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Rabies Virus as a Research Tool and Viral Vaccine Vector

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Abstract

Until recently, single-stranded negative sense RNA viruses (ssNSVs) were one of only a few important human viral pathogens, which could not be created from cDNA. The inability to manipulate their genomes hindered their detailed genetic analysis. A key paper from Conzelmann's laboratory in 1994 changed this with the publication of a method to recover rabies virus (RABV) from cDNA. This discovery not only dramatically changed the broader field of ssNSV biology but also opened a whole new avenue for studying RABV pathogenicity, developing novel RABV vaccines as well as a new generation of RABV-based vaccine vectors, and creating research tools important in neuroscience such as neuronal tracing.

I. RABIES VIRUS AS A RESEARCH TOOL

Neurotropic viruses have become an invaluable tool for neuroscientists in their quest to elucidate the architecture of neuronal networks (Callaway, 2008; Taber *et al.*, 2005; Ugolini, 1995, 2010). Compared to conventional methods of neurotracing, viruses offer the advantage of self-amplification, which ensures equally strong labeling of each cell as the virus passes from one neuron to the next. In addition, viral tracers allow a more specific targeting of cell types. Although numerous neurotropic viruses are known, mostly rabies virus (RABV) and several members of the alphaherpesvirus family have been employed for neuronal tracer studies to date. RABV is particularly suited for this purpose because it is transported in a strictly transsynaptical way with very little spread to nonneuronal cells, at least at early times of the infection. In addition, RABV exhibits exceptionally low cytopathogenicity in infected neurons. In both respects, RABV is on par with or even superior to alphaherpesviruses, including the PRV Bharta isolate. RABV does, however, also have disadvantages compared to herpesviruses. Unlike PRV, RABV is pathogenic to humans and therefore necessitates special safety measures. Also, as RABV is an RNA virus with a strictly cytoplasmic replication cycle, it does not permit the use of cell type-specific promoters to limit marker gene expression to certain cell types. Notwithstanding those limitations, RABV has gained increasing popularity as a tool for neuronal circuit analysis. Here, we present an overview of studies that have employed recombinant and nonrecombinant RABV as neuronal tracers highlighting the technical advancements that have been made in recent years.

Early studies of RABV spread in the central nervous system (CNS) have utilized fixed and street viruses in different animal models mainly to study the basis for differences in viral pathogenesis (Coulon *et al.*, 1989; Gillet *et al.*, 1986; Kucera *et al.*, 1985; Lafay *et al.*, 1991; Smart and Charlton, 1992; Tsiang *et al.*, 1983). These studies clearly showed the strong tropism of RABV for neuronal cells. They also revealed that different types of neurons are not

equally infected. Refractiveness to infection is in part explained by the strength of innervation and receptor density at the presynaptic membrane. Marked differences in cell tropism were observed not only between the pathogenic and attenuated mutant strains of CVS but also between pathogenic street and fixed viruses. These data strongly suggest the preferential use of different receptors by different RABV strains. In fact, several receptors have been reported in the literature, but their relative importance is unclear (Coulon *et al.*, 1998; Jacotot *et al.*, 1999; Lentz *et al.*, 1983; Superti *et al.*, 1986; Thoulouze *et al.*, 1998; Tuffereau *et al.*, 1998). Whether the absence of one or several of these receptors from certain types of neurons renders them refractive to infection by RABV remains to be determined.

The general suitability of RABV as a neuronal tracer was demonstrated in several detailed time-course studies of viral spread in rodent and primate models (Astic *et al.*, 1993; Kelly and Strick, 2000; Ugolini, 1995). These studies noted the exceptional low cytotoxicity of RABV, the strict time-dependence of viral spread to higher order neurons, and the limited spread to nonneuronal cells. Kelly and Strick also carried out a rigorous comparison of different RABV strains, which revealed significant differences in the kinetics of viral spread. The pioneering work carried out by Ugolini and Kelly laid the groundwork for a large number of similar studies that mostly employed CVS-11 alone or in combination with conventional tracers to elucidate the architecture of different neural circuits in monkeys, rats, guinea pigs, and cats (Buttner-Ennever *et al.*, 2002; Clower *et al.*, 2005; Graf *et al.*, 2002; Hashimoto *et al.*, 2010; Hoshi *et al.*, 2005; Iwata *et al.*, 2011; Kelly and Strick, 2003, 2004; Lois *et al.*, 2009; Morcuende *et al.*, 2002; Nassi and Callaway, 2006; Prevosto *et al.*, 2009; Rathelot and Strick, 2006; Rice *et al.*, 2009, 2010; Ugolini *et al.*, 2006; Viemari *et al.*, 2004a,b).

Low cytopathogenicity in neurons and “clockwork-like” kinetics of viral spread are the hallmarks that have made RABV such a widely used neurotracer. However, at least one study has highlighted some variability in both parameters (Ruigrok *et al.*, 2008). The reasons for this variability are not clear, but genetic variability of outbred animals and differences in the early innate and T-independent humoral immune responses among individual animals could play a role. Also, it should not be forgotten that RABV has an inherently higher spontaneous mutation rate and genetic variability than DNA viruses. This and the ability to form defective interfering particles if passaged at high multiplicity of infection (MOI) could easily result in phenotypic changes from the original virus isolate depending on the passage history of the particular virus stock in use. It will probably not be possible to completely eliminate the temporal dispersion in viral spread particularly once the virus has spread to higher order neurons, but it is generally advisable to use infectious doses as high as possible to synchronize timing of the infection and, therefore, increase reproducibility.

