Spread and pathogenic characteristics of a G-deficient rabies virus recombinant: an in vitro and in vivo study

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Rabies virus (RV), a highly neurotropic enveloped virus, is known to spread within the CNS by means of axonal transport. Although the envelope spike glycoprotein (G) of cell-free virions is required for attachment to neuronal receptors and for virus entry, its necessity for transsynaptic spread remains controversial. In this work, a G gene-deficient recombinant RV (SAD ∆G) complemented phenotypically with RV G protein (SAD ∆G+G) has been used to demonstrate the absolute requirement for G in virus transfer from one neuron to another, both in vitro, in neuronal cell cultures (cell line and primary cultures), and in vivo, in murine animal models. By using a model of stereotaxic inoculation into the rat striatum, infection is shown to be restricted to initially infected cells and not transferred to secondary neurons. In mouse as in rat models of infection, the limited infection did not cause any detectable symptoms, suggesting that G-deficient RV recombinants might be valuable as non-pathogenic, single-round vectors for expression of foreign genes.

Rabies virus (RV), a member of the genus Lyssavirus (family Rhabdoviridae), infects mostly neurons within the CNS of mammals. This neurotropism can be explained partly by cellular membrane components that may act as receptors for RV: neural cell adhesion molecule (NCAM) (Thoulouze et al., 1998), p75 nerve growth factor receptor (p75NTR) (Tuffereau et al., 1998) and nicotinic acetylcholine receptor (Lentz et al., 1981). Binding of RV to these receptors is mediated by the viral glycoprotein G, a 505 amino acid protein (Anilionis et al., 1981), present as a homotrimer at the virus surface (Gaudin et al., 1992), which induces a neutralizing antibody response (Wiktor et al., 1973). After internalization, G mediates fusion of the viral envelope with the endosomal membrane (Gaudin et al., 1993). Within the peripheral nerves and CNS, RV spread has been shown to occur through axonal transport in a microtubule network-dependent process (Tsiang, 1979; Gillet et al., 1986; Kucera et al., 1985; Ceccaldi et al., 1989). This allows RV to infect almost all brain neuronal subtypes and to spread centrifugally to peripheral glands (salivary, adrenal, lacrimal etc.) (Charlton, 1988).

The mechanisms of transsynaptic spread of RV remain unclear. Although it has been shown that RV G is responsible for interaction of RV with cells, some authors have hypothesized that RV G would not be necessary for transsynaptic spread of RV nucleocapsid from one neuron to another. On the basis of sequential electron microscope studies, it has been suggested that, in the early phase of infection, before the assembly of full virus particles, the bare viral nucleocapsid could be transported along the axon and subsequently transferred through the synapse into the post-synaptic neuron, without acquiring a G-containing envelope (Gosztonyi, 1994; Gosztonyi et al., 1993). This hypothesis of transsynaptic transfer of bare RV nucleocapsids is based on the fact that full virus particles could never be detected at the sites of synapses in these studies. These conclusions, however, are in contrast with the previously reported role of G in RV spread. Of special interest is the fact that point mutations at position 333 are in these studies. These conclusions, however, are in contrast with the previously reported role of G in RV spread. Of special interest is the fact that point mutations at position 333 are able to modify virus spread within the brain (Coulon et al., 1989; Lafay et al., 1991).

Previously, Mebatsion et al. (1996a) have been able to obtain a recombinant RV mutant deficient for the entire G gene (SAD ∆G) by a reverse genetic approach. This mutant was able to bud spikeless virions from the cell surface of fibroblast cell lines, although with a 30-fold lower efficiency than the parental strain SAD L16, but was unable to produce infectious virus particles. However, this study did not involve neuronal cells, the natural host cells for RV, and the possibility of transsynaptic spread of nucleocapsids was not addressed. In the present study, we investigated the behaviour of the G-deficient

