reviews

molecular pharmaceutics

Vaccine Delivery Methods Using Viral Vectors

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Received September 15, 2006; Revised Manuscript Received November 28, 2006; Accepted November 28, 2006

Abstract: Viral vectors have different capabilities as gene delivery vehicles for vaccines and immunotherapeutics. This review discusses selected viral vector systems and plasmid DNA and provides an overview of their specific characteristics, strengths, and limitations. The features, modes of viral entry and replication, expression of heterologous proteins, issues related to both preexisting and heterologous immunity, and vaccine strategies are discussed for the different vectors. Comparisons of the features and capabilities of the vectors are provided.

Keywords: DNA vaccines; adenovirus; adeno-associated virus; poxvirus; canarypox; MVA; vesicular stomatitis virus; alphavirus; measles; vaccine strategies; preexisting immunity; prime−boost

Introduction

Viruses have evolved highly efficient structures and mechanisms for infecting cells and utilizing the cellular machinery for production of virally encoded proteins. Thus, viral vectors are natural preferred vehicles for heterologous gene delivery for immune responses and have been extensively studied and developed as such. Although not all viruses specifically bind to and infect antigen-presenting cells (APC), this may not be a prerequisite for an effective vaccine or immunotherapeutic vector. The reasons for this include the fact that humoral responses can develop even if the antigen is not made by an APC, and the mechanism of cross-priming, whereby a cell expressing an antigen can transfer the antigen to professional antigen-presenting cells for the generation of MHC Class I-restricted cytolytic T lymphocyte responses.

During the past few years, vectors for efficiently carrying and delivering genes for immunization have been developed. The earliest example of a viral vector dates to the use of vaccinia as a vector for a licensed animal rabies vaccine, discussed below in the poxvirus section. The concept of nucleotide-based DNA plasmid vaccines derives from the demonstration of cellular and protective immunity to microbes.[1] DNA plasmids containing only a promoter, poly A, a selection marker and the gene of interest were made. Also, RNA may serve as a carrier for a construct, which then is assumed to be functioning as mRNA in the cytoplasm only. The efficacy of such constructs has, however, not been clearly proven in man. More complex plasmids and microbial vectors have been developed for several reasons: to ensure efficient delivery to somatic cells, to increase the antigen produced, and to increase their potency by the provision of adjuvants or the activation of innate immune responses.

Attenuated live virus vaccines, whether representing the virus itself (e.g., measles[2]) or a recombinant organism based on the vector (e.g. measles with heterologous gene inserts from other pathogens), appear to be the most effective immunogens, mimicking real-life infection. With certain pathogens, however, such as HIV, it is not acceptable to have even a short course of replication of an attenuated live virus

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10.1021/mp060098+ CCC: $37.00 **© 2007 American Chemical Society**
Published on Web 12/29/2006

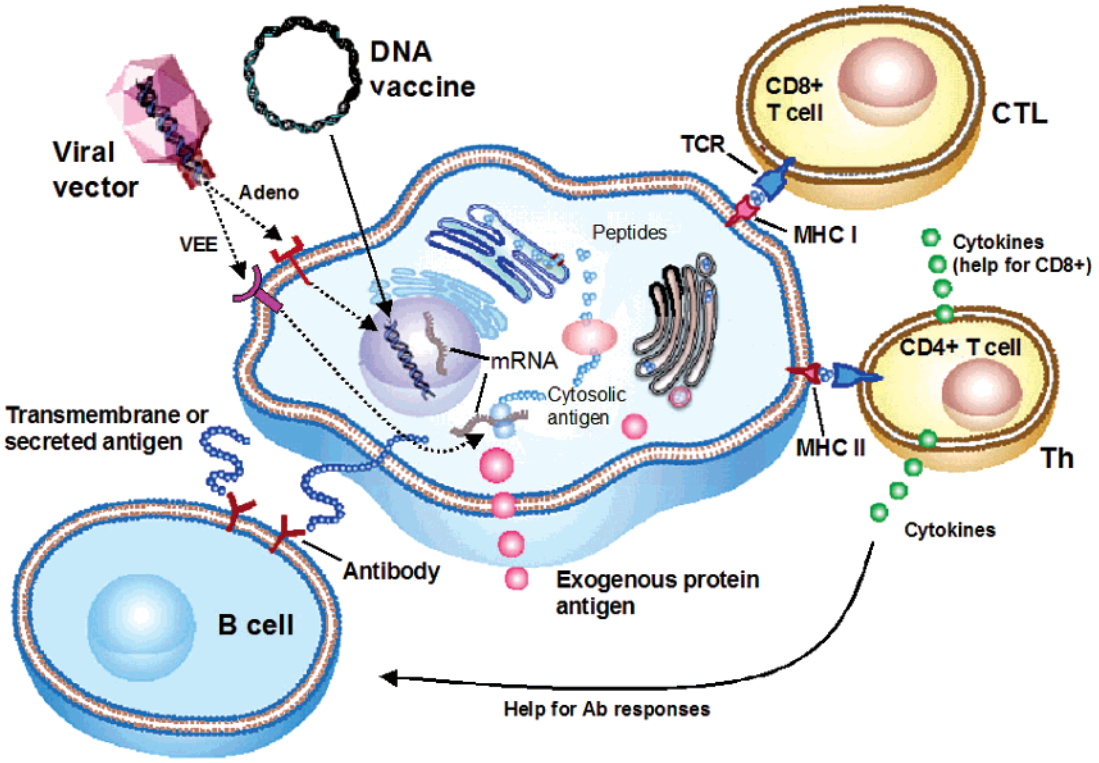

Figure 1. Viral vectors access the cell by specific receptors and replicate or directly express their proteins intracellularly. As examples, the VEE vector, an RNA virus, enters through its cellular receptors and expresses its encoded protein in the cytoplasm. The adenovector, a DNA virus, enters through other receptors; its nucleic acids go to the nucleus, but the genome does not integrate. Plasmid DNA enters the cell nucleus inefficiently when simply injected or more effectively via electroporation. The encoded proteins are then expressed, secreted for activation of B-cells and antibody production, or presented as peptides that bind to MHC class I or class II for cellular immune activation.

in the host, since the vaccine gene(s) may integrate or the vaccine revert to wild-type. Therefore, DNA in plasmids or genes inserted by recombinant techniques into a vector that is considered safe have been developed to avoid these safety concerns.

