Non-segmented negative sense RNA viruses as vectors for vaccine development

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This article intends to cover two aspects of non-segmented negative sense RNA viruses. In the initial section, the strategy employed by these viruses to replicate their genomes is discussed. This would help in understanding the later section in which the use of these viruses as vaccine vectors has been discussed. For the description of the replication strategy which encompasses virus genome transcription and genome replication carried out by the same RNA dependent RNA polymerase complex, a member of the prototype rhabdovirus family – Chandipura virus has been chosen as an example to illustrate the complex nature of the two processes and their regulation. In the discussion on these viruses serving as vectors for carrying vaccine antigen genes, emphasis has been laid on describing the progress made in using the attenuated viruses as vectors and a description of the systems in which the efficiency of immune responses has been tested.

INFECTIOUS disease causing human deaths is an alarming issue for every country. Looking at the statistics provided by the World Health Organization (WHO) it is quite clear that emergence of new viruses along with the transformed strains of older ones is causing pandemics in recent years. Although a great majority of these deaths occur in developing countries, infectious diseases are not confined by international borders and therefore present a substantial threat to populations in all parts of the world. In recent years, the threat posed by infectious diseases has grown. New diseases, unknown in the United States just a decade ago, such as West Nile virus and severe acute respiratory syndrome (SARS), have emerged, and known infectious diseases once considered to have declined have reappeared with increased frequency. Furthermore, there is always the potential for an infectious disease to develop into a widespread outbreak, which could have significant consequences. Spreading of the disease is the regulatory factor of infectious disease. Infectious diseases are mostly spread either with the help of intermediate vectors or by air/water contamination. Vector-borne diseases are sometimes localized due to lack of proper vectors elsewhere. But the global changes in climate along with the ability of pathogens to adapt to similar kind of vectors allow the disease to spread globally. Haematophagous arthropod vectors such as mosquitoes, ticks and flies are responsible for transmitting bacteria, viruses and protozoa between vertebrate hosts, causing deadly diseases such as malaria, dengue fever and trypanosomiasis. In recent years, various viral diseases have posed a great threat to the Indian subcontinent; Japanese encephalitis, the West Nile virus, Chandipura virus (CHPV), avian influenza (H5N1) and swine influenza (H1N1) being prominent among these.

The list of emergent viruses continues to grow. In the early 1990s, there was human immunodeficiency virus (HIV), Ebola, Lassa and others, almost all having jumped from their natural host species to humans. More recently, hepatitis C, Sin Nombre, West Nile, and of course SARS have emerged. A number of important human diseases, both established (mumps, measles, rabies) and emerging (Ebola haemorrhagic fever, Borna disease, Hendra virus, Nipah virus), are caused by viruses with RNA as their genome.

Among all the viruses, the RNA viruses pose special threats to the human population due to their ability to mutate very rapidly. They cause problems to the immune system as well as the vaccination and antiviral drug therapy. One of the orders of such RNA viruses is Mononegavirales, that have non-segmented, negative sense RNA genome. The order includes four families:

- Bornaviridae (Borna disease virus)
- Rhabdoviridae (e.g. rabies virus)
- Filoviridae (e.g. Marburg virus, Ebola virus)
- Paramyxoviridae (e.g. Newcastle disease virus, measles virus).

In order to combat the propagation of viral disease, antiviral drugs and vaccination are the two most potent therapies. Vaccination with live viruses began dated way back in 1983, when recombinant vaccinia virus expressing hepatitis B surface antigen demonstrated specific antibody production in immunized rabbits[1]. In general, live attenuated version of virus is a preferred choice for vaccination due its ability to express a full array of viral

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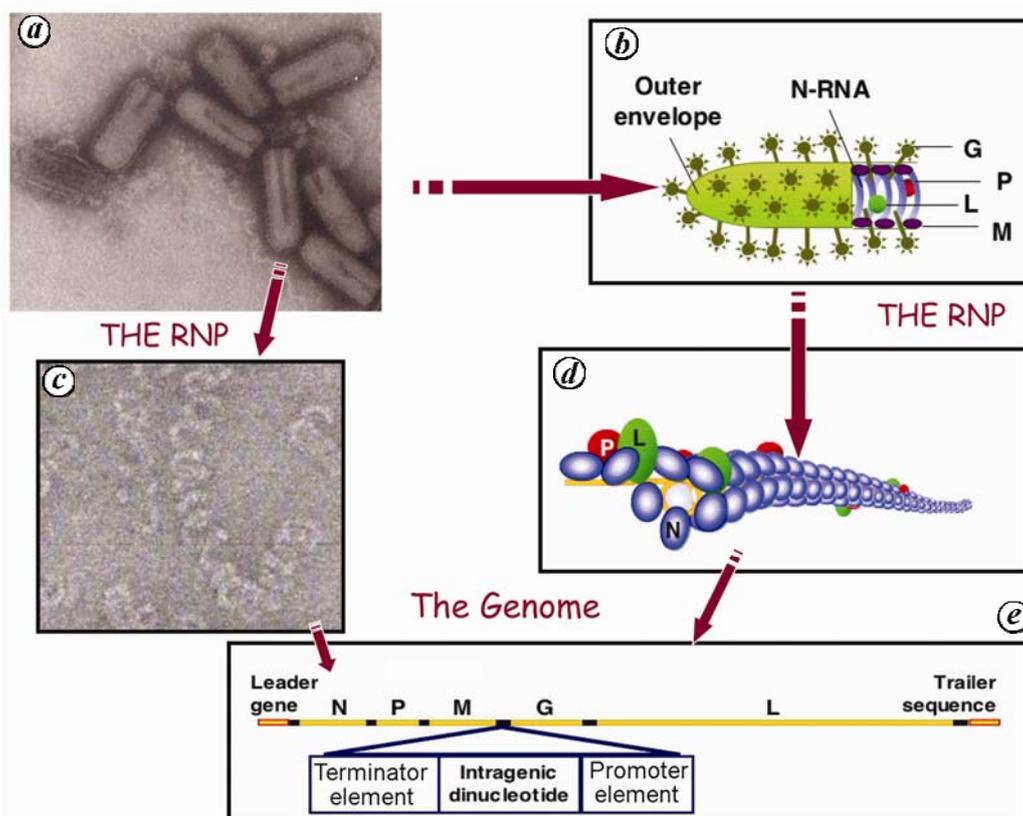
Figure 1. Organization of the rhabdovirus particle. *a*, Electron microscope picture of rhabdovirus. *b*, Cartoon of the assembly of the viral proteins with RNA to form the virus. *c*, EM picture of the RNP particles. *d*, Cartoon of the RNP constituents. *e*, Genome organization of rhabdoviruses.

