Journal of NeuroVirology, 11: 76–81, 2005

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280590900436

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Taylor & Francis Group

Mini-Review—The Rabies Virus

The application of reverse genetics technology in the study of rabies virus (RV) pathogenesis and for the development of novel RV vaccines

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Rabies is a central nervous system (CNS) disease that is almost invariably fatal. Neurotropism, neuroinvasiveness, and transsynaptic spread are the main features that determine the pathogenesis of rabies. Recent advances in rabies virus (RV) research, which made direct genetic manipulations of the RV genome possible, greatly improved the understanding of the role of different viral and host cell factors in the pathogenesis of rabies. Here the authors discuss molecular mechanisms associated with rabies RV infection and its spread to the CNS. *Journal of NeuroVirology* (2005) **11,** 76–81.

Keywords: neuroinvasiveness; neurotropism; pathogenicity; rabies virus; vaccine

Introduction

Rabies is the 10th most common lethal infectious disease, causing approximately 60,000 annual deaths worldwide (Martinez, 2000). The causative agents of rabies belong to the Rhabdoviridae family, Lyssavirus genus, from which rabies virus (RV) is the prototype. RV has a relatively simple modular genome organization and encodes a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a single external surface glycoprotein (G), and a RNA-dependent RNA polymerase (L). Fixed and street RV strains associated with different host species can differ significantly from each other in their ability to invade the central nervous system (CNS) from a peripheral site. For example, whereas virus strains associated with canines are usually transmitted by severe bites that deeply inoculate large amounts of virus into subcutaneous and muscle tissue, bat-associated rabies viruses are likely delivered in comparatively negligible amounts, because bats, especially silver-haired bats (*Lasionycteris noctivigans*), probably do not have the biting power to penetrate deeply human skin (Freeman, 1981). It has been suggested that an epizootic in the silver-haired bat population might reflect adaptation of the virus to this species by either increasing its neuroinvasiveness or altering its tissue tropism, enabling transmission of disease by only a low number of virus particles (Morimoto *et al*, 1996). Such changes of the pathogenicity phenotype, in particular increased neuroinvasiveness, are likely to have public health implications. In fact, silver-haired bat rabies virus (SHBRV) has been identified as the etiological agent of 16 of the 26 indigenous human rabies cases that occurred from 1994 to the present in the United States. None of these 16 cases has been related to any known exposure, further supporting the hypothesis that SHBRV has pathogenic properties distinct from those of the more common canine-associated RVs. Our laboratories are currently working towards the construction of an infectious clone of SHBRV, which will be extremely valuable for further analysis of RV pathogenesis.

The mechanism by which RV infection of neurons causes neurological disease and death in humans and other animals has puzzled investigators for more than a century. It has long been known that human rabies patients show few gross or histopathological lesions that could explain the lethality of rabies (Murphy, 1977). A pathogenic mechanism that might contribute to the profound CNS dysfunction

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This work was supported in part by research grants AI49153 and AI44340 to M.J.S and AI45097 to B.D.

Received 24 March 2004; revised 28 May 2004; accepted 14 June 2004.

characteristic of rabies could be the impairment of neuronal functions as a consequence of virus replication (Tsiang, 1982). Indeed, most clinical signs of rabies are related to abnormal neuronal functions such as altered neurotransmission (Charlton, 1994).

Identification of cellular and viral factors that determine the neurotropism of RV

Neurotropism is defined as the capacity of a virus to infect neurons. Although the molecular basis for the neurotropism of rabies virus has not yet been fully elucidated, several studies identified the glycoprotein (G) as the foremost important element in RV-induced neurological disease(Kawai, 1994). It has been proposed that the neurotropism of RV might be determined by the ability of the RV G to bind to specific neuronal cell surface receptors. For example, experimental evidence has been provided implicating the nicotinic acetylcholine receptor (nAChR) as the attachment site for RV (Lentz *et al*, 1982). However, the observation that RV can also infect neurons *in vivo* that do not express nAChRs indicates that other molecules must act as viral receptors (Tuffereau *et al*, 1998).

