Restriction of poliovirus RNA replication in persistently infected nerve cells

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The aetiology of post-polio syndrome may involve persistence of poliovirus (PV) in the CNS. PV persists in the CNS of infected paralysed mice for over a year after the acute phase of paralytic poliomyelitis. However, infectious PV particles cannot be recovered from homogenates of CNS from paralysed mice after the acute phase of disease, indicating that PV replication is restricted. To identify the molecular mechanism by which PV replication is limited, PV RNA synthesis was analysed by estimating the relative level of genomic (plus-strand) and complementary (minus-strand) PV RNA in the CNS of persistently infected mice. PV RNA replication decreased during the 6 months following onset of paralysis, due mainly to inhibition of plus-strand RNA synthesis. Thus, restriction of PV RNA synthesis may contribute to persistence by limiting virus replication in the mouse CNS. Interestingly, viral RNA replication was similarly inhibited in neuroblastoma IMR-32 cell cultures persistently infected with PV. This in vitro model thus shows that cellular factors play a role in the inhibition of viral RNA synthesis.

Introduction

Poliovirus (PV) is an enterovirus of the Picornaviridae family and is the causal agent of paralytic poliomyelitis, an acute disease of the central nervous system (CNS). The PV genome is a single-stranded RNA of positive polarity, 7500 nucleotides long, composed of a long 5′ non-translated region (NTR) followed by a long open-reading frame and a short polyadenylated 3′ NTR. The PV genome is copied, starting from the 3′ end, to generate a complementary, negative-strand RNA that, in turn, is transcribed into new molecules of positive-strand genomic RNA. Both processes are catalysed by the RNA-dependent RNA polymerase (3Dpol) but a number of other viral proteins and cellular factors also participate in RNA replication (for review, see Xiang et al., 1997). RNA replication is highly asymmetric with the ratio of positive-strand to negative-strand RNA synthesis being greater than 30:1 in human epithelial cell cultures (Andino et al., 1990; Giachetti & Semler, 1991; Lopez-Guerrero et al., 1991; Novak & Kirkegaard, 1991).

In many poliomyelitic patients, a period of decades of clinical stability is followed by the development of a disease called post-polio syndrome, characterized notably by slowly progressive muscle weakness (Dalakas et al., 1995). The presence of PV RNA sequences or PV-related RNA (Leon-Monzon & Dalakas, 1995; Muir et al., 1995; Leparc-Goffart et al., 1996) and of anti-PV IgM antibodies (Sharief et al., 1991) suggests that PV persistence may be involved in this syndrome. Moreover, in human cell cultures of neuronal origin, it has been shown that PV can establish persistent infections (Colbère-Garapin et al., 1989; Pavio et al., 1996).

Poliomyelitis can be experimentally transmitted to monkeys by inoculation of the CNS with any of the three serotypes of PV, and to mice with mouse-adapted PV strains. We previously isolated and characterized a mutant pathogenic for mice, PV-1 Mah-T1022I (Couderc et al., 1993, 1996). With this model, we have shown that PV persists in the CNS of paralysed mice for over a year after the acute disease (Destombes et al., 1997). Moreover, PV plus- and minus-strand RNAs have been detected by RT–nested PCR in the spinal cord of paralysed mice suggesting continuous PV RNA replication in the CNS. However, although PV particles could be detected in mouse motor neurons by electron microscopy...
Table 1. Detection of plus- and minus-strand viral RNA during acute and persistent infection of the mouse CNS by a semi-quantitative RT–nested PCR