Methods for increasing the immunogenicity of a vaccine in addition to alterations of the vector itself include alterations in (a) the route and method of delivery of the gene, (b) the dose administered, (c) the number of immunizations and the timing between them, and, very importantly, (d) the adjuvants that are given as codelivered plasmids, proteins, or other compounds. These means of application have not yet been fully worked out for gene-based or recombinant vector vaccine use in humans. To add further to this complex situation, it has been shown that a mixture of deliveries by priming with one type of immunogen and boosting with one or two different recombinant vectors (or proteins) has been successful in both preclinical and human studies aimed at inducing cellular and humoral immunity.[3,4]

As the delivery of plasmid DNA vaccines is rather unspecific, the uptake of viral vectors is aided by specific cellular receptors, making it, at least theoretically, possible to target a particular cell type (Figure 1). However, viral vectors have traits that both increase and decrease their utility as vaccine vectors, including issues such as induction of innate immune responses, potential safety concerns, and the fact that humans may have preexisting immunity against the virus used as a vector. These issues will be discussed for a variety of viral vectors and plasmid DNA and are summarized in Table 1.

DNA Vaccines

Features. DNA vaccines differ from the other vectors discussed in this review in that they do not utilize another pathogen per se as the delivery vehicle, nor are they derived from viruses. DNA vaccines are comprised of bacterial plasmids, which have been altered to utilize a promoter (generally viral) that functions in mammalian cells, a transcriptional terminator, and possibly other elements needed for the production of the plasmid in bacteria, such as selection markers. Modifications of the genes have included DNA codon optimization, different promoters, and polyAs.[5]

Preexisting Immunity. In contrast to viral vectors, the DNA likely will not generate antivector immune responses, nor are there preexisting immune responses that might interfere with the ability of the plasmid to be used or reused.

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Table 1. Major Advantages and Disadvantages Influencing the Choice of Vaccine Vectors

vector	advantages	disadvantages
plasmid DNA	easy construction, modification, and addition to other vaccines; can act as potent priming; very stable	less immunogenic than anticipated in humans; needs dose increase, adjuvants, electroporation/delivery technology, or addition of heterologous modality boost
poxviruses	can carry large and several foreign antigen (nucleotide) loads; only DNA virus vector replicating in cytoplasm, therefore no risk of integration; experience in humans; no preexisting immunity (fowlpox)	complex construction, constructions not always stable; expensive production; some prior immunogenicity to vector (vaccinia)
adenovirus	effective cellular uptake and protein expression; rapid induction of immunity; stable constructions; experience in humans with natural infection	moderately large foreign antigen (nucleotide) load; immunogenicity to some strains of the virus in the population; no experience in humans of recombinants or chimpanzee adenoviruses; human adenoviruses are oncogenic in animals
adeno-associated virus	can be modified to not carry any original viral genes; proven to be safe and well-tolerated in gene therapy trials	integration considered by some to be a possible risk, but evidence not clear; small foreign antigen (nucleotide) load
vesicular stomatitis virus	high level of expression of inserted genes; low preexisting immunity	safety concerns due to neural tissue receptor
alphaviruses	high expression capacity; RNA virus, integration not possible; can target dendritic cells; no or low preexisting immunity	so far relatively unexplored in humans; small to moderate foreign antigen (nucleotide) load
measles virus	RNA virus, no integration; experience in children as a homologous vaccine; mucosal receptors	preexisting immunity; moderate foreign antigenic (nucleotide) load
poliovirus	experience in children as a homologous vaccine; mucosal receptors	CNS receptors? Recombinants unstable; structure-dependent uptake, sensitive to genetic changes; small foreign antigen (nucleotide) load; preexisting immunity
hepatitis B virus	high level of expression of foreign antigens; stable	preexisting immunity; reverse transcription involved in replication cycle; small foreign antigen (nucleotide) load

Entry and Expression of Foreign Proteins. Whereas viruses have specific structures and mechanisms to enable entry of the virus into a cell by infection, plasmid DNA in its native state is inside its bacterial host and thus does not have a defined mechanism for internalization into the cells, which it transfects, albeit very inefficiently, following direct injection of a plasmid. Indeed, prior to the initial demonstration of the ability of plasmids to be taken up following direct intramuscular injection,[6] it was assumed that plasmid DNA would need formulation or association with particles to transfect cells in vivo. Thus, many of the efforts to increase the potency of DNA vaccines (reviewed in ref 7) have centered upon increasing the rate of transfection of DNA into cells in vivo. Interestingly, the increased rate of transfection may not need to be into professional antigen-presenting cells, because there is evidence that muscle cells transfected with plasmid DNA can express proteins, which then in some form are transferred to professional antigen-presenting cells.[8] The mechanisms whereby unformulated plasmids, such as those injected in saline solutions, gain entry into cells and their nuclei is unknown. However, methodologies for increasing the rate of cellular entry include the use of particles, electroporation,[9] hydrostatic pressure, or propelling forces such as jet streams or particle bombardment. Admixture with lipid solvents or other transfection agents are alternatives that alter cell membranes for uptake of DNA.