While attenuated strains of RABV are less suitable for neuronal tracer studies that require spread of RABV from peripheral nerve endings to higher order neurons in the CNS, they have proven quite useful for a different kind of neuronal tracer approach that aims to limit viral spread to monosynaptically connected neurons (Larsen *et al.*, 2007; Wickersham *et al.*, 2007a,b). This was achieved by deleting the glycoprotein (G) gene from a recombinant clone of the SAD-B19 strain. In addition, the virus was engineered to express GFP. Deletion of the RABV G prevents spread of the virus beyond initially infected cells, while insertion of the *GFP* gene ensures strong labeling of cell bodies, dendrites, and axons which obviates the need for histochemical staining methods to outline the infected neurons (Wickersham *et al.*, 2007a). In essence, this method prevents transsynaptic spread and limits labeling to first-order neurons. Wickersham and Wall then took this approach further and devised an ingenious strategy for targeting the initial infection in a cell-specific manner and limiting viral spread to monosynaptically connected neurons. Cell specificity was achieved by pseudotyping RABV with an avian virus glycoprotein and transfecting target neurons with an expression plasmid for the avian receptor protein TVA. To accomplish monosynaptic spread, the target cells also received an expression plasmid encoding the RABV G. The validity of the approach was first demonstrated *ex vivo* in brain slices transfected with the transcomplementing expression plasmids. In a further improvement of cell-specific targeting a recombinant AAV helper virus was used to express RABV G and TVA in a cre-dependent manner, which now opens a whole new avenue for targeting RABV infection to selected cell types by utilizing Cre-expressing transgenic mice (Wall *et al.*, 2010). This strategy holds great promise for labeling monosynaptic circuits in the mouse model. Unfortunately, such genome engineering approaches are only applicable to small mammals, as genome engineering of primates is impractical due to their long gestation period and high reproductive age.

Viral genome engineering was also instrumental in the first study that employed two different RABVs for dual tracing experiments (Ohara, 2009). To impart neuroinvasiveness and a stronger neurotropism onto an attenuated strain, the RABV G gene of CVS was used to replace the cognate G gene of the HEP-Flury strain. Beta-galactosidase (β -Gal) and GFP were then inserted between the nucleoprotein (N) and phosphoprotein (P) genes to generate two different marker viruses, which were utilized to detect neurons that project to two separate regions in the rat brain. Another elegant study utilized the monosynaptically restricted G-deleted viruses developed by the Callaway laboratory to double label premotor spinal interneurons after bilateral injection of two different marker viruses into the quadriceps muscles of newborn mice (Stepien *et al.*, 2010). As in other studies, little neurodegeneration was observed in infected neurons

until 12 days postinfection. Interestingly, the authors also noted that the efficiency of infection declined dramatically in mice older than 10 days.

While the early pioneering neuronal tracer studies with RABV have utilized nonrecombinant fixed laboratory strains, the future of RABV neuronal tracers clearly lies in the use of recombinant clones. Several factors will have to be considered in these endeavors. The four main features that qualify RABV for neuronal tracer studies are its strong neurotropism, low cytopathogenicity, ability to invade the CNS from the periphery, and wide host range. Neurotropism of RABV is largely determined by the glycoprotein and its receptor specificity (Morimoto *et al.*, 2000). The fact that rabies appears to utilize different cellular receptors and the marked differences in viral spread between different viral strains suggest that the specificity of neuronal labeling can be modified to some extent by constructing recombinant RABVs that express different glycoprotein genes. It is to be expected that this kind of approach will receive a significant boost once the three-dimensional structure of RABV G has been elucidated.

The other main feature that renders RABV suitable for neuronal tracer studies is its neuroinvasiveness. This is a multigenic trait and not solely determined by the RABV G (Faber *et al.*, 2004; Shimizu *et al.*, 2007; Yamada *et al.*, 2006). Although the glycoprotein of a highly pathogenic virus can impart neuroinvasiveness onto an attenuated strain (Ohara *et al.*, 2009b; Tan *et al.*, 2007), the multigenic nature of neuroinvasiveness implies that recombinant viruses that utilize different viral backbones will differ markedly in the kinetics and extent of viral spread.

The glycoprotein also plays an important role in the cytopathogenicity of different RABVs. There is plenty of evidence for a direct relationship between RABV G expression level and cytopathogenicity, and it should be noted that the highly pathogenic CVS strains are considerably less cytopathic than attenuated strains (Morimoto *et al.*, 1999). Cytopathogenicity is, for obvious reasons, of little concern in G-deleted viruses but might become an issue if foreign glycoproteins or other potentially proapoptotic proteins are used to replace the RABV glycoprotein.

With respect to host range, it needs to be kept in mind that different animals vary in their susceptibility to different RABV strains. CVS strains have been successfully used in different animals, but they are particularly suited for rodent models, as they have been developed by serial passaging in mouse brain.

