Recombinant Vectors as Influenza Vaccines

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Abstract The antiquated system used to manufacture the currently licensed inactivated influenza virus vaccines would not be adequate during an influenza virus pandemic. There is currently a search for vaccines that can be developed faster and provide superior, long-lasting immunity to influenza virus as well as other highly pathogenic viruses and bacteria. Recombinant vectors provide a safe and effective method to elicit a strong immune response to a foreign protein or epitope. This review explores the advantages and limitations of several different vectors that are currently being tested, and highlights some of the newer viruses being used as recombinant vectors.

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R.W. Compans and W.A. Orenstein (eds.), *Vaccines for Pandemic Influenza*,
Current Topics in Microbiology and Immunology 333,
DOI 10.1007/978-3-540-92165-3_13,

1 Introduction

While the ideal vaccine would elicit the exact immune response that occurs during natural infection with highly pathogenic influenza virus, expression of influenza virus proteins from live replicating vectors can safely induce a strong humoral and cellular immune response comparable to natural infection (Souza et al. 2005). Recombinant vectors have been developed because it is considered to be too dangerous to vaccinate people with even vastly attenuated forms of dangerous viruses such as highly pathogenic influenza virus or Ebola virus. Vaccination with recombinant vectors offers several advantages over vaccination with inactivated influenza viruses. Inactivated vaccines induce short-lived antibody-mediated immunity, while recombinant vectors elicit a longer-lasting immune response that stimulates both memory B and T cells. Also, the manufacture of the recombinant vaccines entails substantially less risk than growing large quantities of influenza viruses expressing the highly pathogenic hemagglutinin (HA) and neuraminidase (NA) proteins required for inactivation, since the recombinant vectors cannot cause influenza. Many different viruses and bacteria are currently being tested for their ability to function as a good recombinant vector.

2 Qualities of Ideal Vectors

Good vectors should be easy to manipulate genetically, allowing the insertion of large foreign genes or epitopes. The vectors should grow well and be easy to produce in large-scale operations. The foreign proteins should be highly expressed from the vector to elicit the best immune response. Proteins from the vector should not elicit a strong immune response, as this may interfere with the induction of a response to the foreign proteins and also reduce the effectiveness of boosts after initial vaccination. The expression of foreign proteins in the host should be transient and the vector must be fully cleared from the host once the adaptive immune response has commenced. Integration of DNA from the vector into the host genome must not occur, as this can disrupt host genes and possibly lead to the development of cancer. Humans should not have pre-existing antibodies to the vector, as this could prevent replication of the vector and subsequently prevent the induction of an immune response to the foreign proteins. Ideally, the vector should not cause disease symptoms in humans and should be safe even for immunocompromised individuals and young children. Additionally, recombinant vectors that do not require refrigeration would facilitate the distribution of the vaccine to developing nations. Recombinant vectors that can be administered without needles would also aid distribution and enhance vaccination compliance (Babiuk and Tikoo 2000; Barouch and Nabel 2005; Souza et al. 2005; Barouch 2006; Li et al. 2007). Several viruses possess many, but not all, of the characteristics of a useful vector.

3 Newcastle Disease Virus Vectors

Newcastle disease virus (NDV) contains a nonsegmented, single-strand, negative-sense RNA genome and belongs to the family *Paramyxoviridae*. NDV contains six genes that encode seven proteins: nucleocapsid protein (NP), phosphoprotein and V protein (P/V), matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN), and large polymerase (L) protein. As the viral polymerase can disassociate from the viral genome after the transcription of each gene, expression levels of the proteins reduce in a sequential manner from the 3′ to the 5′ end of the genome. Thus, the expression level of a foreign protein can be controlled by its position on the viral genome (Huang et al. 2004). NDV naturally infects avian species, and it is a highly contagious virus with a pathogenicity ranging from avirulent to high levels of mortality (Huang et al. 2003). One determinant of NDV pathogenicity is the cleavage site of the F protein, which is necessary for the fusion of the viral envelope to the cell membrane. NDV strains containing an F protein cleavage site that has several basic amino acids is readily cleaved by numerous cellular proteases in a variety of tissues, leading to wide dissemination of the virus throughout the host organism and high virulence. NDV strains containing an F protein cleavage site that contains fewer basic amino acids is only cleaved by a secreted protease found in the lung, and thus its tropism is limited to the lung, leading to lower pathogenicity (Panda et al. 2004). The ability to easily adjust the pathogenicity of NDV is one of the reasons that NDV is an attractive recombinant vector.

NDV possesses many of the qualities of an ideal vector for use in humans, and several of its properties make it specifically suited as a recombinant vector for pandemic influenza. Since under natural conditions NDV infects only birds, humans do not have pre-existing immunity to NDV. Pre-existing antibodies to the recombinant vector drastically reduce or completely eliminate the formation of immunity to the foreign protein expressed from the vector, and this is one of the major reasons that recombinant vectors are not effective. The fact that birds are a major reservoir for both NDV and highly pathogenic avian influenza virus (HPAI) has led to the development of dual vaccines that can protect poultry against both diseases.

An NDV virus expressing an influenza virus HA, rNDV/B1-HA, was first rescued in 2001 (Nakaya et al. 2001). The influenza virus HA gene from the A/WSN/33 (H1N1) virus had been inserted between the P and M genes of the Hitchner B1 strain, which is avirulent, and this virus has been used as a live vaccine in birds (Russell and Ezeifeka 1995). The genomic structure of a recombinant NDV is illustrated in Fig. 1. The influenza virus HA was confirmed to be incorporated into the viral envelope and to be cleaved. The rNDV/B1-HA showed no pathogenicity in embryonated chicken eggs, which are used to grow large stocks of both influenza virus and NDV for vaccines. Most importantly, vaccination of mice with rNDV/B1-HA conferred complete protection against lethal challenge with A/WSN/33 influenza virus (Nakaya et al. 2001).