Safety Issues. As with other gene delivery systems, the major issue with DNA vaccines is whether the DNA can integrate into the host chromosomes. Plasmid DNA injections in vivo have been studied carefully to rule out the possibility of insertional mutagenesis and oncogenesis. Transfection by plasmid DNA is much less efficient than infection with viruses, thus lowering the likelihood of both homologous and nonhomologous recombination, while also lowering the level of protein expression and related immunogenicity. Recent efforts to increase the number of cells that are transfected by DNA vaccines, utilizing technologies such as electroporation,[9] may increase the potential for integration[10]

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because not only are increasing amounts of DNA transfected into an increasing number of cells but also the mechanism of creating pores in cellular membranes increases the efficiency of nuclear delivery of the plasmid. Because of the association of anti-DNA antibodies with autoimmune disease, DNA vaccines have been extensively evaluated preclinically to see whether any anti-DNA antibodies arise after injection with DNA vaccines. To date, DNA vaccines have been determined to be safe in preclinical and clinical studies.

Adjuvant Effect. Plasmid DNA was initially thought to be simply a vector for delivering the gene encoding the pathogen antigen; however, the bacterial CpG motifs in the sequence are now known to activate the innate immune system via toll-like receptor 9 (reviewed in ref 11). The issue of how DNA vaccine vector backbones can be designed to harness this capability is still under investigation, although a recent study has compared the in vitro and murine in vivo effects of different CpG motifs.[12]

Current Vaccine Strategies: Increasing the Potency of DNA Vaccines and Clinical Studies. DNA vaccines have effectively generated immune responses, protection, or therapy in a variety of preclinical disease models but have generally been disappointing in terms of potency in humans, although two veterinary DNA vaccines have been licensed: for West Nile virus in horses[13] and for infectious hematopoietic necrosis virus for fish.[14,15] Moreover, delivery of DNA coated on gold beads via a gene gun resulted in protective hepatitis B antibody titers in patients who had been nonresponders to the recombinant licensed vaccine.[16]

A variety of new technologies are being developed such as in vivo electroporation[9] and various formulations, including cationic lipids[17] and polylactide-co-glycolide microparticles.[18] Forcing the DNA into cells of either the skin or muscle is also accomplished by the use of specialized devices such as the gene gun noted above, or other propelling devices. The addition of either recombinant or plasmid versions of molecular adjuvants such as cytokines and costimulatory molecules is another approach to increasing the potency of DNA vaccines.

DNA vaccines have been tested clinically for diseases ranging from HIV and malaria to cancer. They are being actively evaluated as a potential approach to pandemic influenza and SARS and for bio-defense because of the ease with which new constructs can be made and the potential ease of manufacture of plasmid versus other types of vaccine. One of the most important strategies has been to combine different types of vaccines in a prime−boost regimen wherein the DNA plasmid serves to prime the immune system for subsequent boosting with a recombinant protein or a viral vector. This strategy, first described for malaria by Hill and colleagues,[19] is under evaluation for HIV utilizing both modified vaccinia Ankara and adenovirus boosts.[20−22] In a recent phase 1 trial, high response rates were noted in healthy persons against a HIV vaccine composed of multigene/multiclade DNA followed by recombinant MVA with heterologous HIV genes.[23]

Adenovirus Vectors

Features of the Wild-Type Virus. Adenoviruses (genus *Mastadenovirus*) are DNA viruses that cause a variety of

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diseases ranging from subclinical infections, in which the person does not even notice the infection, to upper respiratory infections, to diseases of many organ systems, particularly in certain patients with compromised immune systems.[24] Adenoviruses are generally quite species-specific, a feature which has complicated the preclinical studies of adenovectors, and as many humans have been previously infected with adenoviruses (with high rates of infection during childhood), a problem with the development of adenovirus vectors is preexisting immunity against the virus in a serotype-specific manner.

Viral Entry. The breadth of diseases caused by adenoviruses reflects the extensive tropism of the virus. This ability to infect a number of different types of cells is one of the reasons that adenoviruses have been developed as vectors for delivering genes for both vaccine and gene therapy applications (for a review of adenovirus vectors, see refs 25 and 26). Adenoviruses enter cells following binding to a receptor known as CAR, the coxsackie/adenovirus receptor.[27] Internalization, however, also contributes to the tropism of the virus, because it occurs via binding to cellular integrins, notably $\alpha v\beta 5$.[28]

Expression of Foreign Proteins. Adenovectors can be made to be either replication-incompetent (generally by deletion of E1 or deletion of E1 and E3) or replication-competent. An adenovector can package 105% of its genome size into its rigid capsid, and thus, a transgene of 3−4 kb can be included for replication-competent vectors. Replication-incompetent vectors can house transgenes ranging from 7−8 to up to 10 kb depending upon how many original early regions are deleted (reviewed in ref 26).

Safety, Preexisting Immunity, and Adjuvanticity. The key safety concerns with adenovirus relate to the inflammatory response generated by the vector, and a concern about possible integration (as for many gene-based vectors) even though the wild-type virus does not integrate into the genome of the infected cells.[29] There is vast clinical experience with the oral administration of live wild-type adenovirus vaccines, in which more than 100 million military personnel were vaccinated against adenovirus itself. While the immune response directed against the vector may have certain beneficial (i.e., adjuvant) effects for a vaccine, the potential negative aspects include both possible weakened efficiency of the immunization in the presence of antivector immunity and the potential for other deleterious effects of the antivector immunity and inflammatory response. Significant efforts are being made to determine whether the preexisting immunity weakens the effectiveness of the vectors. Adenovirus serotype 5 is the most explored serotype for vaccine vector applications, but the seroprevalence in the world is high against this particular type of the virus.[30,31] Therefore, adenoviruses from strains that have not circulated too widely in the human populations and even chimpanzee strains are under investigation as vaccine and gene therapy vectors. Other novel strategies adopted to avoid preexisting immunity and the possible hampering of vaccine efficacy include the use of virus with altered surface proteins or chimeric virus carrying the surface proteins of another serotype.[32−35]