RABV is readily amenable to genetic manipulation and insertions of additional transcription units due to the modular nature of its genome. This has been amply exploited for the expression of foreign antigens and marker genes like *GFP* and *beta-galactosidase*. Foreign genes up to 6.5 kb have been successfully inserted into the RABV genome (McGettigan *et al.*, 2003a). The maximum coding capacity of RABV is likely considerably higher as the structure of the viral particle does not appear to pose a

significant constraint on the size of foreign genes. Additional transcription cassettes can be placed at different positions, but they will affect genome replication and viral fitness to varying degrees. Insertions between N and P, for example, inhibit viral replication to a larger extent than insertions between G and L, and RABV does not tolerate the insertion of an additional transcription cassette upstream of the N gene. The positioning of foreign genes will also affect their expression level since there is a gradual decrease in transcription level from the N to the L gene (Schnell *et al.*, 2010). For the purpose of neuronal tracer studies, it is probably best to insert additional genes at more downstream positions where they exhibit less of an inhibiting effect on replication. Studies with recombinant variants of N2c (Wirblich and Schnell, 2011), which is characterized by a lower transcription level than attenuated viral strains, show that marker genes placed between G and L are sufficiently amplified to be readily detectable in neuronal cells (Wirblich and Schnell, unpublished). In any case, the effects of gene insertions on viral replication will have to be assessed in detailed time-course experiments for each virus. This is particularly the case if the viruses are to be employed in dual tracing experiments where the window for successful superinfection of neurons with two different viruses is potentially rather short (Ohara *et al.*, 2009a,b).

The technology for constructing and recovering recombinant RABV is still essentially the same as originally reported by Schnell *et al.* (1994). Notable improvements include the use of hammerhead ribozymes to generate an exact 5'-end of the antigenomic RNA and the use of CMV promoters to drive expression of the antigenomic RNA (Inoue *et al.*, 2003; Le Mercier *et al.*, 2002). Recovery has been performed in different cell lines, but 293T and mouse neuroblastoma cells appear to be particularly well suited for this purpose, as they are easily transfected and more permissive for neurotropic strains than baby hamster kidney (BHK) cells. A number of infectious full-length clones are now available, including two clones of bat RABV strains (Faber *et al.*, 2004; Orbanz and Finke, 2010), several clones of fixed attenuated viruses (Huang *et al.*, 2010; Inoue *et al.*, 2003; Ito *et al.*, 2001; Schnell *et al.*, 1994) and infectious clones of the pathogenic Nishigahara and N2c strains (Wirblich and Schnell, 2011; Yamada *et al.*, 2006). The latter should prove particularly valuable for neuronal tracer studies, as N2c is one of the most neurotropic, neuroinvasive strains available, while being one of the least cytopathic.

II. THE NEED FOR NOVEL VACCINES FOR RABV

World health reports estimate that RABV transmitted by infected animals is the cause of an estimated 55,000 human deaths annually (2005). One of the major goals for the treatment and control of RABV infections has

focused on vaccine development. The first recorded vaccine against RABV was an attenuated form administered by Pasteur (1885). Since then, the field has accumulated more research with a better understanding of the pathogenicity of RABV and the immune biology of potential hosts.

Rabies is a disease affecting humans worldwide but its viral life cycle depends on a reservoir in other mammals (Dietzschold *et al.*, 2005; Schnell *et al.*, 2010). Both domestic and wildlife animals such as dogs, raccoons, skunks, mongoose, foxes, and bats can maintain the RABV cycle (Roseveare *et al.*, 2009; Schnell *et al.*, 2010); therefore, vaccine research has also targeted these groups to indirectly protect humans from this fatal disease.

Currently, whole killed (deactivated) RABV virions are used in both pre- and postexposure treatment of RABV in humans and domestic animals (for review, see McGettigan, 2010). As very safe killed RABV vaccines are available, replication-competent RABV vaccines are not considered for human use (McGettigan, 2010). However, the situation is different for the use in animals, especially for vaccination of wildlife. This is because killed virus administered in bait to wild wandering animals would be ineffective. However, orally administered, live-attenuated forms of RABV such as ERA, SAD-B19, SAG-1, and SAG-2 have been used widely in wildlife (more than 85 million doses) and have been successful at nearly eradicating RABV in Western Europe (Anonymous, 2006; Grimm, 2002). Despite their proven efficacy, replication-competent viruses retain the risk of reverting and causing disease (Faber *et al.*, 2005b). As such, research has focused on improving the safety of these vaccines even more while not compromising on their efficacy (see below). This has led to the development of novel vaccines for rabies in wildlife.

III. MODIFIED REPLICATION-COMPETENT RABV AS RABIES VACCINES FOR WILDLIFE

The success of live RABV vaccine regimens in the eradication of rabies in Western Europe indicates promise for this approach, but residual pathogenicity of these vaccines underlies the need to improve on RABV vectors. The reverse genetics technology of RABV (Schnell *et al.*, 1994) has provided the field with a new tool to manipulate the genome of RABV and therefore improve on both safety and efficacy of RABV vaccines (Dietzschold and Schnell, 2002; Dietzschold *et al.*, 2003; Faber *et al.*, 2002; McGettigan *et al.*, 2003b; Morimoto *et al.*, 2001; Pulmanausahakul *et al.*, 2001; Schnell *et al.*, 1994). These efforts include site-directed mutagenesis of viral genes, insertions of proapoptotic and antiviral genes, expression of inflammatory cytokines and chemokines, as well as gene deletions and

duplication of the glycoprotein gene (Cenna *et al.*, 2009; Etessami *et al.*, 2000; Faber *et al.*, 2002, 2005a,b; Gomme *et al.*, 2010; Ito *et al.*, 2005; Kuang *et al.*, 2009; Morimoto *et al.*, 2005; Pulmanausahakul *et al.*, 2001; Shoji *et al.*, 2004; Wen *et al.*, 2010; Wirblich *et al.*, 2008; Zhao *et al.*, 2009, 2010).