antigens. However, in some cases we cannot use live attenuated viruses for immunization, e.g. viruses that do not grow well *in vitro* or the viruses that can exchange genes with circulating viruses. In these cases an alternative mode of vaccination is used which delivers only a component of the viral antigen carried by a vector capable of replicating itself without damaging host and also allows high titer of antibody to antigen so that it can induce immune response. The vector used in this regard is mostly virus derived. Initially the DNA/positive sense RNA viruses are explored for their ability to act as vectors, but the non-segmented negative sense RNA viruses (NNSVs) have not been explored largely due to their inability to incorporate their genome RNA inside the cell culture and also due to lack of recombination mechanisms. But on the contrary, the NNSVs have some features that make them attractive candidates for vectors. Some animal/avian NNSV are antigenically distinct from human pathogens and hence they can infect the entire human population for successful immunization. NNSV can also replicate in cells like Vero cells which can be used in humans. Most importantly, the delivery of the vaccine can be effectively done via intranasal (IN) route and can elicit local immunoglobulin A and systemic immunoglobulin G and cell mediated protective immune response[1]. Genetic exchange

is a rare event in NNSV and lack of recombinations with the circulating viruses make this a great vector.

Advent of reverse genetics changed the whole scenario regarding the use of viral vectors. We can now make cDNA clones of NNSV and transfect the construct within the cell along with some essential viral components to form a functional nucleocapsid. The viral antigen gene can either be integrated in the genome construct or the viral gene can replace one of the NNSV genes making a chimera. Vesicular stomatitis virus (VSV), a member of NNSV, has a number of advantages when used as vaccine vector. It has a very high level of expression associated with a rapid and efficient replication *in vitro*[2]. The VSV glycoprotein has three different serotypes that can be available for sequential immunization[3,4]. In experimental animals, VSV vectors can be administered by IN, intradermal, intramuscular or oral route[5–7]. Also the virus has a regular structure that is stable facilitating purification and manufacturing processes[6].

Since VSV is a prototype virus for NNSVs, which are increasingly being employed as vaccine vectors, the following overview provides the strategy followed in rhabdoviruses, with specific example of CHPV for replication of its genome and also provides some insight into the complex processes of transcription of the genome by
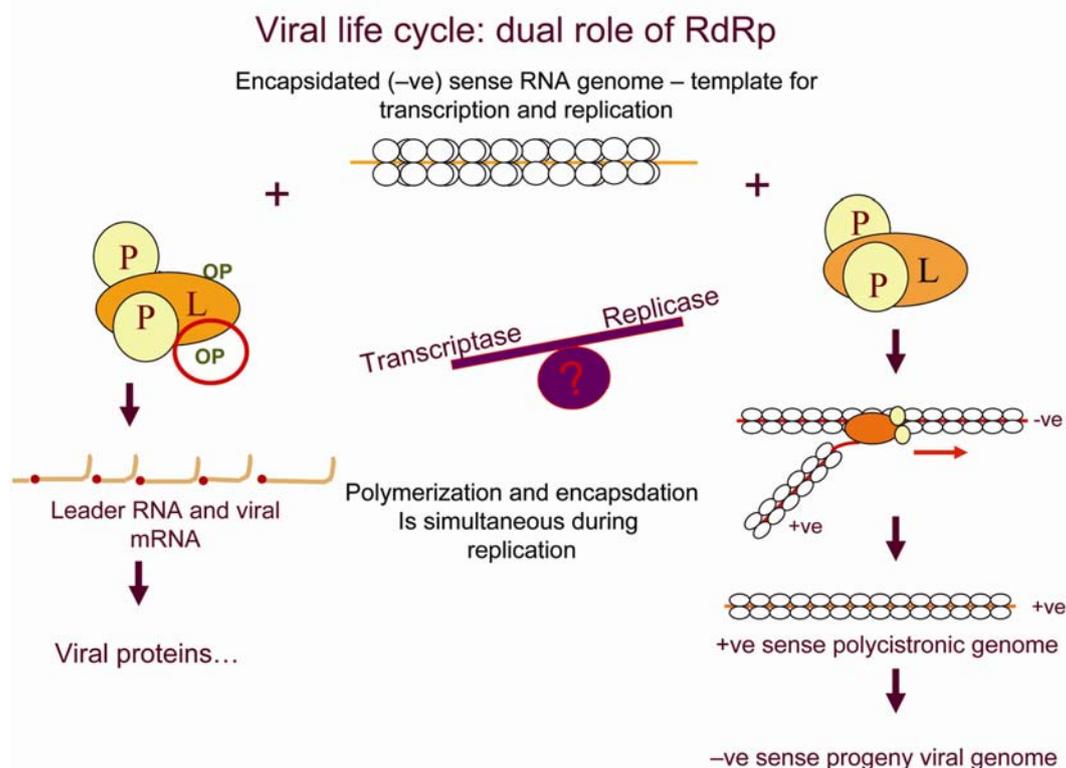
Figure 2. Cartoon depicting the viral life cycle: decision making of viral transcription/replication by viral RdRp with the help of viral P protein.