Current Vaccine Strategies. Adenovectors have been made and tested for a variety of diseases ranging from infectious diseases to cancer, and for human as well as animal applications. The first gene therapy licensed product, Gendicine,[36] is based upon an adenovector encoding tumor suppressor p53 and was approved in 2003 in China for use in the treatment of squamous cell head and neck cancer. For

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vaccines, adenovectors are being developed in efforts to make vaccines for infectious diseases, including HIV (reviewed in ref 37), malaria,[38] SARS,[39,40] and Ebola.[41] Further, a nasal application of an adenovirus expressing an influenza gene has demonstrated safety and immunogenicity in humans.[42] As noted in the DNA Vaccines section, adenoviral vectors are used in not only single-modality vaccine regimens but also mixed-modality prime−boost strategies, for instance, following a prime with plasmid DNA vaccines. Adenovectors are also being evaluated as vaccines for animal diseases such as rabies[43] and foot and mouth disease.[44] An additional approachis to use replication-competent vectors,[45] an attempt that is considered to potentially raise safety issues but which should provide higher levels of gene expression, and hence possibly more potent immune responses.

Adeno-Associated Virus (AAV)

Features of the Wild-Type Virus. The adeno-associated viruses (AAV) are members of the *Dependovirus* genus within the family of Parvoviridae. The virion is small and nonenveloped and surrounds single-stranded DNA molecules of both positive and negative orientation. As the name indicates, the virus is dependent on the co-infection and helper functions of other viruses such as adenovirus and herpesvirus for efficient replication.[46] No human disease has been associated with AAV; instead, it was shown to have beneficial effects for the host as it inhibits the activity of oncogenic viruses such as papilloma virus[47] and adenovirus.[48] AAV exists in several serotypes; type 2 has been most explored as a vector for delivering foreign genetic material.

Viral Entry. The cell tropism of AAV varies between the different serotypes (for a review, see ref 49), thus making a specific serotype more suited for a particular application depending on the target tissue. AAV-2 is able to infect a variety of cells, including liver, lung, muscle, and central nervous system, and is believed to enter cells primarily by binding to heparin sulfate proteoglycans,[50] expressed throughout the body. Upon binding to the target cell receptor, the AAV is internalized by endocytosis, is released in the cytosol upon acidification of the late endosome, and subsequently travels to the nucleus.[46,51]

Expression of Foreign Proteins and Safety Issues. In contrast to other viral vaccine vectors, the AAV can be constructed so that no genes from the original virus are present in the recombinant viral particle. The virion can contain up to 5 kb of inserted foreign genetic material flanked by the AAV inverted terminal repeats (ITRs).[52,53]

AAV has been evaluated primarily as a vector for gene therapy due to its possible ability to integrate into the human genome, a feature that so far has only been shown in vitro

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in human cells.[54,55] Recent data suggest, however, that the wild-type virus instead persists extrachromosomally in infected cells.[56,57] The recombinant virus appears to be safe and well tolerated, and no evidence that it is integrated in vivo in humans has been presented.

Preexisting Immunity. Seroprevalence studies indicate that the majority of the adult population have AAV-2-specific antibodies.[58,59] Since neutralizing antibodies against AAV have been shown to significantly hamper the effects of a subsequent injection of the virus,[60] this will probably be significant in clinical trials, particularly those in which homologous prime−boost regimens using AAV are employed. Thus, the strategy of priming and boosting using different serotypes of the virus has been explored in preclinical models.[61]

Current Vaccine Strategies: Immune Responses and Safety. AAV vectors have been shown to induce antiviral responses upon transduction of cells.[62] However, these responses seem to be of relatively low level, and if they are used as a vaccine vector, it might be beneficial for vaccine-specific immune responses to include molecular adjuvants capable of further activating the innate and specific immune response.

The rAAV has been explored as a vaccine vector in several preclinical animal models.[63,64] In one study, macaques displayed robust immune responses and partial viral control after SIV challenge following a single immunization using rAAV-carrying SIV genes. In addition to several ongoing and completed clinical trials using recombinant AAV-2 for gene therapy, it is currently being evaluated in a phase II trial as a vaccine vector for delivering HIV genes.[65]

Poxviruses

Features of Wild-Type Viruses. The poxviruses comprise a family of complex double-stranded DNA viruses. They belong to the largest family of virus infectious for humans with genomes of 150−300 kb. Smallpox, which is the most virulent one, has been successfully eradicated by vaccination. The DNA replication takes place in the cytoplasm and has even been reported in enucleated cells.

The large complex virion of vaccinia has a genome size of 200 kb and contains enzymes capable of mRNA synthesis. Viruses from the avipox genus are larger, having genomes of up to 260 kb. Canarypox and fowlpox have been utilized, because they infect but do not replicate in human cells. There is thus no concern about preexisting immune responses to these vectors. Variants of avipox members have been used in several preclinical and clinical studies.[66,67]

Viral Entry and Replication. Both enveloped extracellular vaccinia (EEV) and intracellular mature virion (IMV) forms are infectious. IMVs enter by fusing to cell membranes, while EEV entry involves endocytosis in an acidic milieu. Heparin sulfate present on many cells aids in the

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attachment of the virions, and other cellular molecules, less defined as yet, participate in viral entry. In work on these viruses, it was noted that dendritic cells were preferentially infected,[68] and the degree of activation or apoptosis of target cells may be different with EEV and IMV.