RABV G is most often the target for attempted attenuation due to its known role in viral pathogenicity. RABV G is believed to be the main determinant of viral pathogenicity because it determines the viral tropism (for review, see Dietzschold *et al.*, 2008; Schnell *et al.*, 2010) and due to the fact that G is the primary target for virus-neutralizing antibodies (VNA) that prohibit cell-to-cell spread (Dietzschold *et al.*, 1983; Pulmanausahakul *et al.*, 2008). Despite the general success with conventional modified live viruses used to immunize against RABV, these vaccines were not as immunogenic in skunks and dogs. As such, further customized RABV vaccine constructs expressing G from different fixed and street strains of RABV were constructed and tested. Maximum protection in mice was achieved after vaccination with RABV expressing an identical RABV G (Morimoto *et al.*, 2001). Stemming from this, the findings gave credence for vaccine design custom made for groups of wildlife that do not respond to mainstream vaccines.

Several different factors have been employed to increase the immunogenicity of RABV vaccines. Enhanced apoptosis by overexpression of apoptotic genes has been shown to improve RABV immunogenicity (Faber *et al.*, 2002). RABV modified to overexpress cytochrome *c* showed increased apoptosis in primary neurons with a marked reduction in mortality when administered intranasally. Mouse survival had a direct correlation with the induction of VNA. In fact, compared to the control, RABV expressing cytochrome *c* had an effective dose 20-fold lower than the control, vastly improving the vaccine vector (Pulmanausahakul *et al.*, 2001).

Expression of multiple copies of RABV G has led to enhanced immunogenicity and viral attenuation. Neuronal cell lines and primary neurons infected with RABV expressing multiple G proteins showed evidence of increased apoptosis (Faber *et al.*, 2002). Immunogenicity studies with the same constructs resulted in higher antibody titers against RABV G and RABV N. Insertion of triple RABV G was shown to further attenuate the vaccine construct rendering the virus completely apathogenic when injected directly into the brains of immunocompromised and immune-sufficient mice (Faber *et al.*, 2009). RABV expressing triple G protected 5- and 10-day-old mice from intracranial (i.c.) challenge with a lethal RABV infection that killed 100% unvaccinated mice. Immune analysis showed induction of high VNA titers in these mice 21 days postimmunization. Further, postexposure immunization by RABV expressing triple G administered intracranially prevented lethal rabies encephalitis. Of note, the mechanism for viral attenuation secondary to expression of multiple G is not completely understood, and the presented data for the RABV expressing triple G actually showed that the G protein levels were less in the

triple G construct compared to the control RABV at 24 h. However, there has to be some effect of the G protein expression levels on pathogenicity because the control virus (which encodes three G genes but only one with a functional start codon) was nearly as pathogenic as the construct containing only one G. Of note, if higher G expression levels are indeed responsible for the observed RABV attenuation, codon optimization (Wirblich and Schnell, 2011) might be a better approach to increase G levels. This is based on the fact that a single point mutation deleting the start codon of one or two G would result in virus with similar pathogenicity as the parental virus (Faber *et al.*, 2009). However, caution is advised for strategies that are based solely on changes on the expression level of RABV G protein because changes in codon usage (and the resulting changes in G expression levels) do not suffice to render a pathogenic RABV apathogenic (Wirblich and Schnell, 2011).

Another practical way to improve on live RABV constructs is introduction of specific mutations, including those that abolish neurotropism of RABV (Dietzschold *et al.*, 1983). As mentioned above, RABV G determines the tropism of the virus, albeit the spectrum of specific receptors absolutely necessary for infection remains elusive. Mutation of RABV G at position 333, replacing arginine with glutamic acid, attenuated the virus upon i.c. administration (McGettigan *et al.*, 2003b). In addition, random mutations occurring at position 194 of G exchanging asparagine to lysine increased the pathogenicity of the attenuated RABV construct containing the 333 mutation (Faber *et al.*, 2005b). This random mutation provided another residue that has been an excellent target for attenuation of RABV vaccine constructs (Faber *et al.*, 2005b). An alternative approach tested by Mebatsion *et al.* showed that deletion of the conserved dynein light chain 8 (LC8)-binding motif in the RABV P in combination with the RABV G 333 mutation attenuated RABV 30-fold compared to the 333-mutation only RABV when administered in suckling mice (Mebatsion, 2001). Tan *et al.* (2007) confirmed these findings and showed that the deletion of LC8-binding motif in RABV P affects primary transcription of RABV. From these findings, the issue of custom made vaccines and the ability to use this mutation in conjunction with other mutations in specific strains of virus could be used to improve on the safety profile of these vaccines. Site-directed mutagenesis is an excellent method but based only on a very limited number of mutations (single or dual nucleotides) compared to wild-type RABV G; therefore, revertants are possible.

Another approach has taken advantage of immune components by expression of cytokines, chemokines, or hematopoietic factors to increase immunogenicity and decrease pathogenicity. Tumor necrosis factor- α , macrophage inflammatory protein 1- α , granulocyte macrophage cell stimulating factor, RANTES, and IP-10 have been expressed in RABV vectors and their pathogenicity and immunogenicity followed.

This is a modern and intriguing approach that has shown an increase in immunogenicity by manipulating arms of the innate and adaptive immune systems (Faber *et al.*, 2005a; Zhao *et al.*, 2009, 2010). Nevertheless, it also raises concerns about its application: cytokines/chemokines are species specific and the risk of their use in humans and animals is largely unknown, including toxicity and potential to trigger autoimmunity. RABV overexpressing RANTES or IP-10, for example, has been shown to increase the pathogenicity of RABV due to excessive inflammatory cells in the CNS (Zhao *et al.*, 2009). In addition, the same concern as indicated above for the expression of multiple copies of G exists; the exchange of one nucleotide within the ATG start codon of the inserted gene would revert the recombinant RABV to wild-type RABV.