<table>
<thead>
<tr>
<th>(a) PCRD&lt;sub&gt;50&lt;/sub&gt; (log&lt;sub&gt;10&lt;/sub&gt;)*</th>
<th>Time p.p.</th>
<th>Mouse (code)</th>
<th>Plus-strand†</th>
<th>Minus-strand†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>0-1</td>
<td>6·15</td>
<td>4·98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0-2</td>
<td>6·37</td>
<td>5·24</td>
</tr>
<tr>
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<td>10 days</td>
<td>10-1</td>
<td>4·31</td>
<td>3·21</td>
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<td></td>
<td></td>
<td>10-2</td>
<td>3·49</td>
<td>2·45</td>
</tr>
<tr>
<td></td>
<td>1 month</td>
<td>1-1</td>
<td>2·09</td>
<td>1·40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1-2</td>
<td>1·81</td>
<td>1·18</td>
</tr>
<tr>
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<td>3 months</td>
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<td>2·84</td>
<td>2·37</td>
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<td>3-2</td>
<td>2·43</td>
<td>2·08</td>
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<td></td>
<td>6 months</td>
<td>6-1</td>
<td>1·50</td>
<td>1·44</td>
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<td></td>
<td>6-2</td>
<td>1·92</td>
<td>1·69</td>
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<td></td>
<td>12 months</td>
<td>12-1</td>
<td>1·01</td>
<td>1·13</td>
</tr>
<tr>
<td></td>
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<td>12-2</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>(b) PCRD&lt;sub&gt;50&lt;/sub&gt; (log&lt;sub&gt;10&lt;/sub&gt;)‡</th>
<th>Controls§</th>
<th>Amount</th>
<th>Plus-strand†</th>
<th>Minus-strand†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plus RNA</td>
<td>(100 ng)</td>
<td>9·50</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Minus RNA</td>
<td>(100 ng)</td>
<td>—</td>
<td>9·41</td>
</tr>
<tr>
<td></td>
<td>Mix of plus/minus RNA</td>
<td>(1 ng/1 ng)</td>
<td>7·57</td>
<td>7·60</td>
</tr>
<tr>
<td></td>
<td>Mix of plus/minus RNA</td>
<td>(10 ng/1 ng)</td>
<td>8·35</td>
<td>7·42</td>
</tr>
</tbody>
</table>

* Dilution of mouse spinal cord total RNA for which 50% of the RT–nested PCRs were positive.
† The RT–nested PCR procedure was strand-specific with no detectable mispriming of the opposite strand and no self-priming was detected in reactions without added primer.
‡ Dilution of PV RNA for which 50% of the RT–nested PCRs were positive.

for at least 12 months after the onset of paralysis, infectious PV particles could not be recovered from homogenates of CNS from paralysed mice beyond 20 days post-paralysis (p.p.), suggesting that PV replication was restricted (Destombes et al., 1997). Limited replication has been shown with other neurotropic viruses including measles virus, mouse hepatitis virus and Sindbis virus, for which viral genomes were detected although infectious virus could rarely, if ever, be isolated from the CNS during persistent infection (Kyuwa & Stohlman, 1990; Levine & Griffin, 1992; Schneider-Schaulies & ter Meulen, 1992). Similarly, non-productive persistent infection has been described with coxsackievirus B, another enterovirus, during virus-induced chronic myocarditis (Klingel et al., 1992; Tam & Messner, 1999; Reetoo et al., 2000).

One of the main mechanisms by which coxsackieviruses persist in vivo is a restriction of viral RNA replication, possibly as a consequence of inhibition of plus-strand RNA synthesis (Cunningham et al., 1990; Klingel et al., 1992; Andréoletti et al., 1997; Tam & Messner, 1999; Reetoo et al., 2000). In contrast, viral RNA replication is not restricted during persistent infection of the CNS by Theiler’s murine encephalomyelitis virus, another picornavirus, although little infectious virus is detected in the mouse spinal cord (Trottier et al., 2001). The molecular mechanisms of PV persistence in the mouse CNS have not been elucidated.

