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Adaptation of West Nile virus replicons to cells in culture and use of replicon-bearing cells to probe antiviral action

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Abstract

Flaviviruses are emerging threats to public health worldwide. Recently, one flavivirus, West Nile virus (WNV), has caused the largest epidemic of viral encephalitis in US history. Like other flaviviruses, WNV is thought to cause a persistent infection in insect cells, but an acute cytopathic infection of mammalian cells. To study adaptation of WNV to persistently replicate in cell culture and generate a system capable of detecting antiviral compounds in the absence of live virus, we generated subgenomic replicons of WNV and adapted these to persistently replicate in mammalian cells. Here we report that adaptation of these replicons to cell culture results in a reduction of genome copy number, and demonstrate that hamster, monkey, and human cells that stably carry the replicons can be used as surrogates to detect the activity of anti-WNV compounds. Additionally, we have used these cells to investigate the interaction of WNV genomes with interferon (IFN). These studies demonstrated that IFN can cure cells of replicons and that replicon-bearing cells display lower responses to IFN than their IFN-cured derivatives.

Keywords: West Nile virus; *Flavivirus*; Replicon; Antivirals; Ribavirin; Interferon; Persistent infection

Introduction

West Nile virus (WNV) is a member of the *Flavivirus* genus of the family *Flaviviridae*. Members of this genus include several important arthropod-borne pathogens, including dengue virus (DV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), and yellow fever virus (YFV). WN encephalitis, a rare outcome of infection with WNV, was observed periodically in Asia, Africa, the Middle East, and Europe in the years following the original isolation of the virus in 1937 from a woman suffering from a mild fever (Smithburn et al., 1940). Following its introduction into the US in 1999, WNV was responsible for approximately 150 human cases and 18 fatalities through 2001 (Campbell et al., 2002). However, in 2002, the US Centers for Disease Control and Prevention reported over 4000 laboratory-confirmed human infections with greater than 280 deaths and virus activity in over 40 states. While the number of laboratory-confirmed cases rose in 2003, the number of deaths was similar to that reported in 2002. Although there are efforts underway to produce a vaccine to prevent WN encephalitis in man (Monath, 2001), the most important target population for this vaccine (the elderly) is small, development costs are significant, and preparation time for a vaccine that could be used in man is substantial, making antiviral development a useful public health alternative to vaccination.

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The flavivirus genome encodes a single polyprotein, which is proteolytically processed into approximately a dozen functional proteins by both viral and cellular proteases (Chambers et al., 1990). The 5′ terminal 2.5 kb of the viral RNA encodes the three flavivirus structural proteins: capsid (C), membrane (M; which exists in cells as its precursor, prM), and envelope (E). The nonstructural proteins, encoded by the remainder of the polyprotein, are designated NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Chambers et al., 1990). Infectious cDNAs for flaviviruses have been available for over a decade and have been utilized to probe many interesting aspects of viral function. Subgenomic RNAs capable of replicating within cells (replicons) have been reported for Kunjin virus (KV) (Khromykh and Westaway, 1997), WNV (Shi et al., 2002a), YFV (Corver et al., 2003; Molenkamp et al., 2003), DV (Pang et al., 2001), and TBEV (Gehrke et al., 2003). All of these replicons have a similar genetic structure: they include the 5′ untranslated region (UTR), a portion of the C coding region (containing a cyclization domain), a polyprotein encoding NS1 through NS5, and the 3′ UTR. When introduced into cells, these subgenomic RNAs are able to replicate and reporter genes have been inserted into the replicons to follow their replication (Khromykh and Westaway, 1997; Shi et al., 2002a). Interestingly, both KV and WNV replicons are reported to be noncytopathic following their introduction into baby hamster kidney (BHK) cells (Khromykh and Westaway, 1997; Shi et al., 2002a). Vero cells persistently infected with KV replicons have also been reported (Mackenzie et al., 2001). In the case of DV, primate cells transfected with DV replicons were reported to express antigen for up to 8 days without a selectable marker gene (Pang et al., 2001). Recently, Lo et al. (2003b) have reported that BHK cells stably replicating WNV replicons harboring a *Renilla* luciferase gene could be used for identification of antiviral compounds.