In summary, many of these advancements have vastly improved the safety profile and immunogenicity of RABV vaccines. In addition, as more information is gathered on the interaction between RABV and the host, better-tailored vaccines can be designed.

IV. RABV-BASED VECTORS AS VACCINES AGAINST OTHER INFECTIOUS DISEASES

Vaccines have had and do have a great impact on human health and continue to be a mainstay in the prevention and treatment of disease. Scientific research has improved our understanding of the interactions between vaccines and the immune system. Of the different types of vaccine strategies available, viral vectors have been manipulated over the years and proved to be efficacious in the induction of both humoral and cellular immune responses.

This review focuses exclusively on RABV-based vaccines vectors. Of note, several important characteristics render RABV a favorable vaccine delivery platform. Virus recovery is conducted by the reverse genetics system using a cDNA copy of the RABV antigenome (Conzelmann and Schnell, 1994). RABV has a relatively simple genome organization that permits easy manipulation of cDNA by traditional cloning techniques. Foreign genes, such as a vaccine antigenic target, can be stably incorporated into the genome. Stability of a foreign, nonessential gene was exemplified by expression of the bacterial chloramphenicol acetyltransferase (*CAT*) gene inserted into the RABV genome of recovered virus after 25 serial passages in cell culture (Mebatsion *et al.*, 1996a,b). Further, the viral vector sustains its replicative capacity after insertion of multiple, large genes. For instance, infectious virus was recovered after insertion of both the *HIV-1 Pr160* (Gag–Pol precursor) and *HIV-1 Env* genes; a 55% increase in genome size over wild type (McGettigan *et al.*, 2003a).

An important advantage of RABV is that, compared to other viral vectors such as adenovirus, preexisting RABV seropositivity is negligible in the general population. Further, RABV genetic and phenotypic similarity to fellow rhabdovirus, vesicular stomatitis virus (VSV), permits the exchange of the ectodomain of the RABV glycoprotein with that of VSV. This recombinant virus effectively boosts preimmunized individuals as part of a vaccine schedule (Foley *et al.*, 2000; Tan *et al.*, 2005).

Last, attenuation of RABV vectors effectively decreases pathogenicity while maintaining antigen-specific immunogenicity (McGettigan *et al.*, 2003b). This is critical, as vector-specific pathogenicity is a primary concern in live virus vectors. In addition to the use of a less pathogenic vaccine strain of RABV (see below), effective molecular attenuation techniques include gene mutation, deletion, insertion, and rearrangement (Gomme *et al.*, 2010; McGettigan *et al.*, 2003b). Further, the RABV life cycle is exclusively cytoplasmic, so recombination or integration is unlikely to occur.

The vaccine strain of RABV used for development of vaccines is derived from the attenuated SAD B19 strain used for oral immunization of foxes in Europe. SAD B19 was highly attenuated by successive passage in different cell types (Conzelmann *et al.*, 1990). Unlike pathogenic RABV strains, SAD B19 has no or limited ability to invade the CNS from a peripheral inoculation site (Conzelmann *et al.*, 1990). This limited invasion of the CNS by SAD B19 positively correlates with the immunogenicity of the virus. Perhaps the inability to sequester itself in the CNS allows it to be rapidly recognized by the immune system. This is beneficial for vaccine development in that it is both relatively safe and highly immunogenic when administered peripherally. However, even SAD B19 is pathogenic when administered directly to the brain via intranasal route (McGettigan *et al.*, 2003b). Thus, additional molecular strategies to further attenuate the virus have been developed (see below).

A. Human immunodeficiency virus-1

For the past 25 years, scientists have sought to develop a vaccine for human immunodeficiency virus-1 (HIV-1), but this goal remains unrealized. Although the correlates of protection are still uncertain, the current belief in the field is that an effective vaccine candidate should induce both arms of immunity: humoral and cellular (Haut and Ertl, 2009). Studies in monkeys immunized with live-attenuated simian immunodeficiency virus (SIV) showed protection from wild-type strains of homologous SIV (Koff *et al.*, 2006). This finding gave credence to the construction of live viral vaccines for the control of HIV. In recent years, several vaccine vector approaches capable of eliciting this type of immune response, such as DNA, Pox, and adenovirus vectors, have been evaluated preclinically

(see Gomme *et al.*, 2010 and references within). RABV is one such vector with an intracellular life cycle and ability to stably express foreign antigens and, as such, is a probable candidate for an HIV vaccine.

An important study conducted in 2000 showed the immunogenicity of recombinant RABV vectors in animals for the first time. In this study, RABV was engineered to express HIV-1 gp160 envelope protein from either laboratory adapted (NL4-3) or dual-tropic isolate HIV-1 (89.6) (Schnell *et al.*, 2000). This recombinant virus was recovered on BHK cells, and the functionality of the foreign envelope protein was confirmed by a fusion assay in human T cell line, Sup-T1. Immunogenicity was evaluated by monitoring envelope-specific antibody responses in mice inoculated by footpad injection and left either unboosted or boosted with a recombinant gp120/gp41 protein. Only boosted mice seroconverted, and the recombinant virus expressing NL4-3 induced HIV-1 neutralizing antibodies. This demonstrated that RABV can efficiently prime B cells for robust humoral responses. A subsequent study showed that priming alone with these vectors could also induce cytotoxic T lymphocytes (CTL), which are cross-reactive to heterologous HIV-1 envelope proteins (McGettigan *et al.*, 2001a). Together these studies clearly demonstrated the potential RABV vectors have in eliciting a balanced humoral and cellular HIV-1-specific immune response.