Thus, as with many other neurotropic viruses, the identity of an RV receptor remains controversial. A more detailed overview by M. Lafon about RV receptors is published in this issue of *Journal of NeuroVirology*. On the other hand, determinants of RV G have been identified that play a potential role in the interaction of RV with neuronal receptors. Three conformational regions, designated as antigenic site I, II, and III, have been identified in the ectodomain (ED) of RVG. These operationally defined sites contain the epitopes for neutralizing monoclonal antibodies (mAbs) (Lafon *et al*, 1983). We along with other colleagues have demonstrated that the pathogenicity of several fixed rabies virus strains (i.e.,ERA, HEP, CVS) correlates with the presence of a determinant located in antigenic site III of the G protein (Dietzschold *et al*, 1983). Of note, substitution of the arginine (Arg) residue of position 333 in antigenic site III with either glutamine (Gln) or isoleucine (Ile) markedly reduced the pathogenicity of several RV strains (e.g., ERA, HEP) (Dietzschold *et al*, 1983; Seif *et al*, 1985). One possible explanation for the loss of pathogenicity of Arg333 mutants of RV G is an alteration in the fusion activity of RV G. It has been shown that RV G–mediated pH-independent membrane fusion in neuronal cell lines is contingent with the presence of wild-type Arg333 (Morimoto *et al*, 1992). Several investigators have confirmed that nonpathogenic RV strains with a Gln333 RV G have difficulty entering peripheral neurons and stunted spreading shown both *in vitro* and *in vivo* (Coulon *et al*, 1989, 1998; Dietzschold *et al*, 1985; Morimoto *et al*, 2000). More recently, it was observed that RV G determines the pattern of infection in the brain (Yan *et al*, 2002).

The significance of these findings for the natural history of rabies, however, is not clear because the Arg333 mutants of RV G were obtained from tissue culture-adapted strains (e.g., ERA, CVS-11), which despite having an intact antigenic site 3, showed neuroinvasiveness indices (see below) 1,000 to 10,000 times lower than that of most street RV strains, e.g., dog- and bat-associated RV strains (Morimoto *et al*, 2000). Furthermore, phenotypic analyses of recombinant RVs in which the G gene of a nonneuroinvasive and less neurotropic strain was replaced with that obtained from highly neuroinvasive and neurotropic strains revealed that the pathogenicity of the recombinant viruses was, markedly lower than that of the wild-type viruses. Even more puzzling, a Arg333 to Glu mutation in the G protein of the highly (N2c) neurotropic RV strain CVS-N2c did not result in a decrease in pathogenicity (Morimoto *et al*, 2001). However, two lyssaviruses of the phylogroup 2 (Mokola and Lagos bat), which contain the Arg333 Glu mutation in their G protein, displayed a reduced pathogenicity and did not kill mice after intramuscular (i.m.) inoculation. Of note, none of these viruses with Arg333 were isolated and therefore factors other than this mutation might be responsible for the reduced pathogenicity (Badrane *et al*, 2001). Although these finding indicate that RV pathogenicity depends on multiple factors, including several host cell factors and different elements of the RV genome, they also demonstrate the power of reverse genetics technology in delineating pathogenic mechanism. Using this technology we were able to transfer the neurotropism of a highly neurotropic to a less neurotropic RV strain by exchanging the G protein of less neurotropic strain RV strain with that of the pathogenic strain. The failure to completely restore the pathogenicity of an attenuated RV strain by exchanging its G with that of a pathogenic RV indicates that other factors contribute to pathogenicity. These factors can now be studied by introducing specific changes into the genome of well-characterized RV strains.