Recently, NDV recombinant vectors expressing HA genes from HPAI strains have also been generated. NDVs expressing HAs from H5 and H7 influenza virus

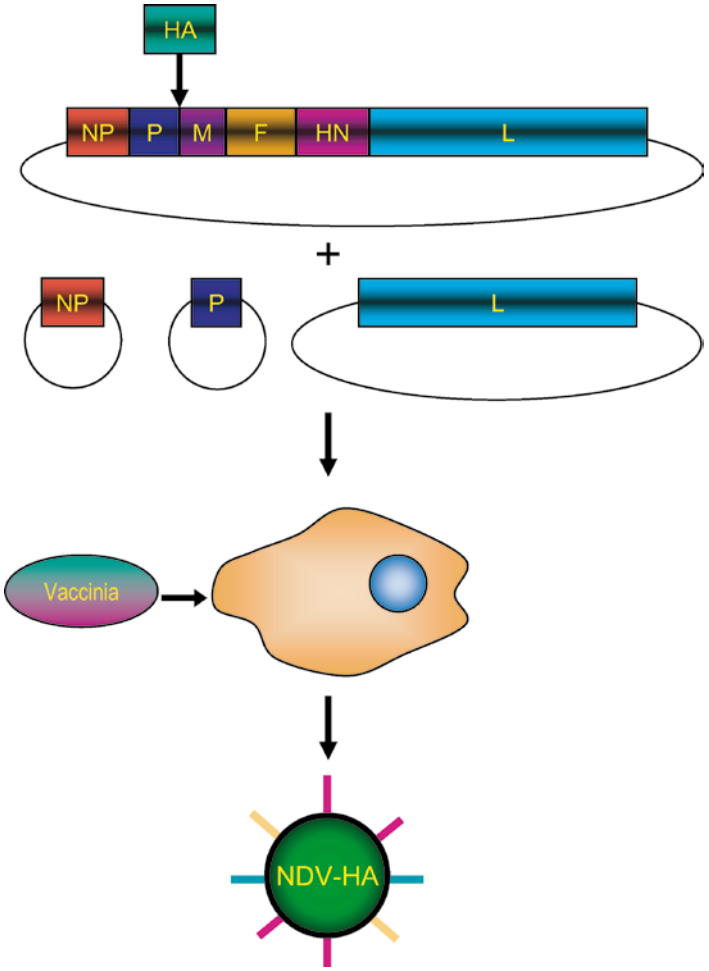

Fig. 1 Rescue of a recombinant NDV vector expressing influenza virus HA protein. The HA gene was cloned between the P and the M gene in a plasmid containing the full-length NDV genome under the control of the T7 promoter that requires the T7 polymerase for expression. Cells were cotransfected with a plasmid containing the full-length NDV-HA genome as well as helper plasmids expressing NP, P, and L from a Pol II promoter. One hour prior to transfection, cells were infected with MVA-T7 vaccinia virus, which had been modified to express the T7 polymerase. The resulting NDV virus expressed NDV F and HN, as well as influenza HA on the virion surface. Conformation of the rescue of NDV-HA virus was determined by sequence analysis, as described in Nakaya et al. (2001)

strains, which have both caused illness and death in humans working with infected birds, were designed to be dual vaccines to protect birds from HPAI and NDV (Park et al. 2006; Veits et al. 2006; Ge et al. 2007). In order to enhance incorporation of H7 into the viral envelope, the ectodomain of H7 was fused to the transmembrane and cytoplasmic domains of the F gene and inserted into the NDV genome between

the P and M genes. The virus was too attenuated to confer full protection after challenge with HPAI, so three basic amino acids were added to the F protein fusion site to enhance viral spread. The resulting virus was still highly attenuated compared to pathogenic strains of NDV, but it did induce 90% protection after stringent challenge with HPAI and 100% protection after challenge with NDV (Park et al. 2006). H5 HAs were cloned into a different nonpathogenic NDV strain, La Sota, and used to vaccinate chickens. Not only were the chickens fully protected after lethal challenge with HPAI and NDV, but the vaccine also prevented the chickens from shedding virus after challenge (Veits et al. 2006; Ge et al. 2007).

NDV has also been used as a recombinant vector for pathogens other than influenza virus. Infectious bursal disease virus (IBDV) causes immunosuppression in poultry, which reduces the effectiveness of vaccines and leaves the animals especially susceptible to other infections. A recombinant NDV expressing the IBDV VP2 protein provided protection against IBDV and NDV in chickens (Huang et al. 2004). Another recombinant NDV was designed to prevent respiratory syncytial virus (RSV), which causes severe respiratory disease in infants and the elderly. A recombinant NDV expressing RSV F protein protected mice against challenge with RSV (Martinez-Sobrido et al. 2006).

Recombinant NDV vaccines for pathogens such as severe acute respiratory syndrome virus (SARS-CoV) and human parainfluenza virus type 3 (HPIV3) have been tested in primates. SARS-CoV caused a worldwide outbreak in 2003 with a mortality rate of about 10%. Vaccination of African green monkeys with an NDV vector expressing SARS-CoV spike protein resulted in a dramatic reduction of viral replication after challenge with SARS-CoV (DiNapoli et al. 2007). An NDV vector expressing HPIV3 HN protein induced levels of antibody comparable to natural infection with HPIV3 in African green monkeys (Bukreyev et al. 2005).

One limitation of NDV as a recombinant vector is that it can be difficult to grow NDVs with long foreign genes or multiple foreign genes inserted into the NDV genome. This shortcoming has recently been overcome by rescuing a recombinant NDV with a bisegmented genome. One segment contains the genes for NP, P, and L, while the other segment contains the genes for M, F, and HN. A recombinant virus expressing GFP from the first segment and SARS-CoV spike protein from the second segment was rescued, demonstrating that NDV can be designed to express multiple foreign proteins and large proteins such as SARS-CoV spike protein (Fig. 2) (Gao et al. 2008).