the same enzyme versus replication of the same genome by the same enzyme.

Rhabdoviruses belong to the mononegalovirales whose linear single-strand genome RNA has negative polarity; i.e. their genome RNA is noninfectious and complementary to functional, virus-specific, positive sense messenger RNAs (mRNAs). These characteristically bullet-shaped viruses are ubiquitous in nature and have a uniquely broad and diverse host range including vertebrates, invertebrates and plants. Two notable and often studied rhabdoviruses are the animal pathogen VSV and the human pathogen rabies virus (RV). One of the unique features of the negative-strand genome RNA of a rhabdovirus is that it serves as the template for both transcription (mRNA synthesis) and replication (genome RNA synthesis). Moreover, due to the packaging of a novel, virus-specified RNA polymerase within rhabdovirions, these viruses serve as one of the model systems to study transcription and replication and their regulation *in vitro* (Figure 1).

CHPV belongs to *Vesiculovirus* genera of the Rhabdoviridae family within the order Mononegaloviridae. The RNA genome serves as a template for both transcription as well as replication for these rhabdoviruses. CHPV genome RNA comprises a short 49 nt leader gene[8], followed by five transcriptional units coding for viral polypeptides separated by intragenic spacer regions and a short non-transcribed 46 nt trailer sequence (t) arranged

in the order 3′ l-N-P-M-G-L-t 5′. Upon infection, the virus releases its genome in host cytosol where it undergoes both transcription as well as replication. The viral RNA dependent RNA polymerase, large protein L, transcribes and replicates with the help of phosphoprotein P. Transcription initiates at the 3′ end of the encapsidated genome, resulting in the synthesis of all the six viral mRNAs with progressive termination at each gene junction[5,6,8]. One of the key features of the rhabdoviruses is the polar nature of transcription. In the replication mode, the same RNA polymerase copies the entire genome to produce a positive stranded polycistronic RNA strand complementary to the whole viral genome. This positive sense RNA is used as template for subsequent synthesis of negative stranded progeny RNAs (Figure 2). Transcription and replication events depend on whether RNA polymerase can read-through the gene boundaries or not. Termination of the products at gene boundaries leads to transcription, whereas anti-termination gives the replication product.

Vesiculovirus transcription is characterized by actino-mycin D resistant synthesis of leader RNA and viral mRNAs. Order of transcription of VSV genes was determined by *in vitro* transcriptional mapping analysis using UV-radiation. Such studies revealed that VSV mRNAs are synthesized in an obligatory sequential manner after polymerase entry at single 3′ end of the genome termini, i.e. at the beginning of leader gene[2,4,8]. Determination of

relative molar ratios of different viral mRNAs within infected cells revealed that their abundance decreased with increasing distance from the 3¢ promoter in an order (N > P > M > G > L), thus implying a mechanism that also ensures polar transcription[8].

Successful *in vitro* reconstitution of CHPV transcription with purified components was shown by Chattopadhyay *et al.*[9] and considered as an important step towards developing mechanistic insight into transcription. When purified, L protein was incubated with N-RNA in a reaction mixture that allows *in vitro* transcription, it was unable to synthesize viral mRNA. However, addition of viral P protein along with L allowed viral mRNA synthesis. These studies confirmed the role of phosphoprotein as an activator of viral transcription[9] and this proposed function was consistent in the observations made in VSV[4]. Unphosphorylated P undergoes phosphorylation with CKII and this phosphorylation induces P to form dimers. This phosphorylated P protein interaction with viral polymerase L results in active transcription[9]. P in its unphosphorylated form is capable of RNA binding and this binding is reversible in nature whereas phosphorylated P is not capable of RNA binding[8]. With the change in phosphorylation state, P regulates viral replication. It can be possible that this dual role of P is due to conformational change induced by phosphorylation. P undergoes phosphorylation in a stepwise manner. First, the serine-62 gets phosphorylated and this phosphorylated P undergoes phosphorylation again either by viral L protein associated kinase (LAK)[10] or by host kinase[10]. The first step of the phosphorylation, i.e. the serine phosphorylation plays the most crucial role in determining P mediated transcription. Mutation in serine does not result in phosphorylation indicating that the conformational change in P protein during the first phosphorylation step allows the second site for phosphorylation. It was proposed that unphosphorylated P protein might play a role in viral replication by changing the polymerase to read-through the gene boundaries. To test this hypothesis, the virus yield was compared in infected cells overexpressing either wild type P protein or the mutant P lacking the CKII phosphorylation site. The virus titer was 100-fold more in the mutant than the wild type P, supporting the hypothesis.

In vitro transcription assay with detergent disrupted virus in presence of either wild type or phosphorylation defective P protein showed that phosphorylation favours the production of small leader RNA whereas unphosphorylated P protein favours the production of higher length RNAs at the expense of the small leader RNA[11].