As evidence in the field continued to promote the importance of cellular immune responses in controlling HIV-1 infection, RABV-based vectors were redesigned to incorporate structural, nonsurface proteins, such as HIV-1 Gag and Pol. Compared to the highly variable envelope protein, Gag is one of the most conserved proteins in HIV-1. In fact, Gag epitopes that are conserved among different HIV-1 clades have been found in individuals infected with HIV-1, suggesting their importance in viral fitness (Durali *et al.*, 1998; McAdam *et al.*, 1998; Rolland *et al.*, 2007). From the perspective of vaccine development, Gag is an attractive vaccine target antigen. McGettigan *et al.* (2001b) generated an RABV expressing HIV-1 Gag. Electron microscopy studies of infected HeLa cells showed that virus-encoded Gag protein manifested into HIV-1-like particles budding from both the plasma membrane and cytoplasmic vacuoles, as previously observed when expressed by other viruses (Karacostas *et al.*, 1989). *In vivo* immunogenicity studies demonstrated that the RABV HIV-1 Gag vector induced Gag-specific CD8⁺ T cells with MHC class I:Gag-specific T cell receptors, CTL activity, and IFN- γ -secretion (McGettigan *et al.*, 2001b).

Having shown that RABV vectors induce both humoral and cellular immune responses *in vivo*, research focus returned to that of vector safety. Besides efficacy, safety is a chief concern in vaccine development. Though the SAD B19 strain used for RABV-based vectors has substantially decreased vector-associated pathogenicity, additional attenuation

techniques were employed. For this approach, RABV vectors expressing HIV-1 Gag were engineered to include either the R333E mutation or a deletion of 43 amino acids of the RABV G cytoplasmic domain, or a combination of both (McGettigan *et al.*, 2003b). The engineered viruses were apathogenic following intracranial challenge in mice compared to the parental strain. Moreover, the Gag-specific cellular immune responses were not decreased by these changes to RABV G (McGettigan *et al.*, 2003b). These immunogenicity studies were encouraging and, as such, the 333 mutation was introduced and tested in a RABV vector-encoding *HIV-1 Pr160* (Gag–Pol precursor) and *HIV-1 Env* genes. Of note, this vector sustained replicative capacity and Gag-specific immunogenicity after a 55% increase in genome size over wild type (McGettigan *et al.*, 2003a).

Vaccine research has also extended to the identification of adjuvants that can further improve or change the phenotype of the immune response to one that can deal with the target pathogen. In HIV vaccine research, live viral vectors have been manipulated to express inflammatory cytokines at the time of viral replication as a way to enhance the immune response. RABV vaccine studies have included IL-2, IL-4, and IFN- β as vaccine adjuvants (McGettigan *et al.*, 2006). Both IL-4 and IL-2 can induce a Th2 response; in addition, IL-2 can stimulate proliferation of both T cells and B cells and, in general, stimulates a Th1 response. IFN- β , however, is an anti-inflammatory cytokine that is thought to skew the response to Th1 and may influence the expansion of CD8 T cells (Faul *et al.*, 2008). RABV vaccine vector coexpressing HIV-1 Gag or Env and either murine IL-2 or IL-4 when tested in mice were highly attenuated. IL-4 expression reduced the cellular immune response to both Gag and Env, but did not significantly improve the humoral response. IL-2, however, did not reduce the cellular immune response but significantly improved the anti-Env humoral immune responses (McGettigan *et al.*, 2006). More recently, it was shown that RABV expressing HIV-1 Gag and IFN- β was less pathogenic than controls. IFN- β expression resulted in 100-fold lower viral replication *in vivo* compared to controls. Even with lower viral replication, IFN- β expression seemed to increase the percentage of activated CD8+ T cells during the primary response (Faul *et al.*, 2008, 2009a,b).

A more novel approach in vaccine design takes advantage of the antigen presentation capacity of dendritic cells (DCs). Earlier studies had shown that RABV could infect and mature human DCs with expression of proinflammatory cytokines via activation of the NF- κ B pathway (Foley *et al.*, 2002; Li *et al.*, 2008). Immunization of mice with RABV-infected DCs stimulated cellular and humoral immune responses in mice. Further, it was shown that RABV-infected DCs, in contrast to inoculations with RABV-based vectors (McKenna *et al.*, 2007; Tan *et al.*, 2005), could be used in a homologous prime-boost approach leading to increased Gag-specific cellular immune responses (Wanjalla *et al.*, 2010).

The final test of the effectiveness of such novel vaccine constructs requires a model in which protection from an AIDS-like infection could be monitored. For this approach, RABV backbone plasmids containing the R333E mutation or whose RABV G ectodomain was replaced with that of VSV were used to construct recombinant RABV expressing SIV Env and Gag or Gag–Pol proteins (Faul *et al.*, 2009a,b; McKenna *et al.*, 2007). These constructs allowed for a heterologous prime-boost vaccine regimen, an approach previously shown to increase the cellular response by about 4.5- to 5.5-fold higher than a prime-only approach (Tan *et al.*, 2005). In two independent monkey studies, rhesus macaques were primed and boosted with RABV constructs (SIV_{mac239}Gag, SIV_{SHIV89.6}Env) (McKenna *et al.*, 2007) or (SIV_{mac239}Gag–Pol and SIV_{mac239}Env) (Faul *et al.*, 2009a,b; McKenna *et al.*, 2007) and challenged with either SHIV-89.6 or the highly pathogenic SIV_{mac251}, respectively (Faul *et al.*, 2009a,b; McKenna *et al.*, 2007). Compared to vector controls, the vaccine groups seroconverted with induction of neutralizing antibodies and CD8+ T cells and the vaccines were protected from an AIDS-like disease. Specifically, the protection against the highly pathogenic SIV_{mac251} strain combined with the lack of any vector-induced pathogenicity indicates great promise for RABV-based vectors as HIV-1 vaccines.