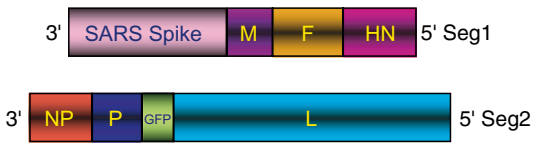

Fig. 2 Expression of two foreign proteins from a bisegmented NDV virus. The nonsegmented NDV genome was divided into two segments to allow the expression of two foreign proteins. Segment 1 contains the M, F, and HN genes as well as the SARS-CoV spike gene. The 3′ and 5′ noncoding regions were added onto the ends of segment 1. Segment 2 contains NP, P, and L genes as well as GFP inserted between the P and L genes. Figure adapted from Gao et al. (2008)

Interestingly, NDV is being tested as a recombinant vector for not only infectious diseases, but also for cancer. It had previously been observed that NDV replicates in human tumor cells much more readily than normal cells. This is believed to occur because cancer cells often have mutations in the interferon pathway, a key host antiviral immune response, while normal cells have an intact interferon pathway. Since NDV is very sensitive to the effects of interferon, it is rapidly eliminated from normal cells. The cancer cells, however, are killed by NDV since they cannot mount an antiviral response. An NDV vector expressing granulocyte/macrophage colony-stimulating factor (GM-CSF), which has been shown to enhance immunity to tumors, stimulates antitumor activity in human cells better than NDV vector alone (Janke et al. 2007). A recombinant NDV vector expressing IL-2 reduced tumor volume and caused a higher remission rate of colon carcinoma tumors in mice compared to NDV alone (Vigil et al. 2007). A new approach involving the expression of tumor-related antibodies from the NDV genome also has promise for cancer therapy (Puhler et al. 2008). Additionally, the safety of NDV has already been demonstrated in humans (Freeman et al. 2006; Laurie et al. 2006). Thus, NDV shows promise of being a valuable recombinant vector in both humans and birds.

4 Vesicular Stomatitis Virus Vectors

Vesicular stomatitis virus (VSV) contains a nonsegmented, single-strand, negative-sense RNA genome and belongs to the family *Rhabdoviridae*. The VSV genome is organized similarly to NDV except that VSV encodes a protein responsible for both fusion and attachment, glycoprotein (G), whereas NDV encodes two separate proteins for these functions, F and HN. VSV normally infects horses, cattle, and swine, where it causes vesicular lesions on the mouth, nose, teats, and hooves. Infection is usually cleared within two weeks without complications. In nature, VSV is spread primarily by arthropod vectors such as sand flies (*Lutzomyia shannoni*) and black flies (*Simulium vittatum*), though transmission by animal-to-animal contact has been reported (Letchworth et al. 1999; Stallknecht et al. 2001; Rodriguez 2002). VSV is considered a good vaccine vector candidate since humans do not have pre-existing antibodies that would interfere with the induction of an immune response to a foreign protein expressed from the virus.

A recombinant VSV expressing the HA of WSN influenza virus strain was rescued and subsequently shown to confer protection in mice after challenge with WSN virus (Kretzschmar et al. 1997; Roberts et al. 1998). VSV expressing HA from A/Hong Kong/156/97, a highly pathogenic H5N1 virus, was shown to elicit neutralizing antibodies in mice and confer protection after challenge with A/Hong Kong/156/97 virus (Fig. 3). Interestingly, this recombinant vector induced cross-reactive neutralizing antibodies to distantly related H5 viruses. Long-term protection was also achieved with this vector, as mice were fully protected after 7.5 months between vaccination and challenge (Schwartz et al. 2007). These experiments demonstrate that VSV may be a suitable recombinant vector for influenza virus HA proteins.