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Fig. 1. Kinetics of infection of standard SAD L16 and SAD ΔG RV on neuronal cell lines (NIE115) and on mouse primary brain cortical cell cultures. Following infection of cell lines and primary cultures at an m.o.i. of 0–01, the spread of infection was monitored by direct immunofluorescence at different days p.i. with a polyclonal rabbit conjugate directed against RV nucleocapsid. Magnification × 350. (A)–(C) SAD ΔG-infected NIE115 cells at days 1 (A), 3 (B) and 4 (C) p.i. (D)–(F) SAD L16-infected N1E115 cells at days 1 (D), 3 (E) and 4 (F) p.i. Arrows in (D) indicate infected cells. (G)–(I) SAD ΔG-infected primary cultures at days 1 (G), 3 (H) and 7 (I) p.i. (J)–(K) SAD L16-infected primary cultures at days 1 (J) and 3 (K) p.i.
mutant on cultured neurons (cell lines or primary cultures) and characterized its pathogenic properties in murine models that allow the study of transsynaptic spread (Gillett et al., 1986; Cercaldi et al., 1989).

The recombinant G-deficient RV mutant (SAD ΔG) was generated previously (Mbatisson et al., 1996a) by deletion of the entire G gene from a full-length infectious RV clone, pSAD L16 (Schnell et al., 1994), which was derived from the attenuated RV strain SAD B19 (Conzelmann et al., 1990; Schneider & Cox, 1983). Stocks of SAD ΔG virus able to perform the first cycle of infection in cell cultures and animals (SAD ΔG + G) were generated in cells that express G protein corresponding to that of SAD L16 from transfected plasmids, as described previously (Mebatsion et al., 1994). The course of infection and spread of SAD ΔG infection were compared in this study with the 'parental' SAD L16 RV.

The infection characteristics of ΔG-RV were investigated in neuronal cell cultures (cell lines and primary cultures) in comparison with the reference RV SAD L16. Murine neuronal cell lines (NIE-115, Cercaldi et al., 1997) were infected with either SAD ΔG or SAD L16 at an m.o.i. of 0.01. The number of infected cells was assessed by immunofluorescence with a polyclonal anti-nucleocapsid conjugate (Sanofi-Diagnostics-Pasteur) as described previously (Atanasiu et al., 1974). The number of infected cells did not increase between days 1 and 4 post-infection (p.i.) when infected with SAD ΔG strain (see Fig. 1A–C), whereas with SAD L16, the entire cell layer displayed infection as soon as day 2 p.i. (Fig. 1D–F). These results were confirmed by counting infected cells from two different culture preparations: whereas with SAD ΔG, the percentage of infected cells versus total cells remained below 1% on days 1, 2, 3 and 4 p.i., a value of 100% infected cells was reached with SAD L16 from day 2 p.i. onwards. Similar kinetics were observed with another murine neuroblastoma cell line, Neuro2a (data not shown). Titration of infectious virus yield on these cultures by the fluorescence forming unit (f.f.u.) method (Bourhy & Sureau, 1990) revealed that cells infected with SAD ΔG (m.o.i. = 1) were unable to release infectious virus on days 2, 3 and 4 p.i. Only on day 1 p.i. could a very low titre (20 f.f.u./ml) be found, probably due to input virus (remaining in the culture medium despite careful removal of the inoculum after infection, or detached from the cell membrane). In contrast, in SAD L16-infected cells, the production of infectious particles increased with time from a value of 10^3 f.f.u./ml as early as day 1 p.i. to nearly 10^7 f.f.u./ml by day 3 p.i. and later.

In order to study infection of primary cultures from mouse brain cortex, cultures were obtained from mouse embryo (E14) brain cortex according to a technique adapted from Tsia R et al. (1991). The cultures were grown in NeuroBasal medium (Boehringer Mannheim) and, 5–7 days after plating, cells were infected with either SAD ΔG or SAD L16 (m.o.i. = 0.1). Infection with SAD ΔG resulted in a pattern of isolated infected cells, whatever the time of infection (from day 1 to 7 p.i.) (Fig. 1G–I). In contrast, infection with SAD L16 resulted in an increase in the number of infected cells with time, as shown in Fig. 1 (J, K). Between days 1 and 3 p.i., the level of infection increased to include the whole extent of neuronal clusters.