Preexisting Immunity. Vaccinia is derived from tissue cultures of poxviruses and has no natural host. It is genetically related to cowpox and variola. After intense vaccine campaigns to eradicate variola, the general population born after 1980 is not vaccinated. Also, the immunity in previously vaccinated individuals has waned to various degrees. Either priming or re-immunization gives rise to potent immune responses to vaccinia antigens. A limitation of the vaccinia vectors was initially attributed to preexisting immunity in those who had been immunized against smallpox. However, with a recombinant vector with a well-expressed inserted gene, this appears to be a less significant problem.[23,69]

Expression of Foreign Proteins. Poxviruses have ideal properties as vectors for foreign genes for two reasons. Their genome is large and sufficiently stable to carry large amounts of foreign materials and still retain transcriptional and translational capacity, and the poxviruses perform their whole life cycle in the cytoplasm of somatic human cells. They carry the early genes and enzymes necessary for their own transcription and translation and therefore do not enter the nucleus, a property that is valuable in vaccine constructions. Strains of vaccinia used to eradicate smallpox have been modified to carry various foreign genes.[70] The use of vaccinia virus as a live recombinant expression vector provides a tool for efforts aimed at developing vaccines against a variety of infectious agents such as malaria, tuberculosis, and HIV. A vaccinia recombinant rabies vaccine for use in animals (Raboral VR-G) was successfully made and licensed. Wild animals are immunized orally by vaccine placed in edible bait, and this vaccine has been distributed worldwide. In these applications, the role of the vaccinia vectors has been both as a competent carrier, with good expression capacity often using promoters of the virus itself, and to provide several immune-enhancing factors encoded by the vector backbone.

Current Vaccine Strategies. Attenuated vaccinia recombinants based on NYVAC[71] and modified vaccinia Ankara (MVA) have entered several clinical trials. Cell culture propagation has led to genomic loss of several vaccinia virulence factor genes by deletion. Foreign genes such as HIV env or gag-pol have been introduced by homologous recombination into these deletions.[72] The clinical trials have indicated that several vectors and additional adjuvants may be needed in humans to obtain good responses to the foreign antigen.[72−75] The use of a DNA vaccine prime followed by modified vaccinia Ankara carrying multiple HIV genes gave very high response rates in a recent phase 1 clinical trial.[23]

There has been substantial clinical progress in the use of pox vectors for malaria, with a phase IIb trial in children in Africa.[38] A NYVAC construct was made to contain eight different stage-related genes from *Plasmodium falciparum* malaria. This construct induced immune responses to all except one of the encoded malaria antigens.[76]

Moreover, MVA and the fowl pox strain F9 have been used for human trials against malaria containing multiple

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epitopes of malaria genes together with the gene for thrombospondin-related adhesive protein, TRAP. Two of five immunized individuals receiving those two pox vectors with malaria genes were protected against a sporozoite challenge.[77] The trial combination was evaluated to reduce parasitic burden by more than 90% and to be safe.

In terms of future possible vaccine strategies, a very interesting finding was made in studies in infant macaques involving SIV genes inserted into ALVAC (a vector based on attenuated canarypox virus) or in MVA. These animals received multiple immunizations during the first 3 weeks of life. Oral challenge with SIV a few weeks later showed complete or partial protection from SIV infection.[78] This suggested that HIV infection occurring during breast-feeding might be aborted by early neonatal immunization with these vectors. Further, even in ovo, the efficacy of a recombinant fowlpox virus harboring genes from Newcastle disease virus (NDV) significantly protected chickens from developing NDV disease.[79] This is remarkable since the vaccine was given in a multivalent vaccine combination that protected also against other dangerous poultry diseases.

Vesicular Stomatitis Virus (VSV)

Features of the Wild-Type Virus. VSV is an arthropod-borne single-stranded negative-sense RNA virus belonging to the family Rhabdoviridae, a family also containing rabies viruses. VSV exists in several serotypes. In humans, the virus causes primarily mild and self-limiting flu-like symptoms,[80] although more serious conditions such as encephalitis have been reported.[81] However, in livestock such as pigs, cows, and horses, the infections manifest as vesicular lesions and the symptoms closely resemble those of foot and mouth disease, making it hard to distinguish between the two infections. Thus, the disease is regarded as a major economical problem in the Americas.[82]

Viral Entry and Replication. Spikes composed of trimers of the viral glycoprotein cover the enveloped and bullet-shaped virion.[80] The cellular receptors for VSV are unknown, although evidence indicates that phosphatidylserine, a component ubiquitously present in plasma membranes, is involved.[83,84] The viral glycoprotein attaches to target cells, resulting in the virus being internalized by endocytosis and released into the cytoplasm upon acidification. The viral membrane subsequently fuses to a cellular vesicle. Replication takes place in the cytoplasm and starts when the viral RNA-dependent RNA polymerase transcribes five subgenomic mRNA which are translated into five viral proteins: nucleocapsid, phosphoprotein, matrix protein, polymerase, and glycoprotein. The virus assembles in two steps and subsequently buds through the host plasma membrane, causing cell death and lysis.[80]

Preexisting Immunity. With the exception of a few areas in the world, the seroprevalence in humans of VSV antibodies is believed to be very low.[80] However, the envelope glycoprotein of VSV efficiently induces neutralizing antibodies[85] capable of preventing reinfection as well as hampering the effect of a subsequent boost using VSV as a vaccine vector.[86] For homologous prime−boost regimes, it has been possible to circumvent this effect by boosting with a second recombinant VSV where the glycoprotein is exchanged for another VSV serotype.[87]

Expression of Foreign Proteins. The 11 kb large genome of VSV permits the insertion of foreign genes in several locations in the genome and allows an insert of at least 4.5 kb.[88] VSV has an advantage over many other viral vectors as it is able to generate viral particles that express and incorporate foreign transmembrane proteins on the surface.[89] In terms of immune competition, another strength of VSV

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is the small genome resulting in relatively few proteins competing with the vaccine antigen for access to antigen presentation pathways.

Adjuvant Effect. From what is known, no proteins encoded naturally by VSV have the capacity to interfere with the host interferon response, and this host response is believed to be highly important for clearing the infection.[90] It is thus reasonable to assume that the interferon response that is activated in target cells during the VSV infection will also act as an adjuvant for vaccine-specific responses to inserted genes.