The role of the RNP particle structure in deciding the transcription of rhabdoviruses may also shed some light. If we compare the genome sequence of the VSV and CHPV, we will find some similarities at the gene termini as well as the intergenic regions. An interesting feature of the nucleotide sequence of the VSV genome is the intergenic sequence. Genes *N*, *NS*, *M*, *G* and *L* contain common sequences at their termini and between each other: 5′CUGUUAGUUUUUUUCAUA3′ (ref. 8). Thus, the signal for termination, polyadenylation and initiation of RNA chains may reside within this sequence. The (U7) stretch clearly signifies the region where the polymerase probably 'chatters' to synthesize poly(A)[7]. Another important feature of the nucleotide sequence of the VSV genome is the end of the *L* gene and the 5′ end of genome RNA. It contains the same termination and polyadenylation signals but lacks the signal for downstream initiation. Therefore, the last 60 nucleotides, or trailer sequence, on the genome remain untranscribed *in vitro*. However, readthrough products containing L mRNA linked to the complement of the end of genome RNA have been detected *in vivo*[12], indicating that the entire genome is transcribed when the transcriptase is in a replicative mode. Another interesting feature is the presence of adenineuridine (AU)-rich sequences sequestered in specified regions of genome RNA[8]. These sequences may be analogs of the TATA boxes which are located upstream of certain genes of eukaryotic DNA and which represent the promoter sites for RNA polymerase II. There are five such boxes in the VSV genome RNA, one of which resides in the middle of the leader template and others which reside near the 3′ ends of genes *N*, *NS*, *M* and *G*. The sequence within the leader template curiously coincides with one of the observed binding sites of the NS protein on the genome RNA[11]. Thus, it may be speculated that the other AU-rich sequences may also represent binding sites of the RNA polymerase complex from which it initiates RNA transcription of downstream genes.

Construction of recombinant vaccinia virus expressing hepatitis B surface antigen by Smith *et al.*[1] in 1983 is the beginning of the era of live viral vectored vaccines. They demonstrated the induction of hepatitis B-specific antibodies in rabbits immunized with the recombinant virus. Subsequently, live-virus vectors were developed with other DNA viruses, such as adenoviruses and herpesviruses, and with positive-strand RNA viruses, such as alphaviruses and flaviviruses. Potentiality of the large group of NNSV has remained unexplored largely due to the lack of infectivity of their genomic RNA in cell culture and the absence of a mechanism for insertion of foreign genes. The NNSV (order *Mononegavirales*) comprise four families: *Paramyxoviridae*, including measles virus (MV) and mumps virus, Sendai virus (SeV), human parainfluenza virus types 1, 2, 3 and 4 (HPIV1 to -4), Newcastle disease virus (NDV), and human respiratory syncytial and metapneumoviruses (HRSV and HMPV); *Rhabdoviridae*, represented by VSV and RV; *Filoviridae*, containing Ebola and Marburg viruses; and *Bornaviridae*, containing Borna disease virus. Schnell *et al.*[13] developed a reverse genetic system in 1994 for rhadoviruses that allowed the recovery of infectious virus entirely from cloned cDNA[2]. This approach was followed for numerous other NSNV[14–22].

Scope of using NSNV as vectors for vaccine development

Live attenuated viral pathogen is the best choice for a vaccine candidate, because it expresses the complete array of antigenic profile and elicits both local and systemic immunity thereby providing solid protection against infection. However, there are several situations in which production of attenuated vaccines becomes impossible and production of vectored vaccine becomes necessary. (i) Attenuation of the virus is not possible; for example, HIV, due to its ability to establish a persistent infection, or Ebola virus, due to its high pathogenicity, (ii) viruses that fail to grow *in vitro* in cell culture system, such as human papillomavirus and hepatitis C virus, (iii) highly pathogenic viruses that pose safety problems during vaccine development, such as SARS coronavirus (SARS-CoV) and Ebola virus, (iv) viruses that are highly labile due to physical instability and loss of infectivity, such as HRSV and (v) viruses that are reported to undergo recombination with circulating viruses, such as CoVs, influenza viruses and enteroviruses. Alternative vaccines that can match with the use of attenuated pathogens as vaccines are to be employed. Viral vectored vaccine may overcome these specific challenges. In addition, they can be tailor-made to include features that might improve efficacy, such as the targeting of dendritic cells[23]. In case of SeV based vector, the antigen gene could be efficiently expressed in dendritic cells by deleting gene for matrix protein. A known major advantage of a vectored vaccine is that it can be rapidly engineered to generate a vaccine against a newly emerged highly pathogenic virus. However in this case, sequence analysis of the new pathogen would identify the likely protective antigen(s), and its cDNA clones would be inserted into a vector for using as vaccine.

NNSV have a number of features that make them good viral vectors, some of them are described here.

- The presence of are numerous well-characterized animal, avian and human NNSV that could serve as vectors. Some avian or animal NNSV, such as NDV and VSV are antigenically distinct from common human pathogens and therefore essentially the entire population should be susceptible to infection and successful immunization. Some of the human NSNV, such as HPIV1 to -3 also have the potential to be used as vaccine vectors in susceptible populations. For example, infants and young children characteristically are immunologically naive to the three HPIV serotypes; thus, attenuated versions of HPIV1 to -3 can be developed as paediatric vaccine vectors.