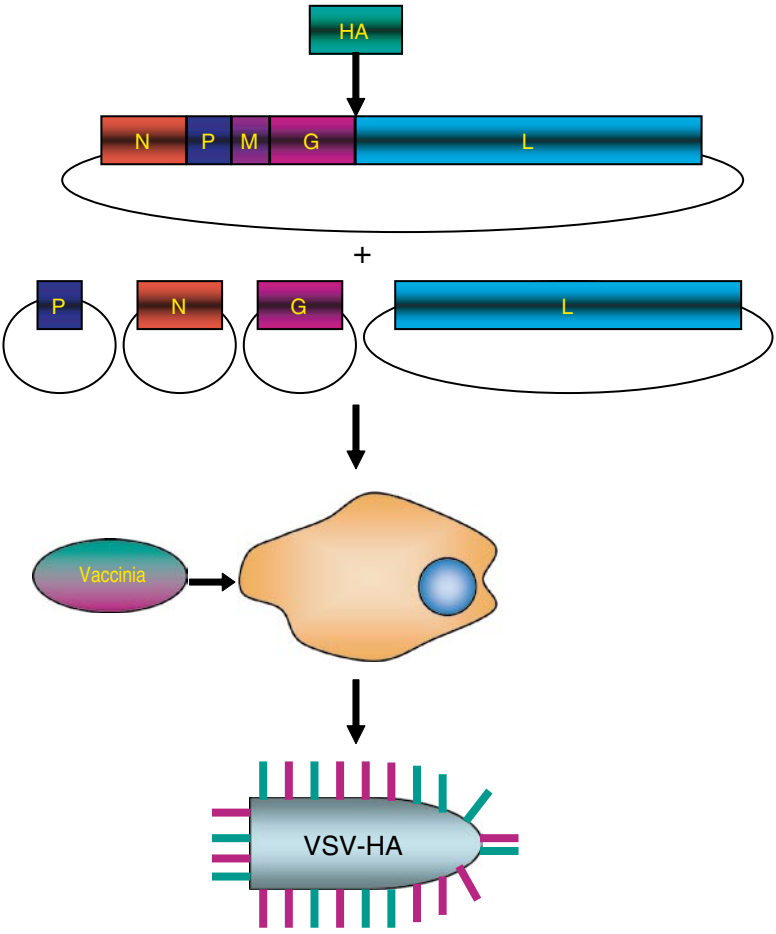

Fig. 3 Rescue of a recombinant VSV vector expressing influenza virus HA protein. The HA gene was cloned between the G and the L gene in a plasmid containing the full-length VSV genome under the control of the T7 promoter. Cells were cotransfected with a plasmid containing the full-length VSV-HA genome as well as helper plasmids expressing NP, P, G, and L from a Pol II promoter. One hour prior to transfection, cells were infected with MVA-T7 vaccinia virus. The resulting VSV virus expressed VSV G, as well as influenza virus HA. Figure adapted from Schwartz et al. (2007)

Recombinant VSV vectors expressing various viral and bacterial proteins of pathogens have also been tested in animal models. VSV vectors expressing the *Yersinia pestis lcrV* gene provided protection in mice from lethal pulmonary challenge with *Yersinia pestis* (Palin et al. 2007). Vaccination with a recombinant VSV expressing SARS spike protein provided protection against challenge with SARS in both young and aged mice (Kapadia et al. 2005; Vogel et al. 2007). VSV vectors are also currently being tested as vaccines and therapeutic agents for HIV

(Johnson et al. 1997; Schnell et al. 1997; Haglund et al. 2000; Rose et al. 2001; Ramsburg et al. 2004; Publicover et al. 2005; Okuma et al. 2006; Cooper et al. 2008). Recombinant VSV vectors have been developed for many other viruses, such as herpes simplex virus type 2, Borna disease virus, Marburg virus, and papillomaviruses. (Daddario-DiCaprio et al. 2006; Natuk et al. 2006; Brandsma et al. 2007a,b; Perez et al. 2007).

One limitation of VSV as a recombinant vector is that it does appear to cause some pathogenicity in humans. Though humans are rarely infected, animal handlers have been exposed to VSV and show symptoms of disease ranging from asymptomatic to fever with myalgia that resolves within a week. Mouse studies of VSV infection demonstrate that the virus can replicate in the olfactory nerve soon after intranasal infection and can then cross the blood–brain barrier. The virus then spreads to many areas of the brain, resulting in neuropathology, hind-limb paralysis, and death (Huneycutt et al. 1994; Bi et al. 1995; Plakhov et al. 1995). VSV infection was also analyzed in nonhuman primates, and it is critical to determine the safety of vectors in this model prior to human use. Macaques inoculated with VSV intranasally shed virus in nasal washes for the first day after infection, but the virus did not cause viremia or enter the central nervous system. However, when macaques were injected with VSV directly into the brain with an intrathalamic injection, the virus spread and caused severe disease symptoms (Johnson et al. 2007).

In order for VSV to be used as a recombinant vector in humans, the virus must be attenuated so that disease symptoms are eliminated. Fortunately, much has already been discovered about the mechanisms of VSV pathogenicity, and so attenuated VSV vectors can be rationally designed. Insertion of the HIV Gag protein into the VSV genome attenuated the virus sufficiently so that it did not cause pathogenesis in macaques, though additional viral attenuation may be necessary for human trials (Johnson et al. 2007). Truncations of the cytoplasmic region of the G protein had been shown to attenuate VSV growth and pathogenesis in mice (Roberts et al. 1998). A recombinant vector containing a G protein deletion and expressing HIV Env protein elicited $CD8^+$ T cell responses comparable to wtVSV expressing Env protein (Publicover et al. 2004).

Another strategy for attenuating VSV expressing HIV proteins includes placing *gag* at the beginning of the genome, which results in a reduction of the expression of the VSV proteins and a reduction of viral replication. Also, the N gene was moved to a further downstream position, which reduces N protein expression and viral replication. The M protein, which has been shown to inhibit the interferon response and induce apoptosis, has also been mutated to reduce VSV pathogenesis (Clarke et al. 2007; Cooper et al. 2008). Applying a combination of these alterations to recombinant VSV vectors expressing Gag protein resulted in a drastic reduction in pathogenesis in mice but still induced a strong immune response to Gag (Cooper et al. 2008). VSV vector replication and pathogenesis can also be eliminated by using VSV mutants that can only complete one cycle of replication. This is achieved by eliminating the VSV G protein from the genome. Since VSV G protein, but not the G gene, is necessary for viral growth, the vector can be grown readily in cell lines that constitutively express VSV G so that G protein can be incorporated into the

viral envelope. In animals, the single-cycle VSV vectors can enter and infect cells and express the viral and foreign proteins in its genome, but viral assembly cannot occur because the G protein is not synthesized. A VSV single-cycle vector expressing HIV Env was demonstrated to produce an immune response to Env that was similar to the response elicited by replicating VSV vectors expressing Env (Publicover et al. 2005). VSV vectors with attenuating mutations are being investigated carefully so that they can be safely administered to humans without side effects.

Similar to NDV, VSV is also being analyzed as a therapeutic cancer agent based on the observation that VSV replicates and induces apoptosis in cancer cells more readily than normal cells (Barber 2004). VSV is also very sensitive to the effects of interferon, and tumor cells without an intact interferon pathway are rapidly killed by VSV. VSV vectors expressing the cytokine IL-12 or the chemokine inhibitor equine herpes virus-1 glycoprotein were able to enhance tumor reduction of squamous cell carcinoma and hepatocellular carcinoma, respectively, in mice (Shin et al. 2007; Altomonte et al. 2008). Thus, VSV vectors show the potential to function as vaccines for infectious diseases as well as cancer.