Safety and Current Vaccination Strategy. Of major concern for the use of VSV as a vaccine vector are the early reports of neuropathology observed in mice following intranasal delivery of the virus.[91] There is also one known case of encephalitis in a human following infection with wild-type VSV.[81] However, current vaccine strategies exploit attenuated versions of the virus, and in prime−boost strategies in non-human primates, the recombinant viruses have been shown to be safe after both intranasal and intramuscular immunization.[92,93] In preclinical experiments where non-human primates were immunized with recombinant VSV carrying genes from SIV and HIV, it was shown that the vaccinees displayed a significantly better clinical outcome than control animals following challenge with SHIV89.6P.[94,95] The improved safety of the attenuated VSV vector together with quite promising preclinical results indicates that this viral vector is getting closer to a human phase I clinical trial.

Measles

Wild-Type Virus. Measles belongs to the genus *Morbillivirus*es in the Paramyxovirus family. Paramyxoviruses are enveloped nonsegmented RNA viruses of ∼16 kb. Measles is an exanthematous childhood disease with potentially serious sequaelae, particularly in immunosuppressed or malnourished individuals. Today, measles vaccination coverage is estimated to prevent over 50 million cases and 4−5 million deaths per year world wide and the goal of the World Health Organization (WHO) is eradication by continued and accelerated vaccine programs.

In natural infection, the virus spreads by aerosolization and replicates in bronchial endothelium. It then spreads via dendritic cells to lymphatic tissues, monocytes in blood, and dermal endothelial cells. From these, it accesses local keratinocytes in skin. In vivo, the infection may result in giant cells with up to 100 nuclei in lymphoid tissue. Up to 6 months after an acute measles infection, the human host has a certain general immunosuppression, but a specific long-lasting immune response to the measles virus.

Viral Entry. Virion proteins of the surface include hemagglutinin and fusion proteins, which contribute to attachment and formation of syncytia of infected cells. Measles virus uses the CD46 receptor as its major target. The CD46 molecule is also a regulatory protein of the human complement cascade. CDw 150 (SLAM) is expressed on T-cells, B-cells, and dendritic cells and acts as one of the coreceptors.[96] Measles virus therefore infects macrophages and dendritic cells. Measles virus and vectors replicate in the cytoplasm with a low likelihood of integration or other changes in host DNA.

Preexisting Immunity. More than 90% of the human population have had measles or have been vaccinated during early childhood. The presence of antibody and cellular immunity does not preclude a substantial boost by vaccination 10 years after the primary infection or vaccination.

Safety, Virus Vaccines, and Vectors. Vaccine strains grow in chick embryo, dog kidney, and human diploid cells. The vaccine strains have become attenuated due to numerous mutations, and these vaccines are very stable. Stability is important, particularly since plus-strand recombinant RNA

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viruses have been reported to delete recombinantly inserted genes. There has been no reversion to pathogenicity, despite years of immunization of children, and measles is expected to become eradicated within the next 10−20 years.

Current Vaccine Strategies. A measles virus-based vector is seen to be perhaps most useful in developing countries in early childhood when protection against both measles and HIV is needed. Development of vectors based on measles is attractive, due to its long-term potential use in millions of children in various parts of the world.

Molecular cassettes have been prepared by reverse genetics from two stable measles vaccine strains, Edmonston and Schwartz. DNA cloning sites have been introduced, permitting introduction of several foreign genes.[97] Three sites have been reported to be suitable for cloning, indicating that several genes from one microbe (e.g., HIV env, gag, and pol) or genes from different microbes might be expressed by one virion carrier. The env antigen of HIV did not become incorporated into the recombinant viral surface, indicating that the vaccine can retain its tropism.[97] For the time being, measles-based vectors can be efficient bi- or trivalent pediatric vaccines carrying their own genes and one or two foreign genes such as from HIV or HBV.

Measles virus vectors have also been delivered successfully to the mucosa via the intranasal and intraperitoneal routes.[98] Protection against West Nile virus challenge was reported in mice.[99]

Strong and enduring neutralizing antibody and cellular responses were reported in mice and macaques given a measles virus recombinant vaccine with an HIV subtype B env insert.[100] Mutated env sequences devoid of the variable loops were also constructed. In transgenic mice expressing the measles virus receptor CD46, it was shown that high antibody titers as well as cellular immunity of CD4+ and CD8+ origin was induced, directed both to the measles antigens and to HIV env.[97] Clinical vaccine trials testing measles vectors have not yet been reported. On the other hand, the measles vaccination program has proven the measles vaccine safe and immunogenic in children, also after mucosal nasal application of an aerosol.[101]

Alphavirus Vectors

Features of the Wild-Type Virus. Alphaviruses are arthropod-borne viruses of the Togavirus family. The icosahedral nucleocapsid is enveloped and contains a single-stranded positive-sense RNA genome of approximately 11.7 kb. The envelope has two glycoproteins (E1 and E2) responsible for attachment and penetration of the host cell. Alphavirus infection in vertebrate cells is cytolytic and transient and may cause a wide array of symptoms ranging from very mild to severe disease.

Viral Entry and Replication. The complete replicative cycle of alphaviruses occurs in the cytoplasm of infected cells. After the genome has been delivered to the cytoplasm of the host cell, expression is segregated into two steps. Immediately following infection, the viral replicase genes are translated from the parental genome and catalyze the synthesis of a complete negative-sense copy of the genome. This serves as a template for the synthesis of progeny plus-stranded genomes as well as for excessive synthesis of subgenomic mRNA from the viral 26S promoter.[102] The subgenomic mRNA encodes the alphavirus structural proteins.

Expression of Foreign Proteins. Recombinant viruses can be produced by replacing the structural protein genes of the virus with a transgene and supplying the structural protein genes in trans.[103,104] The result is the assembly and release of virus particles containing only the replicon RNA encoding the gene to be expressed.