To investigate whether restriction of viral RNA synthesis contributes to the decrease in the viral load in the mouse CNS, we analysed the relative levels of genomic and complementary PV RNAs during both the acute phase of poliomyelitis and the subsequent persistent infection. Female OF1 (Ifa-Credo) mice, 28 days old, were inoculated intracerebrally with $3 	imes 10^7$ pf.u. of the mouse-adapted strain PV-1/Mah-T1022I. Total RNA was extracted from mouse spinal cords during acute (day 0 and day 10 p.p.) and persistent (1–12 months p.p.) phases of PV infection using the RNA PLUS kit (Bioprobe) according to the manufacturer’s instructions. Total RNA was resuspended in RNase-free $H_2O$ (250 ng/µl). A strand-specific semi-quantitative RT–nested PCR method was used to assay viral RNA in samples of total RNA. Briefly, serial dilutions (1/3) of total RNA suspension were subjected to eight independent RT–
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Fig. 1. Plus- to minus-strand PV RNA ratio during acute (day 0 and day 10 p.p.) and persistent (1 to 12 months p.p.) infection of the mouse spinal cord. The ratios are those of the PCRd50 for plus-strand RNA to that for minus-strand RNA presented in Table 1. Controls correspond to mixes of plus- and minus-strand PV RNA transcribed in vitro with known ratios of 10 (R10) and 1 (R1). For the PV RNA ratio in mice, each bar represents one PV-infected mouse and the mouse codes are indicated above the bar.

nested PCRs to detect plus- and minus-strand RNA, as described (Destombes et al., 1997) and PCR products were visualized after electrophoresis on agarose gel. The dilutions of total RNA from mouse spinal cords for which 50% of the RT–nested PCR (PCRd50) were positive were calculated according to the Reed and Muench end-point method (Reed & Muench, 1938) (Table 1a). Negative controls involved testing total RNA (1 µg) prepared from mock-infected mice, and no PCR product was detected with the primers used, as previously illustrated (Destombes et al., 1997). To validate the method, the PCRd50 of known amounts of PV RNA of each polarity transcribed in vitro, as well as those of mixes of plus- and minus-strand PV RNA with known ratios (10 and 1) were determined (Table 1b). The ratios of the PCRd50 for plus-strand RNA to that for minus-strand RNA in the mixes were close to the expected values (8–5 and 0–9, respectively) (Fig. 1).

The values of PCRd50 obtained with RNA from PV-infected mice indicated a sharp decline in both plus- and minus-strand RNA abundance after the acute phase of disease (Table 1a). During the acute phase of infection, positive-strand viral RNA was present in excess over the negative strand with a plus- to minus-strand ratio characteristic of a productive infection (ratio of about 15:1) (Fig. 1). Although this ratio is lower than in human epithelial HeLa cells (Andino et al., 1990; Giachetti & Semler, 1991; Lopez-Guerrero et al., 1991; Novak & Kirkegaard, 1991), it is similar to that observed during a single growth cycle in human neuroblastoma IMR-32 cell cultures infected with PV (Lopez-Guerrero et al., 1991). In contrast, 1 month p.p., when infectious virus can no longer be recovered (Destombes et al., 1997), the ratio of plus- to minus-strand RNA in the mouse CNS was less than half that during the acute phase. This ratio was even lower 6 months p.p., with abnormal equivalent levels of plus- and minus-strand RNAs. The ratio seemed to be stable thereafter. These results indicate that PV RNA synthesis was restricted in the spinal cord of mice during persistent infection and this restriction was mostly due to an inhibition of plus-strand RNA synthesis. This semi-quantitative RT–nested PCR method was not sufficiently quantitative to allow precise determination of the number of PV RNA molecules in samples. However, we could estimate the approximate copy number of PV RNA molecules from the values obtained with PV RNA controls, transcribed in vitro. The approximate copy number of PV RNA molecules per µg of total RNA in the mouse CNS declined from about 10^10 to 10^8 for plus-strand RNA and from about 10^9 to 10^7 for minus-strand RNA, from day 0 to 12 months p.p. The decline in viral RNA yields with time is paralleled by the decrease in the viral load previously observed (Destombes et al., 1997), suggesting that inhibition of PV RNA synthesis may be one of the mechanisms by which virus replication is restricted during persistent PV infection in the mouse CNS. Similarly, during coxsackievirus non-productive, persistent infections in the heart and skeletal muscle of mice, coxsackievirus RNA replication is restricted and equal amounts of positive- and negative-strand RNA are detected. This is consistent with defective positive-strand RNA synthesis (Klingel et al., 1992; Andreoletti et al., 1997). Furthermore, it has been shown that coxsackievirus RNA persists in a double-stranded form in skeletal muscle in mice afflicted by chronic inflammatory myopathy (Tam & Messner, 1999).