5 Influenza Virus Vectors

There has been recent interest in using influenza virus itself as a recombinant vector to protect against highly virulent influenza virus strains as well as other pathogens. Influenza viruses need to be highly attenuated for use as a vector, and several strategies for attenuating influenza viruses have been successful. An attenuated cold-adapted strain was generated by growing influenza virus at 25°C in primary chick kidney cells, and is currently licensed for use in humans (Cox et al. 1988). An attenuated cold-adapted strain that was generated in embryonated chicken eggs grown at low temperatures is also being used in horses (Youngner et al. 2001). Reduction of virulence is also observed by influenza viruses containing deletions of the NS1 protein, the viral protein responsible for inhibiting the innate interferon response (Garcia-Sastre et al. 1998; Talon et al. 2000). Influenza virus vaccines containing deletions of the M2 gene, which are necessary for virus uncoating, are currently being tested (Watanabe et al. 2007). Thus, there are likely many ways to sufficiently attenuate influenza virus.

Several influenza virus vectors have shown promising results in animal models. A recombinant influenza virus containing the NDV HN ectodomain in place of the influenza virus NA ectodomain administered in ovo provided protection in chickens against both influenza virus and NDV after a lethal challenge (Fig. 4) (Steel et al. 2008). Millions of people die each year after infection with *Mycobacterium tuberculosis*, and an effective vaccine is urgently needed. Vaccination with influenza virus that expresses a truncated NS1 protein and the ESAT-6 protein of *Mycobacterium tuberculosis* from the NS gene segment provides protection in mice and guinea pigs from lethal challenge with the bacteria (Sereinig et al. 2006; Stukova et al. 2006). Recombinant influenza viruses expressing portions of *Bacillus anthracis* proteins

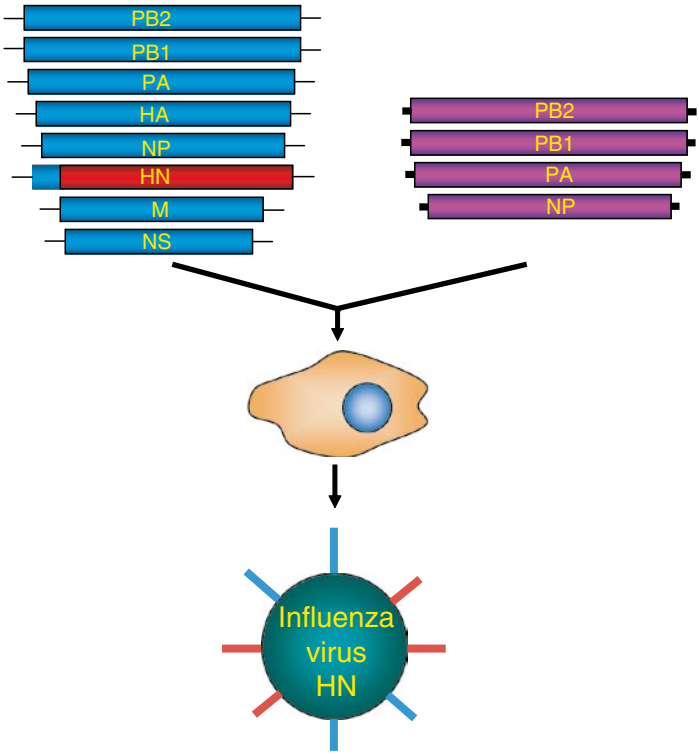

Fig. 4 Rescue of a recombinant influenza virus expressing NDV HN. The ectodomain of influenza virus NA was replaced with the ectodomain of NDV HN. Eight of the plasmids contained a Pol I promoter (*left*) and four of the plasmids contained a Pol II promoter (*right*). Cells were transfected with the 12 plasmids and a recombinant virus expressing NDV HN was rescued, as described in Steel et al. (2008)

fused to the influenza virus HA protein elicited antibodies against both *Bacillus anthracis* and influenza virus (Li et al. 2005). Protection in mice was achieved after vaccination with an influenza virus expressing *Chlamydia trachomatis* epitopes in the NA protein (He et al. 2007). Mice vaccinated with an influenza virus expressing an epitope of *Pseudomonas aeruginosa*, which is the leading cause of mortality in cystic fibrosis patients, were fully protected after challenge (Gilleland et al. 2000). Chimeric influenza viruses have been developed to express HIV epitopes. After intranasal administration, these vectors induce a long-lasting mucosal antibody response in not only the respiratory tract, but also the genital tract (Li et al. 1993; Muster et al. 1994, 1995; Palese et al. 1997; Gonzalo et al. 1999; Gherardi et al. 2003; Nakaya et al. 2003). The encouraging results obtained using influenza virus vectors thus far demonstrate the need for further research in this field.

While the live cold-adapted influenza virus vaccine has been successfully administered to millions of individuals, safety must be carefully considered in the

development of future live-attenuated influenza virus vaccines. Live-attenuated influenza virus vaccines must be designed so that a pathogenic virus could not result from reassortment of the vaccine with a circulating influenza virus strain. This issue was addressed for the cold-adapted vaccine by demonstrating that three internal genes contained attenuating mutations, making it unlikely that reassortment would lead to a virulent viral strain (Cox et al. 1986, 1988; Jin et al. 2003).

The issue of vector-mediated immunity is a concern for most live vaccines, but influenza virus rapidly evolves due to antigen drift. Live-attenuated influenza virus vaccines, like the cold-adapted vaccine, must be reformulated each year to reflect the newly emerged strains. Just as people can be infected multiple times with different strains of influenza virus, a recombinant vector based on influenza virus could be repeatedly administered if it was designed using different antigenic variants of influenza virus.