- Attenuation strategies for NNSV have been studied. In some cases, for example, animal or avian viruses are attenuated in primates due to a natural host range restriction. In other cases, mutations that cause attenuation can be generated by reverse genetics before using as vectors. Thus, it should be possible to attenuate each viral vector expressing a foreign viral protective antigen such that it becomes safe and at the same time helps in eliciting desired immune response against the target antigen for the target population.

- Several NSNV that have the potential to emerge as vaccine vectors replicate efficiently in known well-characterized cell lines, such as Vero cells that are qualified for human and veterinary use which is a requirement for vaccine manufacture.

- The efficient IN route of infection for most of the NNSV helps in efficient induction of local immunoglobulin A and systemic immunoglobulin G antibody and cell-mediated protective immune responses. This helps in the development of needle-free IN vaccines which could be administered easily and safely, facilitating immunization in the context of an outbreak or epidemic. IN vaccines are particularly useful against viruses like SARS-CoV and influenza, that spread via the respiratory tract.

- NNSV replicate in the cytoplasm and hence there is no risk of integration of its genome with the host genome, obviating concerns about cellular transformation.

- Gene exchange has not been observed in nature in NNSV[24], indicating that the use of NNSV based vectored vaccines in open populations would not pose a threat of recombination between vaccine virus and circulating viruses.

- The lack of recombination also contributes to the stability of the inserted foreign gene.

- NNSV encode 5–11 separate proteins, while large DNA viruses, such as poxviruses, encode as many as 200 proteins. Several proteins expressed by these complex viruses modulate or antagonize host immune responses, which might decrease vaccine effectiveness. However there are reports that one or two proteins that are encoded by most of the NNSV interfere with the host interferon system[25,26], but these can often be mutated or deleted[27–29]. MV is one NNSV that is significantly immunosuppressive (mediated by a mechanism other than interferon antagonism), making it a less attractive vector candidate. In addition, the use of a complex vector that expresses a large number of proteins might be less desirable, since these vector antigens might dilute the desired antibody and cell-mediated response directed against the foreign antigen and lead to a reduction of the overall memory T-cell pool[29].

- The genomes of NNSV consist of genes that for the most part are non-overlapping and are expressed as separate mRNAs, thus constituting a modular organization that can be readily manipulated for the insertion and stable maintenance of foreign inserts.

Preparation of infectious clones

The first and foremost step while producing vectors from negative strand RNA viruses is generation of infectious cDNA clones. NNSV can be rescued entirely from cloned cDNA by transfecting cultured cells with plasmids encoding the components of a nucleocapsid, i.e. (i) full-length genomic or antigenomic RNA and (ii) the proteins involved in transcription and replication, namely the nucleoprotein (N or NP), phosphoprotein (P) and the large polymerase protein (L). However, in the case of HRSV and Filoviridae members, Marburg or Ebola viruses; in addition to components of nucleocapsid, the transcription elongation factor M2-1 or transcription activator factor VP30 respectively[30–32] are needed for recovery. It is essential that transfection has to be done into a cell line that can support replication of the particular virus. The expression of the delivered plasmids can be done by bacteriophage T7 RNA polymerase that can be delivered in any of the three ways, i.e. by coinfection with a recombinant vaccinia virus expressing T7 polymerase[33]; the use of cells that constitutively express T7 polymerase (presently baby hamster kidney cell line is available); or by co-transfection of a plasmid encoding T7 polymerase[34]. The third method is ideal and preferable, because it allows the recovery of viruses in a cell line approved for vaccine production (e.g. Vero or BHK) without adding any component/s that needs regulatory approval (e.g. vaccinia virus). Intracellular expression of the genomic RNA and protein components of nucleocapsid results in the assembly of a viral nucleocapsid which is competent for gene expression and genome replication, resulting in production of infectious virus. Once the infective cDNA is available, several strategies can be employed to generate vaccines. Some of the most important ones are described here.

Production of vaccines using replication-competent NNSV

The simplest strategy is to express one or more foreign antigens from genes that have been added to a complete, replication-competent NNSV[35]. The NNSV vector must be sufficiently attenuated to be safe for administration to humans. In the case of NNSV, such as NDV, that are attenuated in primates due to a natural host restriction[36], the wild-type virus can be used directly as vector. Other NNSV will require attenuation by reverse genetics before use as potential vectors in humans. For example, VSV is not a common human pathogen, but VSV infection is associated with disease which may not be serious in humans[37]. Thus, attenuation is needed before VSV can be used as a vaccine vector. Similarly HPIV3, which can cause respiratory infection in humans, needs to be attenuated and validated.

Attenuation of NNSV can be achieved by employing various strategies. The viral gene order can be rearranged, which results in a suboptimal level of gene expression and thereby reduces replication efficiency[38]. Point mutations can be introduced in the known region by reverse genetics[38,39]. Mutations can also be 'transferred' between related viruses by reverse genetics using sequence alignments as a guide[40–42]. Deleting or silencing nonessential accessory genes, including those encoding interferon antagonist proteins, often yields attenuated derivatives[43–45]. In case of rhabdoviruses, an effective attenuation of replication without a significant reduction of immunogenicity could be achieved by partial truncation of the G glycoprotein cytoplasmic tail[28,46–48]. The insertion of foreign genes for expression frequently has an attenuating effect by itself. Attenuation can also be achieved by swapping internal protein genes between related human and animal or avian viruses to introduce host range restriction[49,50].