Mechanisms determining the neuroinvasiveness of RV

Neuroinvasiveness is defined as the capacity of a virus to invade the central nervous system (CNS) from a peripheral site (Figure 1). Viruses, including RV, are obligate intracellular parasites that have devised a panel of techniques that permit them to utilize host cell machinery for their benefit (Poranen *et al*, 2002). Both entry and the migration to the proper host cell compartment are necessary for efficient viral replication, assembly, and budding (Sodeik, 2000). Thus it is not surprising that a wide variety of viruses, especially neurotropic viruses, seem to have acquired common schemes that enable them

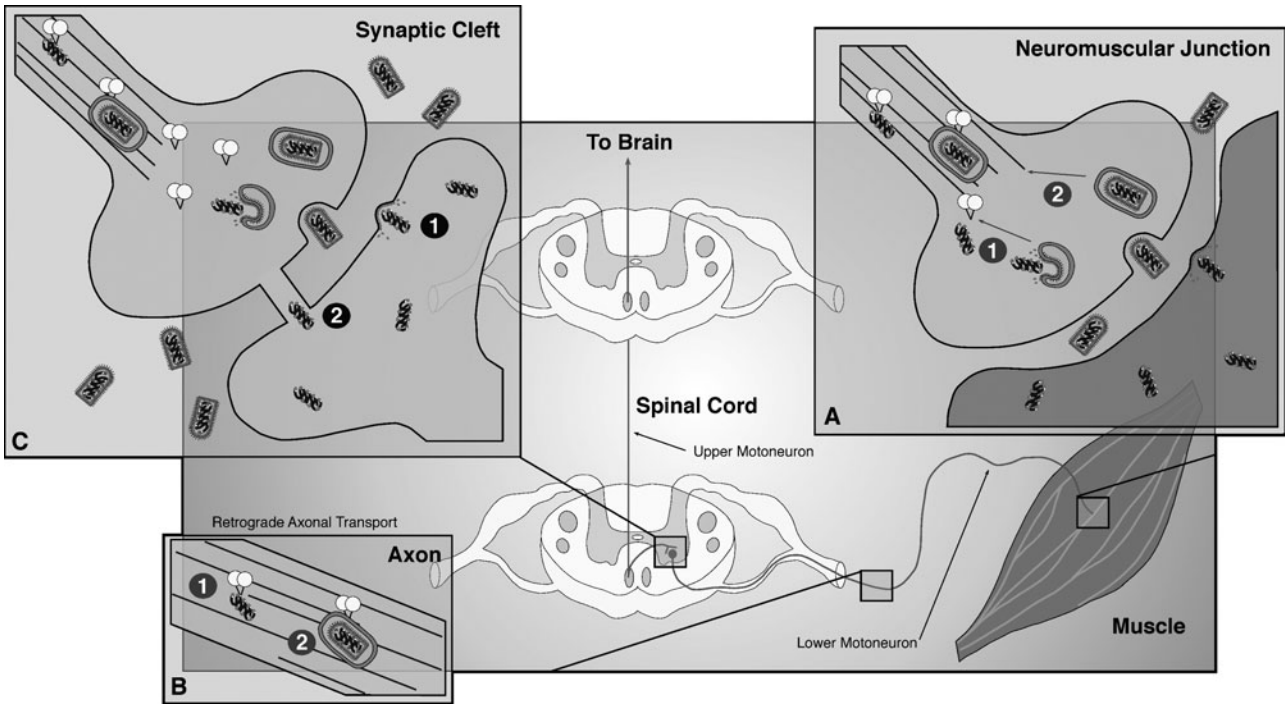

Figure 1 Migration of RV from the periphery to the CNS—proposed models. **(A)** Mature RV virions can target neurons directly or after local propagation in adjacent muscle cells at neuromuscular junctions through receptor-mediated endocytosis and are internalized in endosomes. RV could initiate retrograde axonal transport by following models: Model 1: (**A**, 1) The ribonucleoprotein, RNP, is first released into the cytoplasm via pH-dependent fusion of the viral envelope and the endosomal membrane, interacting with the dynein motor protein via the LC8 binding motif on its phosphoprotein and finally transported as an RNP (**B**, 1) along the axons. Model 2: An alternate pathway may allow an intact endosome-containing RV (**A**, 2) to attach to the dynein motor protein in an unknown mechanism and be shuttled towards the neuronal soma as an endosome (**B**, 2). **(C)** Transsynaptic spread could occur either by (1) budding off, acquiring a viral envelope and reinfecting another neuron (model 1) or through an unknown means in which an RNP (2) can directly enter an adjacent neuron without exiting into the extracellular environment (model 2).