6 Adenovirus Vectors

Adenoviruses are nonenveloped DNA viruses that have been thoroughly explored for their potential use as recombinant vaccine vectors. Adenoviruses were originally identified as one of the causes of acute respiratory infections. Infection with adenoviruses has also been associated with conjunctivitis and gastroenteritis in infants. While adenovirus infection usually results in mild disease symptoms that are promptly resolved, adenovirus infection of immunocompromised individuals can result in severe disease symptoms, such as pneumonia, encephalitis, and even death (Krilov 2005). Adenovirus is often chosen as a recombinant vaccine vector to express foreign proteins because a live vaccine was administered to US military personal for over two decades with no incidence of significant side effects (Souza et al. 2005). This oral vaccine consisted of the two most prevalent strains of adenovirus among military personal, Ad4 and Ad7, contained in a capsule coated to prevent the release of the viruses until they reached the intestines (Howell et al. 1998; Lichtenstein and Wold 2004). Because of its potential to cause illness, many of the adenovirus vectors currently being developed and tested are replication defective and cannot spread cell-to-cell. Replication-defective vectors often have deletions of the E1 portion of the viral genome, since this region is necessary for the initiation of viral replication (Souza et al. 2005).

In addition to its extensive record as a military vaccine, adenovirus offers several advantages as a recombinant vaccine vector. The viral genome is relatively easy to manipulate and the virus grows to high titers. Adenovirus can by lyophilized, after which it does not need refrigeration (Souza et al. 2005). Because of these reasons, adenovirus has been one of the most popular recombinant vectors, and pharmaceutical companies have chosen to test adenovirus vectors in clinical trials.

Several groups have demonstrated that recombinant adenovirus vectors expressing influenza virus proteins can protect animals after challenge. Adenovirus vectors expressing HA and NP of an H3N2 swine influenza virus fully protected swine

after a lethal challenge (Wesley et al. 2004). A recombinant adenovirus vector expressing HA of HPAI H5N1 induced both cellular and humoral immunity in mice, and the vaccine was completely protective in both mice and chickens after lethal challenge (Gao et al. 2006). An adenovirus vector expressing HA from a H9N2 strain that was used to vaccinate chickens in ovo provided complete protection against lethal challenge with an H5N2 strain and partial protection against an H5N1 strain (Toro et al. 2007). Long-lasting immunity after vaccination with adenovirus vector expressing HA from an H5N1 virus provides protection after lethal challenge for at least one year (Hoelscher et al. 2007).

Adenovirus vectors are currently being examined as possible vaccines for a variety of viruses. Adenovirus vectors, which have been shown to prevent disease after challenge, include those expressing herpes simplex virus and measles virus H, N, and F proteins (McDermott et al. 1989; Fooks et al. 1998; Sharpe et al. 2002). Perhaps the most famous adenovirus vector is the Merck-sponsored HIV vaccine V520 that recently went into clinical trials. The vaccine consisted of adenovirus vectors containing HIV *nef*, *gag*, and *pol* genes (Steinbrook 2007; Sekaly 2008). The vaccine was administered as three injections at zero, two, and six months. The clinical trials were halted early because it became clear that not only was the vaccine failing to prevent HIV infection, but the individuals given the vaccine also had a higher rate of HIV infection than those given the placebo (Sekaly 2008). This devastating failure necessitates a thorough analysis of what went wrong so that it will not be repeated in future trials. It is clear that a major problem is that many people have been exposed to the Ad5 strain used as the vaccine vector. About half of the individuals in western countries have antibodies to Ad5, and about 95% of people in developing countries have antibodies. The presence of pre-existing antibodies likely led to a rapid memory immune response that prevented the development of an immune response to the HIV proteins expressed from the vectors. What was unexpected and is not yet fully explained is that individuals that had been previously exposed to adenovirus before the vaccinations were more susceptible to HIV infection. Other researchers have been testing less common strains of adenovirus to use as vectors in hopes of circumventing the pre-existing immunity problems (Hofmann et al. 1999; Reddy et al. 1999; Farina et al. 2001).

Surprisingly, this was not the first time that adenovirus vectors have unexpectedly harmed clinical trial participants. Adenovirus vectors have been used in gene therapy trials as well as for therapy against cancer, and it was during a gene therapy trial that a participant died of an inflammatory response after receiving a high dose of vector (3.8×10^{13} virus particles) (Lehrman 1999; Marshall 1999). Another disadvantage of using this vector is that the adenovirus genome is DNA, and there is a risk that viral DNA may disrupt host genes and possibly cause cancer. Some adenovirus strains can cause cancer in laboratory animals (Trentin et al. 1962). Even though adenoviruses have not been shown to cause human cancer, it is possible that some cancer cases may arise after the vaccination of a large population. In light of the fiascos involving this vector, it is difficult to foresee high enthusiasm for adenovirus vectors in the future when other vectors seem more promising.

7 Venezuelan Equine Encephalitis Virus Vectors

Venezuelan equine encephalitis virus (VEE) is an RNA virus that primarily infects equines in Central and South America. In contrast to adenovirus, most humans do not have pre-existing antibodies to VEE that could interfere with the vaccine (Davis et al. 1996). Like VSV, VEE is transmitted by insects. Strains of VEE range in pathogenicity from avirulent to causing acute encephalitis and death in equines. Humans can be infected as well and usually only develop mild symptoms, but human deaths have occurred (Weaver et al. 2004). Thus, VEE must be highly attenuated in order to be used as a vaccine vector. Many of the VEE vaccines being tested use a viral replicon particle (VRP) that is capable of infecting cells but cannot spread throughout the host. Foreign proteins are expressed at high levels from the VRP vectors. One major advantage of using this vector it that the VEE targets antigen-presenting cells in the draining lymph node, so the foreign antigen is presented directly to the site where the adaptive immune response begins (Davis et al. 1996, 2002; Charles et al. 1997). Another advantage is that VEE vaccines can also induce an IgA mucosal immune response, even after subcutaneous injection of the vaccine (Charles et al. 1997). Since many pathogens, including HIV, initially invade mucosal surfaces, the induction of mucosal immunity by a vaccine is highly desirable.