Production of chimeric viruses

An alternative method to deliver foreign antigen is to replace the major protective surface antigen(s) of the vector with that of the pathogenic virus of interest. Such antigenic chimeric virus can be a viable vaccine vector provided the surface proteins of foreign origin are effectively incorporated into the virion particles and becomes functional in the vector background.

Several notable examples are based on VSV in which the VSV G glycoprotein is replaced by the haemagglutinin (HA) glycoprotein of influenza virus[48] or the respective glycoprotein of Ebola, Marburg or Lassa virus[51,52] and Borna disease, a fatal immune-mediated neurological disease in sheep and horse caused by Borna disease virus[53]. The resulting chimeras were attenuated *in vitro* compared to the VSV parent, suggestive of a degree of incompatibility between vector proteins and foreign glycoproteins thereby increasing the safety of the vaccine. Although attenuated, the chimeras were competent for *in vivo* replication and were immunogenic and protective in mice or nonhuman primates[48,51,52,54]. However, not all VSV-based chimeras are replication competent. For example, chimeras in which the VSV G protein was replaced with the G or F protein of HRSV were not viable, even though each HRSV protein was incorporated into the VSV particle, and even though F alone is sufficient to confer efficient infectivity and growth to HRSV *in vitro*[55,56].

A noteworthy antigenic chimeric virus is the replacement of the *F* and *HN* surface glycoprotein genes of bovine PIV3 (BPIV3), which is attenuated in humans due to a natural host range restriction, with the *F* and *HN* genes from its closely related human counterpart HPIV3. This has the effect of combining the host range restriction

of BPIV3 with the major antigenic determinants of HPIV3, resulting in an attenuated virus bovine–human PIV3 (B/HPIV3) that replicates with undiminished efficiency *in vitro*, retains the host range attenuation phenotype in nonhuman primates, and is under clinical trials as a vaccine against HPIV3 (refs 57–59).

However, substitution of the HN and F glycoproteins of HPIV1 into an HPIV3 backbone resulted in a chimera that replicated with wild-type efficiency *in vitro* and *in vivo*[41,59]. The lack of attenuation associated with chimerization in this case reflects the close relationship between these two viruses. The substitution of the HN and F glycoproteins of the more-distantly related HPIV2 (genus *Rubulavirus*) into HPIV3 did not yield a viable chimeric virus. However, when the HN and F proteins of HPIV2 were modified to replace their cytoplasmic domains with the corresponding ones of HPIV3, viable antigenic chimeras that replicated efficiently *in vitro* could be recovered, although they were attenuated *in vivo*[60]. Thus, the ability of a heterologous glycoprotein to function efficiently in an antigenic chimera may depend on the fit of its cytoplasmic domain into the vector background.

Production of chimeric virus expressing additional foreign genes

Antigenic chimeric viruses also can be used to express additional antigens from added genes of immunological importance. Thus one vaccine can be developed against several diseases. For example, the B/HPIV3 chimera has been used to express protective antigens of HRSV or HMPV from added genes[61–63]. These applications would yield a bivalent vaccine against HPIV3 and HRSV or HPIV3 and HMPV respectively, each based on a single virus. B/HPIV3 expressing the HRSV F glycoprotein is currently in clinical trials by Med Immune, Inc.

Specific viral vaccine vectors

Newcastle disease virus

NDV (family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Avulavirus*) is an avian pathogen which naturally occurs as three pathotypes: velogenic strains, which cause systemic infections with a high level of mortality; mesogenic strains, which cause systemic infections of intermediate severity and lentogenic strains, which cause mild infections that are largely limited to the respiratory tract and which are used as live attenuated vaccines against NDV for poultry[64]. One of the primary determinants of virulence is the cleavage activation phenotype of the F protein precursor, which in highly virulent strains is cleaved by ubiquitous intracellular proteases, providing the potential for pantropic replication, and which in nonvirulent strains is cleaved by a secretory protease,

restricting replication to mucosal surfaces. NDV usually does not infect humans, but occasionally causes conjunctivitis in bird handlers[65,66]. IN and IT inoculation of the respiratory tract of African green and rhesus monkeys with a mesogenic or lentogenic strain of NDV resulted in attenuation[36] due to a natural host-range restriction.

NDV shares only a low level of amino acid sequence identity with human paramyxoviruses and is distinct antigenically, suggesting that the human population would be susceptible to vaccination with an NDV-based vector. Studies with mice showed that NDV expressing protective antigens of influenza A virus, HRSV or simian immunodeficiency virus (SIV) were immunogenic and, in the first two cases, induced protection against challenge[19,67,68]. However rodents are not good models for evaluation due to phylogenetic distance. Recently, recombinant lentogenic and mesogenic strains of NDV expressing HPIV3 HN protein from an added gene were evaluated by IN and IT immunization of the respiratory tract of African green monkeys and rhesus monkeys. After one dose, an efficient HPIV3-specific serum antibody response was detected; after the second dose, it became equal to or greater than that induced by HPIV3 infection[36]. Thus, NDV warrants further evaluation as a potential vector for human use. NDV is also being actively developed as a vaccine vector for veterinary use in which the vector itself serves as a needed poultry vaccine[69–71]. NDV is classified as serotype 1 of the avian paramyxoviruses of genus *Avulavirus*; eight other serotypes, which cause disease of various levels of severity in different species of birds have been identified[72]. These can also be evaluated as possible vectors for human use.