To investigate whether PV variants with altered replication properties arise during PV persistence in the mouse CNS, we analysed the genome of PV present in the spinal cord of a mouse 6 months p.p. The entire RNA genome was amplified as
a series of six overlapping fragments, including the 5' and 3' proximal extremities, and sequenced. Surprisingly, we found no deletion or point mutation with reference to the consensus sequence of the viral genome (data not shown). Although we cannot rule out the role of nucleic acid modification such as methylation, this result seems to indicate that the inhibition of viral RNA synthesis in the mouse CNS is not due to viral factors. Similar results have been reported in mice with coxsackievirus-induced chronic myopathy where virus persistence in muscle was not associated with evolution of the viral genome (Tam & Messner, 1999). Thus, host factors are presumably the main factors responsible for the restriction of the synthesis of positive-strand RNA during persistent PV infection in the mouse CNS.

As PV is able to persist in human neuroblastoma cell cultures with a reduced virus yield (Colbère-Garapin et al., 1989), we investigated whether PV RNA replication was similarly inhibited during PV persistence in vitro. IMR-32 cell cultures persistently infected with the mouse-adapted mutant PV-1/Mah-T1022I were established as previously described (Colbère-Garapin et al., 1989). A persistent infection was also established with the parental PV-1/Mahoney to test whether, although unlikely, the evolution of viral RNA synthesis was dependent on the substitution that confers the mouse-adapted phenotype to PV-1/Mah-T1022I (Thr 22 of VP1 to Ile). During the 2 weeks following infection, a large proportion of the cells died. Thereafter a few colonies appeared. After about 1 month, cells reached confluency and the virus yield dropped to about 1 TCID$_{50}$ per cell per week (data not shown), compared to the virus yield in freshly infected IMR-32 (about 1000 TCID$_{50}$ per cell 12 hours post-infection). The ratio of PV plus- to minus-strand RNA in persistently infected cell cultures was followed from 12 h to 6 months post-infection (p.i.). The PV plus- and minus-strand RNAs were detected independently by a strand-specific slot blot method. This hybridization technique was chosen because with large amounts of viral RNA it is more accurate than the semi-quantitative method, which would require too many dilutions for such RNA samples. Briefly, serial dilutions (1/5) of total RNA preparations (40 ng/µl) were denatured with formaldehyde and each dilution was blotted on to two separate nylon membranes (Hybond-N$,^\text{®}$, Amersham Pharmacia Biotech) using a Minifold II slot blot apparatus (Schleicher & Schuell) and fixed by UV. Both membranes were prehybridized at 68 °C for 2 h in 6 × SSC, 5 × Denhardt’s solution, 0.1% SDS. Probes specific for either plus- or minus-strand PV RNA were obtained from a 1.5 kb fragment of PV-1/Mahoney cDNA (positions 2232–3677) by using the Ribobsp enzyme Combination System in vitro transcription kit (Promega). The specific activity of the $^{32}$P-labelled probes was at least $2 \times 10^{8}$ c.p.m./µg. One membrane was hybridized in the same buffer containing the plus-strand specific riboprobe at 68 °C overnight and the second in the same conditions with the minus-strand specific riboprobe. The membranes were washed twice in 2 × SSC, 0.1% SDS at 68 °C for 30 min, twice in 0.2 × SSC, 0.1% SDS at 68 °C for 30 min and exposed to a PhosphorImager Storm 820 (Molecular Dynamics). Slot blot signal densities were quantified as average intensity of all the pixels in the spot, in arbitrary units, using the Image Quant software (Molecular Dynamics). Slot blot experiments included a range of known amounts of PV RNA of each polarity transcribed in vitro. Serial dilutions of total RNA prepared from mock-infected cells as described above were used as negative controls. The background was less than 10% of the signal at each respective dilution and was subtracted from experimental values before calculating the ratios of plus- to minus-strand RNA in PV persistently infected IMR-32 cells. To validate the method, mixes of plus- and minus-strand PV RNA transcribed in vitro with known ratios of 10 and 1 were tested: the results obtained were as expected (11 and 1, respectively) (Fig. 2).