to *attach* to the cellular microtubule network and employ it as a means of movement (Bearer *et al*, 2000; Martinez-Moreno *et al*, 2003; Smith *et al*, 2001; Sodeik *et al*, 1997; Suikkanen *et al*, 2002). For instance, non-neurotropic viruses posses elements that interact with the cytoskeleton for efficient navigation in the host cell as observed in vaccinia virus (VV) (Geada *et al*, 2001; Hollinshead *et al*, 2001; Ploubidou *et al*, 2000; Rietdorf *et al*, 2001; Ward and Moss, 2001), adenovirus (AV) (Leopold *et al*, 2000; Suomalainen *et al*, 1999), retrovirus (Martinez-Moreno *et al*, 2003; Petit *et al*, 2003), and canine parvovirus (CPV) (Suikkanen *et al*, 2002). Furthermore, the microtubule network (Sodeik *et al*, 1997) has been shown to be critical in herpes simplex virus (HSV) axonal migration (Dohner 2002; Smith 2001) via its tegument protein (Bearer *et al*, 2000), whereas the poliovirus (PV) receptor CD155 and its interaction with a dynein light chain, Tctex-1, may characterize the neuroinvasive nature of the virus (Mueller *et al*, 2002; Ohka *et al*, 1998). Hence, the host cell microtubule network along with its accessory proteins likely plays a crucial function in a virus life cycle (Sodeik, 2000) (Figure 1).

Unlike HSV and PV, RV causes a fatal neurological disease with the highest case-fatality ratio among infectious diseases (Hemachudha *et al*, 2002; Jackson, 2002, 2003; Rupprecht *et al*, 2002). The pathogenicity of a particular RV strain correlates with its neuroinvasiveness as a highly pathogenic RV is always highly neuroinvasive. As listed above, the rabies virus G protein is a major contributor to the pathogenicity of the virus (Dietzschold *et al*, 1983, 1985; Morimoto *et al*, 1999, 2000; Seif *et al*, 1985). Several G-associated pathogenic mechanisms have been identified: (i) G must interact effectively with cell surface molecules that can mediate rapid virus uptake (Lentz *et al*, 1987); (ii) G must interact optimally with the RNA-N-P-M complex for efficient virus budding (Mebatsion *et al*, 1997, 1999; Morimoto *et al*, 2000); and (iii) expression levels of G must be controlled to prevent functional impairment of the infected neuron (Morimoto *et al*, 1999). As discussed above, the rapid uptake of RV by axon terminals is a prerequisite for the initial infection of neurons. However, rapid virus entry into neurons alone is not sufficient for an effective neuronal virus spread within the CNS. Based on the observation that induction of apoptosis in primary neurons depends on the expression level of RV G and that the pathogenicity of a particular RV inversely correlates with its ability to induce apoptosis, Morimoto *et al*

concluded that the structural integrity of the neuronal network must be preserved to facilitate the neuronal spread to and within the CNS (Morimoto *et al*, 1999). The conclusion that overexpression of RV G causes apoptosis was confirmed using a recombinant RV that contained two RV G genes instead of one. This recombinant virus overexpressed RV G and was more potent in inducing apoptosis than a recombinant RV having only a single G gene (Faber *et al*, 2002).

Of note, RVs expressing higher RV G levels are more immunogenic in the infected host and therefore the virus is probably cleared before it reaches the CNS. By contrast, RV strains expressing RV G at a lower level than attenuated vaccine strains do not induce apoptosis and fail to trigger a protective immune response.