We next studied the affects of infection in vivo. Intracerebral infection of mice (BALB/c, 20 g) with 3000 f.f.u. per animal induced 100% mortality with SAD L16 (mean time to death 9–65 days, n = 20), whereas no mortality was observed in mice infected with SAD ΔG + G (n = 22). In SAD L16-infected mice, clinical signs (paralysis, cachexia and bristling) were observed from 1 to 3 days before death, whereas no changes could be observed during SAD ΔG infection. To assess more precisely whether SAD ΔG infection could have an effect on behaviour, a running-wheel activity test (Ottenweller et al., 1998) was performed. As shown in Fig. 2, SAD ΔG infection did not induce any change in activity compared to uninfected controls, whereas SAD L16-infected mice showed a dramatic decrease in running-wheel activity as soon as day 5 p.i.

The extent of virus spread within the brain was evaluated by immunofluorescence on frozen sections (16 μm thick). In the early stages of infection (until day 4 p.i.), no difference was seen between L16 and ΔG strains, both infecting a reduced number of cells within the striatum, hippocampus, thalamus and cortex (Fig. 3A, B). At later stages, the spread of infection throughout all main brain structures was observed in the brains of SAD L16-infected mice and, by day 7 p.i., some brain structures such as the hippocampus were widely infected (Fig. 3 C). On the contrary, no spread of infection from the initially infected target cells was observed in the SAD ΔG-infected mice. Rather, a decrease in the number of infected cells was...
observed and, at day 7 p.i., a reduced number of virus inclusions could be detected. For example, see the paucity of virus inclusions at day 7 p.i. in the hippocampus (Fig. 3D) compared with SAD L16 (Fig. 3C). With SAD ΔG RV, this decrease in immunoreactivity for viral proteins continued over time (rare inclusions on day 10 p.i.; data not shown).

In order to assess the axonal transport of SAD ΔG and transsynaptic spread, a model of stereotaxic inoculation in the rat striatum was used (Gillet et al., 1986; Ceccaldi et al., 1989). Briefly, rats (male Wistar, 150 g, CER Janvier) were anaesthetized and placed in a stereotaxic frame. Virus inoculum (10^5 f.f.u. of either SAD L16 or SAD ΔG per animal) was inoculated

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**Fig. 3.** (A)–(D) SAD L16 RV and SAD ΔG infection of mouse brain structures after intracerebral inoculation. Virus inclusions were detected on brain cryosections by direct immunofluorescence with a rabbit polyclonal conjugate raised against RV nucleocapsid at different days p.i. Magnification × 700. (A) SAD L16, brain cortex, day 4 p.i. (B) SAD ΔG, brain cortex, day 4 p.i. (C) SAD L16, hippocampus, day 7 p.i. (D) SAD ΔG, hippocampus, day 7 p.i. (E) Distribution of infected neurons (direct immunofluorescence on frozen sections) in different brain areas of the rat on days 4 and 7 p.i. after stereotaxic inoculation of either SAD L16 or SAD ΔG RV into the striatum. Abbreviations: STRI, striatum; CORT, cortex; THAL, thalamus; HIPP, hippocampus; CERE, cerebellum; SU NI, substantia nigra (pars compacta). Brain sections (30 sections per animal; three animals per point) were examined on levels A6790 µ and A2180 µ of the stereotaxic atlas of König & Klippel (1974) and the mean number of infected cells per slide was scored as follows: 0, no infection; +, 1–5 infected neurons per section; ++, 5–15 infected neurons per section; ++++ , 15–30 infected neurons per section.
into the striatum according to coordinates in the atlas of König & Klippel (1974). Rats were sacrificed at different days p.i. (three per day) and the brains were rapidly removed and frozen. The first infected cells to be detected by immunofluorescence on frozen sections (20 µm thickness) until day 3 p.i. were in the substantia nigra (pars compacta), thalamus, cortex and hippocampus. A similar pattern of infection was observed with both strains during these first stages of infection (Fig. 3). However, whereas infection with SAD L16 spread to other brain areas and increased with time until death (days 8–10 p.i.), no such increase was observed with SAD ΔG. Actually, a slight decrease could be seen at late stages for SAD ΔG in some structures (e.g. thalamus and substantia nigra); this phenomenon of virus clearance needs additional studies which are under way.