Preexisting Immunity. Alphaviruses are zoonotic, mosquito-borne viruses endemic to certain geographical regions and occur naturally in the human population only during infrequent epidemics. Therefore, preexisting antivector immunity is unlikely to be a problem. If a primary immunization with an alphavirus-vectored immunogen is boosted with the same vector, vector immunity may become a problem. No direct evidence in preclinical studies in mice has, however, thus far been presented indicating that preexisting antivector immunity would hamper the efficacy of the boosting immunization. In primates, little information about preexisting immunity is available, and negative effects due to pre-existing immunity cannot be completely ruled out.

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Alphavirus Vaccine Vector Systems and Mechanisms of Immunity. Vaccine vectors based on Sindbis (SIN), Semliki Forest virus (SFV), and Venezuelan equine encephalitis virus (VEE) efficiently induce both cellular and humoral immune responses to the vectored immunogen and have been shown to confer protection to experimental challenge in various animal models, including influenza virus, Marburg virus, and various cancers.[104−108] The biology of the alphaviruses is likely responsible for the high immunogenicity of these vectors. Transgenes under the viral promoter are, for instance, expressed to a high level,[103] and ≥10-fold increases in the level of protein expression have been reported to be produced as compared to expression from the CMV promoter. This is due to the self-replicating nature of the viral RNA and efficient shutoff of translation of host cell mRNA by the viral replicase.

During viral replication, alphaviruses have both single- and double-stranded RNA intermediates that can be recognized by various pathogen-recognition receptors (PRR) such as TLR-3, TLR-7/8, RIG-I, and MDA-5.[109] Recognition of viral RNA by these molecules leads to a massive innate immune response and is characterized by extensive release of type I interferons.[110] Another attractive attribute of alphavirus vectors is effective delivery as compared to, for instance, naked DNA, and their potential ability to target dendritic cells (DC) in vivo.[111,112] Antigen expression by professional antigen-presenting cells (APC) such as DC is considered advantageous by many investigators for effective immune induction. In addition, alphaviruses are known to induce apoptosis of transduced cells.[102] Apoptosis is an important mechanism for transfer of viral antigens to, and cross-priming of, dendritic cells.[113] Indeed, cross-priming via apoptosis has been shown to be crucial to the effect of vectored antigens in DNA-based SIN vectors.[114]

Current Vaccine Strategies. Several groups have immunized non-human primates with SFV- and VEE-based vectors encoding HIV-1 antigens in the ongoing effort to produce an effective HIV vaccine.[115−117] These studies have induced immunological responses capable of reducing viral loads after challenge, and several clinical trials are under way.

In a report from a phase I trial, a VEE-based HIV vaccine was shown to be safe, but immunological responses were modest. Responses may, however, have been hampered by a low dosage and a suboptimal route of administration.[118]

Other alphavirus-based approaches for vaccination comprise immunization with cDNA encoding the replicon RNA or with transcribed replicon RNA, as described for SFV and SIN.[119,120] In addition, it has recently been shown that SFV vectors encoding an irrelevant antigen and VEE vectors without any transgene may act as very potent adjuvants to

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codelivered proteins promoting a Th1-type antibody response systemically and/or mucosally.[121,122]

Other Vaccine Vectors

Hepatitis B virus (HBV) has a genome of ~3 kb and a complicated replication cycle involving reverse transcription. HBV infects preferentially liver cells via the transferring receptor, endonexin, and asialoglycoprotein receptor. HBV transmits through sexual contacts and contaminated blood products. More than 500 million people have had the infection or are chronic carriers of the virus. Extensive vaccination programs have contributed immensely to the decrease in the incidence of hepatitis, which in turn should decrease the future incidence of liver cancer, one of the long-term consequences of this infection.

Viruslike particles based on HBV can be used as vaccination vehicles. Because of its limited genomic size, HBV has constraints on the size of inserted genes, and the stability of inserted gene products has been an issue. Woo et al. exchanged strong hepatitis B surface antigen (HbsAg) epitopes for epitopes from respiratory syncytial virus and human papilloma virus. This approach generated CD8+ T-cell responses to the relevant antigen.[123] Although the HBV as a vector can carry only limited information, such chimeric vectors might be useful for stable epitopes in nonvariable proteins such as those found to be associated with certain cancers.

Poliovirus-based vectors have been developed since the early 1990s.[124] Poliovirus, being a picornavirus, has a positive-strand RNA genome of ~8 kb and exists in three different serotypes. The natural infection is spread orally, mainly by contaminated water. Poliovirus infects mucosally in the oropharynx and gut via the poliovirus receptor CD155, a member of the superimmunoglobulin family. It can also be neurotropic and may cause paralysis. The poliovirus is sensitive to correct structural conformation for its receptor-mediated uptake and therefore can harbor only smaller amounts of foreign genes in recombinant constructions. Moreover, poor genetic stability has limited their use as recombinant vaccines, although more stable constructs have been made.[125] Potent oral and killed systemic vaccines have almost eradicated the disease. Nearly the whole world population thus has humoral immunity to the virus, which is sensitive to neutralization by antibodies. Therefore, mucosal immunization during infancy might be the exclusive possibility for this type of vector.