VEE VRPs expressing HA from an H5N1 influenza virus were used to successfully protect two-week-old chickens from lethal challenge (Fig. 5) (Schultz-Cherry et al. 2000). VEE vaccines have also been developed for many other agents, including SIV, HIV, Lassa virus, Norwalk virus, *Borrelia burgdorferi* (the causative agent of Lyme disease), SARS-CoV, cowpox virus, dengue virus, and RSV (Caley et al. 1997, 1999; Pushko et al. 1997; Davis et al. 2000; Baric et al. 2002; Harrington et al. 2002; Gipson et al. 2003; Johnston et al. 2005; Deming et al. 2006; Cecil et al. 2007; Mok et al. 2007; Thornburg et al. 2007; White et al. 2007). Further testing will determine whether VEE vaccine vectors are safe and efficacious in humans.

8 Poxvirus Vectors

Poxviruses, DNA viruses with large genomes, have been studied as recombinant vectors after the successful eradication of smallpox using vaccinia virus. Vaccinia viruses possess several properties of an ideal vector (Panicali et al. 1983): they are easy and inexpensive to manufacture, can be lyophilized, can accommodate large inserts of foreign DNA, and can induce both mucosal and systemic immunity after oral administration (Gherardi and Esteban 2005; Souza et al. 2005). A major drawback of vaccinia vectors is that a large segment of the population has pre-existing immunity to vaccinia from the smallpox eradication program, which would interfere with the induction of an immune response to a foreign protein

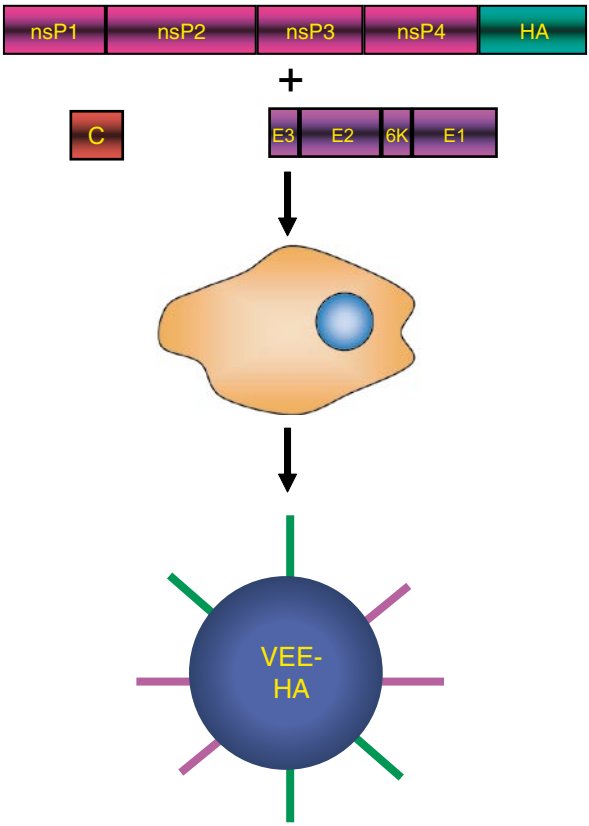

Fig. 5 Rescue of VEE replicon expressing influenza virus HA from three messenger RNAs. Influenza virus HA was cloned into a plasmid containing the VEE nonstructural genes. Helper plasmids were prepared containing the capsid genes and glycoprotein genes. All plasmids were linearized and transcribed into mRNA. The mRNA was transfected into cells and a recombinant VEE replicon expressing influenza virus HA was rescued. Figure adapted from Pushko et al. (1997)

expressed from vaccinia virus. To overcome this, similar poxviruses from other species that do not cross-react with vaccinia virus, such as canarypox virus and fowlpox virus, are currently being tested (Johnson et al. 2005; Bublot et al. 2006). However, it appears that these recombinant vectors induce a weaker immune response to foreign proteins compared to recombinant vaccinia vectors (Zhang et al. 2007). A canarypox vaccine encoding HIV gp120 failed phase 2 clinical trials in humans since it failed to elicit a strong cellular immune response (Russell et al. 2007). Also, vaccinees can spread vaccinia virus to other individuals, which is especially dangerous for immunocompromised individuals. To address this, replication-defective attenuated vaccinia viruses, such as the Ankara strain, are being evaluated for recombinant vector potential, though these attenuated strains do

cross-react with vaccinia virus, making it less likely that they will ultimately be successful (Souza et al. 2005).

9 Live Attenuated Measles Viruses as Recombinant Vectors

Another strategy involves altering currently licensed vaccines—such as the live attenuated measles virus vaccine—to express foreign proteins, in the hope that there would be a strong immune response to both measles virus and a foreign protein (Zuniga et al. 2007). The measles virus vaccine is highly efficacious in infants and has an excellent safety record. A measles virus vector expressing West Nile virus glycoprotein protected mice against a lethal challenge with West Nile virus (Despres et al. 2005). The only disadvantage of this vector is that most of the human population has already been vaccinated and has pre-existing immunity to measles virus. However, mice and macaques were vaccinated with the measles virus vaccine, and after 12 months were vaccinated with the measles virus vaccine expressing HIV gp140. The animals developed antibody titers to HIV that were similar to the antibody titers in naïve animals (Lorin et al. 2004). While more work is required to substantiate these results in order to recommend using this vector in humans with pre-existing measles virus immunity, at the very least this is a promising method for vaccinating naïve infants against both measles virus and another pathogen.