B. Hepatitis C virus

Hepatitis C virus (HCV) affects 120–170 million people worldwide and is the most common cause for liver disease requiring transplantation in adults (Tellinghuisen *et al.*, 2007). Most of these cases are chronically infected and 20% develop severe liver disease, including cirrhosis and hepatocellular carcinoma. A major obstacle in the treatment and control of HCV lies in the error-prone replicative machinery, which increases the genetic variability of HCV within an individual, resulting in quasispecies. Antiviral therapies have helped manage the disease; however, they have several disadvantages, including a low rate of response and toxicity. Similar to HIV, the correlates of protection against HCV are not well understood. There is, however, an appreciation of the need to induce neutralizing antibodies (nAb) and CD4+ and CD8+ T cells to multiple HCV antigens. However, for the humoral response, it remains largely unknown which epitopes are important for nAb to target leading to sterilizing immunity (Tellinghuisen *et al.*, 2007; von Hahn *et al.*, 2007).

RABV vaccine constructs against HCV were constructed and tested in mice and shown to induce both a humoral and cellular response. A recombinant RABV construct expressing chimeric E2 containing the CD4 transmembrane and RABV G cytoplasmic domain was shown to allow cell surface expression of E2. Moreover, the chimeric E2 was incorporated into RABV virions. For the humoral studies, these killed

RABV particles were administered to mice in a prime-boost regimen and shown to induce detectable antibodies against HCV-E2 after boost as measured by ELISA. Mice primed with live RABV construct cloned to express both HCV envelope proteins (E1 and E2), which upon expression interact in a noncovalent heterodimeric complex retained in the ER, were shown to mount a cellular immune response capable of lysing cells pulsed with an HCV-specific peptide (Siler *et al.*, 2002).

C. Severe acute respiratory syndrome

The global impact of emergent infectious diseases has become a topic of interest to vaccine developers and researchers (Faber *et al.*, 2005c). Severe acute respiratory syndrome (SARS) is one such disease whose causative agent is a coronavirus named SARS-CoV. From a vaccine standpoint, SARS-CoV may be an important pathogen despite the fast decline in SARS reported cases due to existence of animal reservoirs such as raccoon dogs and the Chinese ferret badger. It is thought that antibodies against SARS-CoV spike (S) protein are neutralizing and therefore a potential target for vaccines (see Faber *et al.*, 2005c and references within). For the vaccine studies, live-attenuated recombinant RABV containing the R333E mutation expressing SARS-CoV S protein was shown to induce high neutralizing antibodies in mice (Faber *et al.*, 2005c). The translation of these studies to wildlife reservoirs using live RABV may be possible based on the efficacy of live RABV vaccines in the eradication of RABV reservoirs in wildlife.

V. SAFETY: GENERATING SAFER RABV VACCINES AND VECTORS FOR USE IN HUMANS

Safety is a major concern in the development of vaccines, especially where live replication-competent vaccines are considered due to the likelihood of revertants or residual vector pathogenicity. There are several methods that have been used to improve the safety of RABV vaccines while maintaining their immunogenicity (see Wirblich and Schnell, 2011 and references within).

VI. REPLICATION-DEFICIENT OR SINGLE-CYCLE RABV

Despite the great improvements to replication-competent RABV, there are still potential safety concerns associated with the use of live viruses for widespread immunization of humans. Even highly attenuated RABV can be lethal following intracranial inoculation, at least in the

immunodeficient host. In order to address such safety concerns, viruses have been further attenuated by complete genome deletion of an essential gene(s) that renders the vector unable to complete its viral life cycle. This attenuation strategy has been used on many viral backbones: adenovirus (Ad), vaccinia virus (VV), canarypox virus (CPV), herpes simplex virus (HSV), VSV, and RABV (Bozac *et al.*, 2006; Cenna *et al.*, 2008, 2009; Coulibaly *et al.*, 2005; Gomme *et al.*, 2010; Peng *et al.*, 2005; Publicover *et al.*, 2005; Russell *et al.*, 2007).