Chimeric viruses, in which a whole gene fragment is exchanged, may serve in the very potent viral delivery of foreign genes and yet provide a sufficient attenuation to prevent adverse effects of the vector. This approach has been tested within the flavivirus family, where one flavivirus has been utilized to harbor genes from dengue, tick borne encephalitis, and Japanese encephalitis virus.[126−128] For instance, a recombinant yellow fever virus vector with West Nile virus inserts elicited neutralizing antibodies and cellular immunity to West Nile virus in healthy individuals.[129]

Recently, vaccine vectors based on the Australian West Nile virus subtype Kunjin (KUN) have been described (reviewed in ref 130). Subtype KUN is considerably less pathogenic than the New York stain of the West Nile virus.[131] As the virus belongs to the family of flaviviruses, it has a positive-sense single-stranded genome, thus resembling alphaviruses, and KUN replicons can be delivered as DNA, RNA, or viruslike particles. The entire replication cycle of

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the virus takes place in the cytoplasm of the host cell and results in prolonged high-level gene expression. Unlike most alphavirus replicon systems, KUN replicons are, however, noncytopathic; expression levels can be sustained for longer periods of time, and transduced cells may also be able to divide. KUN replicons have been used to induce long-lived and protective CTL responses against experimental challenge with West Nile virus and tumors expressing human papillomavirus 16 proteins.[132,133]

Conclusions

Viral vectors offer several advantages and challenges, as compared to naked nucleic acids and traditional vaccines for vaccine delivery and vaccine production. In addition, the number of vector systems that are being used in clinical trials and preclinical experiments, or are under development, is rapidly growing. Therefore, this review attempts to give an overview of viral vectors to help guide selection of the appropriate vector for delivery of an antigen-encoding gene. Some of the unique properties of the vectors discussed here are listed in Table 1. Understanding these properties is essential for selecting the optimal vector for the antigen and vaccination strategy of choice.

Naturally, prior to the selection of a vaccination strategy, it is of utmost importance to verify that the chosen vector can correctly express the desired gene product, epitopes, or gene fragments in vitro and in vivo. Another consideration to take into account is the genetic stability of the vector: will the desired gene be correctly expressed by all or most of the vector-transduced cells in vivo?

Initially, an increased level of antigen expression and efficient targeted delivery were the driving forces behind the development of viral vectors. One way to obtain an increased overall level of antigen expression was to ensure a more efficient delivery of the genetic material so that more cells in the vaccinee would express the antigen. In addition, many viral vectors, including viral replicons based on alpha- and flaviviruses, produce significantly increased amounts of antigens compared to an antigen expressed from a CMV promoter. Moreover, using a viral vector delivery, the antigen gene could be targeted to a professional antigen-presenting cell or to a specific tissue. For instance, several viruses target DCs; these include members of the alphavirus genus and the poxvirus family, and they may thus elicit direct priming of an immune response. Other viruses, such as adenovirus, polio and measles virus infect preferentially via mucosal surfaces. Vectors based on these viruses may therefore be particularly suitable for mucosal administration as well as induction of mucosal immunity. To date, however, most vectored vaccine delivery has been via either the intramuscular or subcutaneous route, even though the properties of the vector would actually be in favor of another route of administration. This is an avenue for future investigations, and it is not unlikely that more efficient routes of administration may exist for vector-based vaccines already in clinical trials. Some vectors can be manipulated to better target a certain cell type or to escape from preexisting immunity. For instance, VSV-based vectors can be engineered to incorporate foreign surface glycoproteins, and the spike protein of adenovirus can be replaced with that of another adenovirus.

A very important factor for the choice of vaccine vector is the capacity of the vector to carry foreign genetic material. Several constraints may act on the genetic load for any given vector. For instance, nonenveloped viruses such as poliovirus, adenovirus, and AAV often have a rigid capsid structure that does not allow packaging of more genetic material than the wild-type virus without interrupting the interaction with the viral receptors. Some viruses with larger genomes may, however, be able to carry a larger genetic load, but vector construction may in turn be more cumbersome. In addition, genetic stability of some larger vectors such as poxvirus-based vectors has sometimes been an issue.

Furthermore, when selecting a viral vector, one should consider the life cycle of the wild-type virus. For some applications, especially in gene replacement therapy, a persisting or integrating vector, such as AAV or a retrovirus, could be beneficial. If the application is cancer immunization with an oncogene, the same vector choice may prove detrimental and a vector with an entirely cytoplasmatic replication cycle may be the one of choice to minimize the risk of integration. If the vector also induces cell death or apoptosis of the transduced cell, safety may even be improved further.

Importantly, some viral vectors as well as naked nucleic acids can activate innate immunity through pattern recognition receptors and may act as adjuvants to the delivered antigen. Pattern recognition leads in general to type I interferon production and an inflammatory response, which links the innate response to adaptive immunity. It has been proposed by Pulendran and co-workers that the more pattern recognition receptors are activated, the more potent the immune induction by the vaccine.[134] Examples of pattern recognition with an impact on vaccination are DNA vaccines containing hypomethylated bacterial DNA CpG dinucleotides that are recognized through Toll-like receptor 9 (TLR9). There is evidence that this is also true for herpes simplex

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2-derived DNA.[135] Many RNA viruses have a double-stranded RNA replication intermediate, which may activate TLR3 as well as the cytoplasmic helicases RIG-I and MDA-5. In contrast, many viruses have evolved mechanisms for interfering with pattern recognition and inflammatory responses. Better vectors that do not interfere with pattern recognition and inflammatory responses may be constructed as we gather information about the biology of each vector and the immune response each induces. For instance, the attenuation in MVA is a result of a loss of a large portion of the genome encoding immunoregulatory genes.

For clinical applications, especially for childhood vaccination, the vector choice may also be influenced by previous clinical experience with the vector that either has demonstrated the safety and immunogenicity of the virus or has resulted in a preexisting immune response against the vector. The experience may then have to be weighed against the potency of the vector to induce relevant immune responses. Therefore, vaccine vectors based on poliovirus and measles virus vaccine strains may be the first to have success in the clinic.

In conclusion, viral vectors may offer considerable increases in vaccine potency and may generate safe vaccines against malaria, tuberculosis, HIV/AIDS, influenza, and various cancers. Much effort must, however, still be invested in characterization, safety, and efficient manufacturing of viral vectors before mass vaccination with recombinant viral vectors will play a significant role in the fight against modern-day plagues.

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