10 Other Recombinant Vectors

A current vaccine strategy under development is the use of bacteria as delivery vehicles of foreign antigens. Attenuated strains of intracellular bacteria such as *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* are being engineered as recombinant vectors (Schoen et al. 2004; Cheminay and Hensel 2008; Schoen et al. 2008). While intracellular, the bacteria remain in a membrane-bound vesicle inside the host cell, which prevents foreign proteins expressed by bacteria from entering the host cytosol, a necessary step for antigens to be presented to the immune system. Two mechanisms of antigen delivery to combat this problem have been tested in bacterial vectors. One involves synthesis of the foreign protein inside the bacteria and release of the foreign protein into the human cell by the bacterial type III secretory pathway (Panthel et al. 2008). Because proteins must be unfolded prior to being secreted, foreign proteins with high stability cannot be completely unfolded and are unable to exit the bacterial cell. It has been demonstrated that the removal of small stabilizing domains in HIV proteins can allow these large foreign proteins to be secreted by the type III pathway (Chen et al. 2006). However, the complexity of the bacterial genome and the difficulty of secreting foreign proteins will limit the use of this system.

Another mechanism of antigen delivery by bacterial vectors involves the release of DNA encoding a foreign protein into the host cell, essentially a DNA vaccine delivered by a live bacterial organism. This strategy includes transforming bacteria with a naked DNA vector that encodes a foreign gene. Bacteria that target antigen-presenting cells, such as *Listeria monocytogenes* that targets dendritic cells, must be used for this technique. The chosen bacteria have to be highly attenuated and designed to lyse upon host cell entry. Once the bacteria are lysed, the DNA vector enters the cytosol and then transports to the nucleus, where it is transcribed. After being translated in the cytoplasm, the antigens can be processed to be presented on both MHC I and MHC II molecules in order to stimulate humoral and cellular immunity (Mollenkopf et al. 2001; Weiss 2003; Schoen et al. 2008). While this method shows promise, as with viral vectors, pre-existing immunity to bacterial vectors does appear to inhibit the production of an immune response to foreign proteins (Sevil Domenech et al. 2007).

A new area of recombinant vector research has been focusing on using transgenic plants as delivery vectors. Plants are safe and inexpensive vectors, can easily be grown in large quantities, are stable at room temperature, and can be designed to express many antigens (Webster et al. 2005). Expression of HIV antigens in plants has been reported, and these vaccine vectors are currently being evaluated for their efficacy (Yusibov et al. 1997; Marusic et al. 2001).

11 Conclusions

The growing interest in using recombinant vectors as vaccines for influenza virus and other dangerous pathogens reflects the reality that these vaccines have substantial advantages over most other types of vaccines. While some recombinant vectors appear to be more encouraging than others, ideally it is hoped that several different vectors will ultimately be used to vaccinate against different diseases. An important hurdle to overcome in the development of recombinant vectors is the problem of pre-existing immunity to many of the vectors being tested. The issue of pre-existing immunity must also be addressed for recombinant viruses that humans currently do not have immunity against. This is because people have to be vaccinated multiple times for influenza virus, as the viral HA protein mutates. If a strong immune response is generated against the vector after the first vaccination, the vector may not be able to replicate sufficiently after successive administrations. This would prevent the formation of an immune response to the mutated HAs, leaving the individual vulnerable to infection with the altered influenza viruses. This is being addressed for VSV by the generation of vectors that express different serotypes of VSV G protein that do not cross-react. Recombinant VSV vectors expressing HIV Env elicit a strong immune response to Env, and subsequent vaccination with different VSV vectors expressing HIV Env and other G proteins can be used to boost the initial immune response (Rose et al. 2000). Further work in this area is needed to overcome this limitation of recombinant vectors. A comparison of the viral vectors is shown in Table 1. Overall, recombinant vectors provide a

Table 1 Comparison of the viral vectors

Vector	Advantages	Disadvantages	References
NDV	RNA genome; humans do not have pre-existing immunity; multiple foreign genes can be expressed from bisegmented genome; can be constructed for avian and human use; phase I trials demonstrate safety in humans	Requires refrigeration	Freeman et al. (2006); Laurie et al. (2006); Park et al. (2006); Veits et al. (2006); Gao et al. (2008)
VSV	RNA genome; humans do not have pre-existing immunity	Vector safety in humans not yet established; requires refrigeration	Roberts and Rose (1999); Clarke et al. (2006)
Influenza virus	RNA genome; can be constructed for avian and human use	Segmented genome that could reassort; humans have pre-existing immunity; requires refrigeration	Garcia-Sastre and Palese (1995); Steel et al. (2008)
Adenovirus	Can be lyophilized; does not require refrigeration	DNA genome (may alter host genome); humans have pre-existing immunity; clinical trial failure	Lehrman (1999); Marshall (1999); Souza et al. (2005); Steinbrook (2007); Sekaly (2008)
VEE	RNA genome; humans do not have pre-existing immunity; targets APC cells; can induce mucosal immunity after s.c. injection	Vector safety in humans is currently being studied	Davis et al. (1996); Charles et al. (1997); Davis et al. (2002)
Poxviruses	Can be lyophilized; does not require refrigeration; can accommodate at least 30 KB of foreign DNA	DNA genome (may alter host genome); humans have pre-existing immunity	Gherardi and Esteban (2005); Souza et al. (2005); Vijaysri et al. (2008)
Measles virus	RNA genome; excellent safety record in humans	Humans have pre-existing immunity	Zuniga et al. (2007)

safe and effective mechanism for eliciting humoral and cellular immunity to the most dangerous pathogens on the planet.

Acknowledgements Work done in the laboratory of the authors was funded by NIH grants U54 AI057158 (Northeast Biodefense Center) and U01 AI070469 (Live Attenuated Vaccines for Epidemic and Pandemic Flu), and with Bill and Melinda Gates Foundation grant 38648.

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