Beside the importance of RV G for neuroinvasiveness, current progress in RV research has identified RV P as an alternate contributor to the axonal transport of RV within neurons (Figure 1A). Using the yeast two-hybrid system, two groups initially found that the cytoplasmic dynein light chain, LC8, interacted strongly with RV P (Jacob *et al*, 2000; Raux *et al*, 2000) via a conserved (K/R)*X*TQT motif (Lo *et al*, 2001). Shortly afterwards, coimmunoprecipitation assays revealed the LC8 binding sequences in RV P, and mutagenesis experiments revealed that RV P–LC8 interaction is not required for transcription (Poisson *et al*, 2001). In an attempt to define the functional significance of the RV P–LC8 association, one recent study demonstrated that the deletion of the LC8 binding from an already attenuated RV vaccine strain, SAD-D29, decreased the LD_{50} by 30-fold in 2-day-old suckling mice. Nonetheless, the observation that the attenuated RV still kills at doses higher than 10^2 foci forming units (ffu) when introduced peripherally indicates that the LC8 binding site may not be the sole factor in the retrograde axoplasmic flow of RV (Mebatsion, 2001). However, the use of suckling mice implies that attenuation of the RV not containing the LC8 binding motif was not due to changes in immune response strongly suggesting a modified transport to the CNS (Figure 1A).

As described above, several characteristics of RV G are important. The direct involvement of RV G in axonal transport was recently suggested by Mazarakis *et al*, who showed that pseudotyping of a lentivirus, equine infectious anemia virus (EIAV), with RVG alone conferred it the ability to travel from the gastrocnemius muscle to the spinal cord (Figure 1C, 2). Conversely, the same vector pseudotyped with vesicular stomatitis virus glycoprotein (VSV G) did not get transported to the spinal cord (Mazarakis *et al*, 2001). In contrast, results from Ceccaldi's group clearly indicate that a ΔG RV *trans*-complemented with RVG was restricted to the initially infected neuron after sterotaxic inoculation into the rat striatum and the ΔG-RV was unable to infect any secondary cell. As a result, there is contention over the identity of the driving force responsible for the retrograde transport of RV, whether it is the LC8 binding site on the RVP, the RVG or a combination of both (Figure 1C, 1 or 2).

Transsynaptic spread of RV

Another key factor in rabies pathogenesis is the ability of RV to pass through synapses, thereby utilizing the neuronal network to propagate within the CNS. Based on the failure to detect mature virions in synapses, it has been hypothesized that RV G might be dispensable for the transsynaptic propagation of RV (Gosztonyi, 1994) (Figure 1C, 2). However, the reduced spread of RV antigenic site 3 mutants within the nervous system indicates that a functionally intact G protein is absolutely essential for the axonal/transsynaptic spread of a lethal rabies virus infection in adult animals (Figure 1C, 1). In addition, Etessami *et al* showed in a rodent model system that a G-deleted RV (ΔG), which has been *trans*-complemented with RVG, is able to infect neuronal cells but is unable to spread to secondary neurons (Etessami *et al*, 2000). These findings demonstrate how closely neurotropism and neuroinvasiveness of pathogenic RVs are interconnected. Whereas the ability to spread from a peripheral site to the CNS is certainly not solely the function of RV G, a G protein from a highly neurotropic RV is probably also advantageous for the spread from a primary to a secondary neuron and therefore also important for neuroinvasiveness.

Outlook

It has become evident that the application of reverse genetics allows the construction of modified RVs (Schnell *et al*, 1994) that are highly attenuated and (McGettigan *et al*, 2003), therefore, have a great potential as live vaccines for wildlife or as vaccine vehicles for other human infectious diseases such as the human immunodeficiency virus (HIV) or hepatitis C (HCV) (Dietzschold *et al*, 2003; Faber *et al*, 2002; Foley *et al*, 2000, 2002; McGettigan *et al*, 2001a, 2001b, 2001c, 2003; Morimoto *et al*, 2000, 2001). As indicated above, molecular biology and the analysis of RV pathogenicity made great progress during the last 5 years and several parameters of RV pathogenicity are now better defined (Morimoto *et al*, 2000, 2001). The coming years will show whether the combination of several attenuation markers in a single RV will result in safer and more effective RV vectors. In addition, our laboratories made progress on the development of an infectious cDNA clone of SHBRV and currently have a highly attenuated and a highly pathogenic RV strain available for further pathogenicity studies. Targeted exchange of genes and regulatory elements between these two RV strains will open a whole new field of RV pathogenicity research.

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