There have been a number of reports indicating that antiviral compounds can control flavivirus infections, supporting the contention that antiviral drugs could be useful in combating the emerging public health threat produced by WNV infection. Several nucleoside analogues and compounds that interfere with nucleoside synthesis have been shown to have anti-flavivirus activity in vitro (Anderson and Rahal, 2002; Huggins, 1989; Huggins et al., 1984; Jordan et al., 2000; Markland et al., 2000; Morrey et al., 2002). However, studies of these types of compounds in vivo are limited, and studies with the best-characterized compound in this group, ribavirin, failed to demonstrate any effect on DV titers (Malinoski et al., 1990) or any alteration in signs of yellow fever (Huggins, 1989) in monkeys. Interferon (IFN) has also been shown to be effective against many different flaviviruses in vitro (Anderson and Rahal, 2002; Diamond and Harris, 2001; Luby, 1975; Yasuda et al., 2000). Additionally, several IFN-inducing compounds, such as poly-dI-dC, have been useful in treating flavivirus disease in animals (Haahr, 1971; Harrington et al., 1977; Luby, 1975; Sarzotti et al., 1989; Stephen et al., 1977; Taylor et al., 1980). However, IFN or IFN-inducing drugs are only effective if delivered immediately before, or soon after the onset of infection. Therefore, it appears that these compounds may only be effective during the amplification of virus that precedes the virus' transit of the blood–brain barrier, in the case of encephalitic viruses (Brooks and Phillpotts, 1999; Taylor et al., 1980), or entry into the liver, in the case of YFV (Stephen et al., 1977). Furthermore, a recent study of the clinical effectiveness of IFN α 2a in JE patients failed to demonstrate any benefit from the therapy (Solomon et al., 2003).

Despite the apparent limitation for use of IFN in treating flavivirus infection, IFN action is clearly important in determining the outcome of flavivirus infections. Recently, works on Murray Valley encephalitis virus (MVEV; Lobigs et al., 2003) and DV (Johnson and Roehrig, 1999; Shresta et al., 2004) have demonstrated that mice with deficits in the IFN α/β receptor are more susceptible to flavivirus infection. Moreover, work by Munoz-Jordan et al. (2003) indicated that several DV proteins, when expressed individually, could alter the ability of IFN to activate an antiviral response in human cells. This study pointed in particular to NS2A, NS4A, and NS4B as expressing activities that could protect viral replication from the IFN-induced antiviral defense mechanism of the host cell (Munoz-Jordan et al., 2003).

Here we describe the generation of an infectious cDNA and replicon for WNV from a human isolate of WNV and demonstrate that multiple cell types can be generated that persistently carry the replicon. In our derivation of these cells, we discovered that during the establishment of persistence, replicon levels were reduced in these cells. Furthermore, in some cell types, the generation of cell lines harboring replicons was more difficult, suggesting that an equilibrium between the replicon genome and the host cell was required to produce stable cell lines. Following isolation of replicon-bearing cell lines, these cells stably maintained the replicons for dozens of passages, and these lines could be used to evaluate antiviral activity of both nucleoside analogues and IFN. These latter results are particularly interesting, since they indicate that replicon expression did not prevent IFN from eliminating the replicon from these cells. However, evaluation of the antiviral action of IFN in replicon-bearing cells demonstrated that relative to WT and IFN-cured cells, the replicon-bearing cells displayed a reduced response to IFN that is consistent with the recent report that DV nonstructural proteins can inhibit IFN activation of its target genes (Munoz-Jordan et al., 2003).

Results

Cloning of a replicon and infectious cDNA from a human isolate of WNV

RNA extracted from WNV-infected Vero cells was reverse transcribed and fragments were amplified following