen in the context of a distinct vector. This vaccination scheme induces synergistic enhancement of immunity to the target antigen reflected in an increased number of antigen-specific T cells, selective enrichment of high avidity T-cells and increased efficacy against pathogen challenge (185). Recently, several studies have demonstrated the efficacy of prime-boost vaccination strategies in generating cellular immunity to a variety of pathogens. These include, Mycobacterium tuberculosis (45, 137), HIV (21, 188) and simian immunodeficiency virus (153), malaria (28, 170), Leishmania (25, 138), Ebola virus (107), hepatitis C virus (38, 128), herpes simplex virus (49, 160), human papillomavirus (47, 95), hepatitis B virus (110), and Japanese encephalitis virus (86). Thus, heterologous prime-boost vaccination strategies with use of DNA vaccines and viral vectors generate high levels of both CD4+ and CD8+ T-cell mediated immunity, which is currently viewed as crucial for protection against a variety of pathogens, including HIV.

**Conclusions:**

Last decades have brought remarkable advances in the field of molecular biology and gene sequencing that help to uncover the mechanisms of interactions between microorganisms and the host immune system. This progress has significantly facilitated the development of rationally-
designed vaccines for both prophylaxis and therapy of a wide range of diseases. Currently, the field of vaccine development has a set of novel powerful tools. This arsenal of tools includes reverse vaccinology, which allows identification of potential vaccine antigens without the need for cultivating the pathogen, as well as a wide variety of vectors that allow increase and manipulation of the immune response towards the desired type of immunity. Some successful steps have been made to solve the key problems associated with the relatively low immunogenicity of DNA vaccines and pre-existing immunity to viral vectors. These steps include development of a DNA prime and recombinant viral vector boost strategy, which may help circumvent pre-existing immunity to the viral vector, and generation of different methods that improve the antigen expression by vaccine vectors and presentation of expressed antigens to the immune system. Some of these new vaccine strategies are being translated into clinical trials.

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43