VII. POTENTIAL NOVEL HUMAN RABIES VACCINES BASED ON REPLICATION-DEFICIENT RABV

Deleting RABV genes *P* or *M* generated replication-deficient RABV (Cenna *et al.*, 2008, 2009), so termed because they lack viral components that are required for a complete viral life cycle. RABV *P* is a phosphoprotein cofactor to the viral RNA polymerase, and its deletion severely hinders intracellular replication (Cenna *et al.*, 2009). However, due to the role of *M* in assembly and budding, *M*-deleted virus is structurally impaired forming mainly cell-associated rod-shaped particles instead of the typical bullet-shaped particles. *M* also has a role in regulating the balance between transcription and replication, and as such, *M*-deleted virus may be impaired at the level of viral replication. These effects on virion formation reduce infectious titers as much as 500,000-fold (Mebatsion *et al.*, 1999). In addition, deletion of *P* and *M* may have additional, unknown effects on viral fitness that contribute to their immunogenicity. McGettigan *et al.* generated a *P*-deleted replication-deficient RABV for potential use in pre- or postexposure vaccine regimens for prevention of human rabies infections (Cenna *et al.*, 2008). A likely candidate would induce IgG2a antibodies for their potent antiviral effector functions. Current regimens use inactivated RABV particles that require several doses to be effective. In a head-to-head comparison of live *P*-deleted RABV and inactivated RABV, they found mice immunized with *P*-deleted had 10-fold greater survival and a proportionately greater IgG2a response after lethal challenge than mice immunized with inactivated virus (Cenna *et al.*, 2008). A later study showed *M*-deleted RABV is even more potent than *P*-deleted in mice, inducing greater IgG and VNA titers and protecting 100% of lethally challenged mice even at immunization titers as low as 10^3 foci-forming units (Cenna *et al.*, 2009). Notably, neither *P*- or *M*-deleted virus induced clinical signs of rabies, nor were they found in the brain or spinal cord following intramuscular injection of immunocompromised RAG2 knockout mice (Cenna *et al.*, 2008, 2009).

Replication impaired RABV vectors lacking RABV *M*, but expressing RABV *G* proteins, were shown to be safe and immunogenic in both mice

and nonhuman primates. In fact, the M-deleted RABV had fourfold higher VNA titers 10 days after inoculation compared to a commercially available killed RABV vaccine. One hundred and eighty days later, the monkeys that received M-deleted RABV maintained higher VNA titers with antibodies shown to have a higher avidity than the killed HDCV (Cenna *et al.*, 2009). These data strongly demonstrate the potential for replication-deficient RABV to replace current pre- and postexposure RABV vaccines.

VIII. REPLICATION-DEFICIENT/SINGLE-CYCLE RABV AS VACCINE VECTOR

The G-deleted RABVs are termed “single-cycle” or “spread-deficient” because they lack viral components that are required for viral spread or infectivity. RABV G has a critical role in the attachment and entry of the virus into host cells, which makes G one of the most important determinants of viral pathogenicity (Dietzschold *et al.*, 1983, 2008; Pulmanausahakul *et al.*, 2008). Particles lacking G undergo one complete cycle of intracellular replication and produce progeny that are unable to spread (Mebatsion *et al.*, 1996a,b), as shown by infection of single neurons following intracranial inoculation of a G-deleted RABV (Etessami *et al.*, 2000; Wickersham *et al.*, 2007a). Virus particles lacking G are still capable of budding, although at a 30-fold lower efficiency (Mebatsion *et al.*, 1996a,b). These virions, however, are incapable of attachment and entry into a secondary host cell. Gomme *et al.* (2010) generated a G-deleted RABV-encoding HIV-1 Gag for development of an HIV-1 vaccine. Compared to the replication-competent parental virus, G-deleted RABV generated lower RABV-specific antibody responses but equivalent HIV-1 Gag-specific CD8⁺ T cell responses. Moreover, these responses were enhanced by a heterologous boost with a G-deleted RABV complemented with VSV glycoprotein. This shows that single-cycle RABV is a promising platform for safe, live viral vaccines and further studies will analyze if similar responses can be induced in nonhuman primates.

IX. KILLED RABV–RABV PROTEINS AS CARRIERS OF FOREIGN ANTIGENS

The simplicity and plasticity of the RABV genome is one of the many advantages as a vaccine vector. The genome is amenable to inclusion of whole foreign antigens as RABV protein chimeras. Both RABV G and N proteins have been tested as carriers of foreign antigens and proven to be immunogenic when applied as live or killed vaccines (Koser *et al.*, 2004;

Langley *et al.*, 2010; Smith *et al.*, 2006). There are several possible advantages to using RABV proteins as carriers: (i) RABV G as a carrier expressing a foreign envelope protein combined with the RABV-CD allows insertion of the foreign gene into the RABV virion (Mebatsion and Conzelmann, 1996; Mebatsion *et al.*, 1997; Smith *et al.*, 2006); (ii) in this setting, immunogenic epitopes may be presented in an organized structure which may increase their immunogenicity; and (iii) depending on the carrier (RABV N) and foreign antigen, forming the chimera could stabilize the antigen allowing longer expression and having an impact on the immunogenicity (Koser *et al.*, 2004).

The viability and immunogenicity of RABV constructs containing G or N fusion proteins have been extensively studied (Koser *et al.*, 2004; Siler *et al.*, 2002; Smith *et al.*, 2006). RNPs obtained from recombinant RABV constructs with GFP fused to RABV N were used to immunize mice in a prime-boost regimen. In comparison to the controls that were immunized with GFP, mice immunized with RNP had significantly higher antibodies against GFP, which seemed dependent on CD4⁺ T cell response, because no GFP-specific antibodies were detected after depletion of CD4⁺ T cells (Koser *et al.*, 2004).

RABV G protein as a carrier for foreign antigen has also been shown to be efficacious in inducing immune responses in mice and monkeys (McKenna *et al.*, 2003, 2004; Smith *et al.*, 2006). As a vaccine strategy, it is employed where a substantial humoral response is known to be a good correlate of protection. RABV G chimeras carrying the *Bacillus anthracis* protective antigen (PA) had sufficient incorporation of PA in the virions. Both live and killed viral particles induced anti-PA antibodies in mice that were detectable postprime and increased after boost (Smith *et al.*, 2006).

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