The present data indicate that RV G is absolutely required for production of infectious RV particles from cultured neurons (primary cultures or cell lines). Mebatsion et al. (1996a) have previously demonstrated that infection of fibroblast cell lines with the same G-deficient RV did not produce infectious virus, although budding of spikeless particles occurred. These data are in agreement with previous work emphasizing the role of G in virus attachment to the cell membrane (Iwasaki et al., 1973; Perrin et al., 1982) and recognition of host-cell membrane receptors (Wunner et al., 1984). Some of these receptors have been reported, such as the nicotinic acetylcholine receptor (Lentz et al., 1982), NCAM (Thoulouze et al., 1998) and p75NTR (Tuffereau et al., 1998). In addition, other membrane components such as phospholipids could play a role (Superti et al., 1984). In spite of the obvious involvement of these structures in virus attachment to the cell membrane, it remained unclear whether G would be absolutely required in the case of transsynaptic passage of RV. This hypothesis was based mainly on the fact that RV, which uses axonal transport to spread within the CNS (Kucera et al., 1985; Gillet et al., 1986; Cecaldi et al., 1989), has never been visualized as full virus particles at the site of synaptic junctions in sequential electron microscope studies (Gosztonyi et al., 1993). Thus, it was speculated that the bare RV nucleocapsid could be transported along axons and further transferred to the subsequent neuron without the involvement of any envelope structure (Gosztonyi, 1986, 1994; Gosztonyi et al., 1993). Such a mode of transmission, which is in contrast with the previously reported role of RV G in host cell entry, is problematic in terms of membrane traffic but could be explained by the phenomenon of spinule formation and the possible existence of perforated synapses (Calverley & Jones, 1990). However, the present results, which use a model of axonal transport following virus inoculation into the striatum, clearly indicate that no transsynaptic spread of RV occurred in the absence of G. Actually, after infection of regions that are connected anatomically to the inoculation site, such as substantia nigra (pars compacta), cortex, thalamus and hippocampus (Gillet et al., 1986; Robertson & Travers, 1975; Veening et al., 1980) with G-complemented SAD ΔG, no subsequent transfer of RV to other areas occurred. In contrast, although SAD L16 produced the same pattern of infection as SAD ΔG at first, this was followed by the spread of infection to other areas not connected directly to the striatum (e.g. cerebellum).

As a consequence of the absence of transsynaptic transfer, G-deficient recombinant RV shows restricted spread within the CNS. This reduced infection of the brain is not accompanied by the classical rabies clinical signs (e.g. locomotor activity changes) that occur with the parental strain. These results are in line with previous findings stating that modification of RV G is able to change the pathogenic characteristics of RV. A single amino acid change within G has been shown to reduce the neurovirulence of RV (Dietzschold et al., 1985; Coulon et al., 1989). Here, we have shown that deletion of the entire G gene results in non-pathogenic virus restricted to a single round of infection and unable to infect secondary cells, even in the brain, where transsynaptic transmission of nucleocapsid was suspected to represent a possible means of spread. We have shown previously that recombinant pathogenic RV can be used as a vector to express foreign genes in brain neurons (Cecaldi et al., 1998; Etessami et al., 2000). Elimination of virus-induced pathogenicity of RV by deletion of the G gene thus allows RV to be considered as a safe one-step replication vector not able to spread further in the brain, in addition to the previously reported neurotropic virus vectors (Goins et al., 1997; Hermens et al., 1997). Moreover, it has been shown that RV-derived pseudotype vectors can be targeted specifically to cells other than neurons (by envelope switching), considerably extending the range for vector applications (Mebatsion & Conzelmann, 1996; Mebatsion et al., 1997). Further investigations are now in progress to delineate the kinetics and mechanisms of virus clearance, which occurs with the G-deficient virus after infection of the first-order target cells, and to study the expression of foreign genes inserted into the RV genome (Mebatsion et al., 1996b) and targeted to neurons (Cecaldi et al., 1998; Etessami et al., 2000).

The technical assistance of P. Weber is acknowledged. R.E. is the recipient of a French Ministry of Foreign Affairs grant (no. 95/8104). B.N. was supported by a DVA Educational Detail and by NATO. This work was supported financially by the Direction de la Recherche et des Etudes Techniques (contract no. 95/178) and the Deutsche Forschungsgemeinschaft (SFB455/A3).

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Received 25 February 2000; Accepted 1 June 2000