Vesicular stomatitis virus

VSV (family *Rhabdoviridae*, genus *Vesiculovirus*) is a natural pathogen of cattle, horses and swine that causes disease characterized by severe vesiculation and/or ulceration of tongue, oral tissues, feet and teats[73]. The infection of mice and cotton rats with VSV by the IN route results in significant replication of the virus in brain, encephalitis and high mortality[74–76]. VSV is not usually a human pathogen. Most human infections that do occur (from contact with infected animals) are believed to be asymptomatic but sometimes result in illness characterized by chills, myalgia and nausea[77–79]. A case of encephalitis associated with VSV infection in a 3-year-old child has also been documented[80].

VSV has a number of very advantageous features as vaccine vector; it is the simplest NSNV with extremely efficient replication and high level of expression *in vitro*. The general population in most areas of the world lacks immunity to VSV and thus, presumably, is susceptible to immunization. Recombinant VSV bearing the G glycoprotein from three different serotypes is available for

sequential immunization[81,82]. In experimental animals, VSV vectors have been very effective when administered by the IN, IM, intradermal or oral routes[83–85].

The major drawback for VSV is that, at present, there is little or no experience with its administration to humans and, thus, it is not clear whether serious complications may occur in some individuals. The central nervous system involvement observed in rodents and a human case warrants caution. Also it is not known how immunogenic and protective VSV vectors will behave in humans, either as antigenic chimeric viruses or as vectors expressing added foreign genes, although results with experimental animals are promising. Recombinant VSV appears to be inherently attenuated, presumably due to chance mutations, and a number of strategies exist for further attenuating the virus[3]. There is possibility that VSV-based vaccines will be evaluated clinically in the near future and, thus, detailed information on its replication, pathogenesis and immunogenicity needs to be evaluated.

Though VSV is somewhat of an unknown entity at present with regard to human administration, it is believed that its most feasible first use will be as a vector to control outbreaks of highly pathogenic agents. This is because, the vaccination is done only for healthy adults, the number of vaccines will be relatively small, and the high risk of the pathogen would outweigh some potential risk of residual virulence of the vector[3]. As described earlier, antigenic chimeras based on VSV expressing the GP glycoprotein of the haemorrhagic fever agents Ebola, Marburg or Lassa viruses have been constructed. A single IM immunization of cynomolgus macaques with the constructs was well tolerated and induced antibody and cellular immune responses specific to the foreign GP, which protected the animals against a subsequent challenge with a high dose of each respective virus[52,54]. VSV has also been used to express the SARS-CoV S spike glycoprotein from an added gene, and a single IN immunization of mice provided complete protection against an IN challenge with SARS-CoV[85]. As experience with VSV in humans is gained and safety and immunogenicity are established, another use in outbreak control would be to immunize against highly pathogenic H1N1 influenza or pathogenic H5N1 viruses in areas where those viruses are endemic or during pandemic situations. VSV bearing the HA glycoprotein of human influenza A virus was highly immunogenic and protective against an otherwise lethal challenge in mice[86]. In this application, the lack of recombination associated with NNSV provides a major safety advantage thus making prospective immunization feasible.

VSV has also been evaluated as a vaccine vector against a number of prevalent human viruses, including papillomavirus[84,87], hepatitis C virus[88] and HIV[81–83,89]. In particular, the three VSV glycoprotein exchange vectors were each engineered to express the SIV Gag protein and HIV-1 envelope protein from added genes. Three sequential immunizations of rhesus monkeys by the combined IM and oral routes induced a vigorous HIV-specific cytotoxic T lymphocyte (CTL) response and protection against a subsequent challenge with simian–human immunodeficiency virus (SHIV)[81]. This is an important line of experiments, since effective immunization against HIV poses the most daunting challenge to vaccinology.

VSV has also been developed as a vector against common paediatric pathogens, such as HRSV[55] and MV[79,90]. However the use of these vaccines seems to be limited, since it would be preferable to avoid mass inoculation of infants with a virus that they otherwise would rarely encounter and for which immunoprophylaxis is not needed. This view might change if VSV is found to be substantially more effective in humans than live attenuated versions for these paediatric pathogens. However, the use of VSV in a mass vaccination probably would interfere with its subsequent use against other pathogens.

Rabies virus

RV (family Rhabdoviridae, genus *Lyssavirus*) causes deadly neurological diseases in numerous animal species and humans. Several highly attenuated strains of the virus lacking neurovirulence in experimental animals have been developed[46,91–93]. Studies with mice have demonstrated the immunogenicity of RV vectors expressing the HIV envelope or Gag protein, or SARS-CoV S protein, from added genes[94–98]. Since RV infection in humans is rare, most of the population would be susceptible to immunization with an RV-based vector. However, whether attenuated derivatives could be used in humans is unclear, given the high neurovirulence of the parent virus.

Sendai virus

SeV (family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Respirovirus*) is a parainfluenza virus and shares a moderate level of sequence and antigenic relatedness with HPIV1 and virulent to mice. This antigenic relatedness is helpful in developing paediatric vaccine against HPIV1 on the premise that SeV will be attenuated in humans due to a host range restriction[99–101]. SeV has also been evaluated as a vector to express the HRSV G glycoprotein gene. This bivalent vaccine against HPIV1 and HRSV upon IN inoculation of cotton rats induced HRSV-neutralizing antibodies and a high level of protection against a subsequent challenge with HRSV[102]. However, SeV replicated nearly as efficiently as wild-type HPIV1 in African green monkeys and chimpanzees, raising doubts as to whether it will be satisfactorily attenuated in unmodified form for use in seronegative humans[100].

SeV expressing the SIV Gag gene was tested for immunogenicity and protective efficacy against SIV challenge. A group of 8 macaques was primed with a plasmid

DNA expressing SIV Gag and boosted with the SeV-Gag construct. Upon challenge with SIV by the intravenous route, 5 weeks after vaccination; 5 out of 8 vaccinated animals showed undetectable plasma viraemia[103]. The use of a recombinant SeV expressing SIV Gag for immunization of macaques chronically infected with SIV resulted in the induction of an effective Gag-specific CTL response[104]. However, the use of SeV as a vaccine vector for the adult human population may pose problems due to its antigenic relatedness to HPIV1 and presence of antibodies against HPIV1. Indeed, IN immunization of healthy human adult volunteers with SeV resulted in an increase in virus-specific antibodies in only three out of nine individuals, suggesting restricted replication due to previous exposure to HPIV1[101]. Therefore, further studies are needed to evaluate the level of attenuation and immunogenicity of SeV-based vaccines in HPIV1 seronegative and seropositive humans.

Human parainfluenza viruses

The HPIVs belong to family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Respirovirus* (HPIV1 and -3), and genus *Rubulavirus* (HPIV2 and -4). HPIVs cause respiratory illness in infants and children ranging from common cold to pneumonia and is prevalent worldwide. Live attenuated derivatives of HPIV1, -2 and -3 have been developed by reverse genetics for use as IN paediatric vaccines, whereas HPIV4 I is a less serious pathogen[42,105]. Thus, in contrast to VSV, there is extensive experience with infection of humans by HPIVs. HPIVs have a larger genome (15 kb vs 11 kb) than VSV. They are larger and pleomorphic, and encode two or more additional proteins. They replicate at least 10 to 100-fold less efficiently in cell culture. HPIVs infect and cause disease only in the respiratory tract and do not spread (significantly) to other tissues which limits HPIV-based vectors to IN administration. However this is a positive aspect with respect to safety. Upon IN immunization, it induces both strong systemic and local immunity.

The HPIVs are ideally suitable vectors for paediatric pathogens, especially for those that cause respiratory tract infections and use the tract as a portal of entry, e.g. measles virus, HRSV and HMPV. Since vaccines for HPIVs are needed, insertion of a protective antigen gene of another paediatric virus creates a bivalent vaccine, thus simplifying administration. For example, topical immunization of the respiratory tract of rhesus monkeys with an attenuated version of HPIV3 expressing the HA glycoprotein of MV efficiently induced systemic neutralizing antibody responses specific for both MV and HPIV3 (refs 106 and 107). This method is beneficial in two ways: (i) The existing measles vaccine is a live attenuated virus that is administered IM and is very sensitive to neutralization by maternal antibodies thereby necessitating a delay in administration until 12–15 months of age with the result that some individuals become susceptible before the vaccination is received. An HPIV3-vectored vaccine is not neutralized by MV-specific antibodies and can efficiently infect and immunize young infants at an earlier age. (ii) The vaccine eliminates concerns of persistence or partial reversion of the live attenuated measles vaccine virus, facilitating a global effort to eradicate MV. However, it may take some time to replace the established, current live attenuated measles vaccine.

Similarly attenuated chimeric B/HPIV3 expressing the protective F or G protein of HRSV or the F protein of HMPV was shown to be immunogenic against both the vector and the vectored antigen in rodents and nonhuman primates[61–63,108]. In this case, the use of an HPIV-vectored vaccine results in a higher level of antigen expression and avoids the poor growth and instability of HRSV and HMPV. The attenuated B/HPIV3 virus expressing HRSV F is presently in clinical trials as a live IN vaccine against HPIV3 and HRSV. Vectored vaccine based on HPIV3 would be most suitable for immunization within the first six months of life, whereas vectors based on HPIV1 and -2 would be most suited for immunization between six months and one year of age.

The HPIVs also have been evaluated as vectors against highly pathogenic viruses that can infect via the respiratory tract. IN and IT immunization of African green monkeys with an attenuated B/HPIV3 virus expressing the spike (S) protein of the respiratory pathogen SARS-CoV resulted in the induction of a systemic neutralizing antibody response against SARS-CoV and conferred protection against IN and IT challenge with SARS-CoV[109]. HPIV3 was also used to express the GP glycoprotein of Ebola virus to evaluate the efficacy of IN immunization against a virus that causes a severe systemic infection. IN immunization of guinea pigs with this recombinant induced a systemic Ebola virus-specific antibody response and conferred complete protection against a lethal dose of guinea pig-adapted Ebola virus administered intraperitoneally[110]; the construct is now being evaluated in nonhuman primates. These vaccine candidates could be developed for use as paediatric vaccines against SARS-CoV and Ebola virus. However, its efficacy in adults due to natural infection with HPIV3 needs to be studied. Use of nonhuman paramyxoviruses, such as NDV as a vector may solve this problem.

MV is an important member of the *Morbillivirus* genus in the *Paramyxoviridae* family. Attenuated measles vaccine induces a life-long immunity which persists for a period as long as 25 years[111]. In addition, MV shows genetic stability upon multiple *in vitro* passages. The envelope glycoprotein gene of HIV has been cloned into MV vector and this has been shown to elicit high levels of CD8+ and CD4+ cells upon single immunization. In addition, the antibodies generated show virus neutralizing antibody titres[112].

In conclusion, while the results of immune response experiments with NNSV vectored vaccines are highly encouraging, this field is wide open for designing better-priming vectors as well as for studying long term, robust immune responses elicited by combinations of differently primed and boosted vaccines.

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