The ratios of plus- to minus-strand RNA in PV persistently infected IMR-32 cells were calculated (Fig. 2). For both persistent infections, the plus- to minus-strand RNA ratio in freshly infected IMR-32 cell cultures (12 h p.i.) was 11:1. During the first month of infection, the virus ratio declined substantially (4:1), then more modestly between 1 and 6 months p.i. (2:1). Thus, the plus- to minus-strand RNA ratios of both mouse-adapted mutant PV-1/Mah-T1022I and the parental strain PV-1/Mahoney decreased in persistently infected IMR-32 cells. The copy number of PV RNA molecules was estimated approximately by comparison with the range of known amounts of PV RNA of each polarity transcribed in vitro. The copy number of PV RNA molecules per µg of total RNA in PV persistently infected IMR-32 cells declined from about $10^{11}$ to $10^{9}$ for plus-strand RNA and from about $10^{10}$ to $10^{8}$ for minus-strand RNA, between 12 h and 6 months p.i. The decline in copy number of plus-strand RNA molecules between day 0 and 6 months was by a factor of about 1000 and correlated with that of TCID$_{50}$. Consistent with the findings...
in the mouse CNS, PV RNA replication was restricted by inhibition of plus-strand RNA synthesis in neuroblastoma cell cultures. Although interactions between PV and its receptor seem to play a crucial role in PV persistence in several cell systems (Colberè-Garapin et al., 1998; Pavio et al., 2000), the restriction of PV RNA replication could be an additional factor involved in the persistent infection.

Since host factors are presumably the main factors responsible for restricting the synthesis of positive-strand RNA during persistent PV infection in the mouse CNS, we investigated whether cellular factors were involved in the inhibition of PV RNA synthesis during virus persistence in IMR-32 cells. First, we cured IMR-32 cell cultures persistently infected with PV-1/Mahoney for 6 months (IMR-32/PV-cured) by three passages in the presence of anti-PV-1 rabbit serum. Complete curing of a culture was confirmed by testing for infectivity from cells disrupted by a freeze–thaw step and for viral RNA by RT–PCR amplification with viral primers.

Persistently infected cells can be cured by treatment with anti-PV rabbit serum and this may suggest that continuous reinfections are required to maintain PV persistence in IMR-32 cells. Although we have found chromatolysis of motor neurons as well as inflammatory cell infiltration in the mouse CNS at all time points investigated after the onset of paralysis, even at 12 months p.p. (Destombes et al., 1997), it is difficult to know whether the persistent PV infection involves continuous reinfection of nerve cells.

RNA synthesis of PV-1/Mahoney during a single growth cycle was compared in IMR-32 and in IMR-32/PV-cured cells. Cells were infected at an m.o.i. of 10 TCID<sub>50</sub> and the ratio of plus- to minus-strand RNA was evaluated by slot blot assays from 2 to 24 h.p.i. The maximal values of the plus- to minus-strand RNA ratios during the single growth cycle experiments in both IMR-32 and IMR-32/PV-cured cell cultures (6 h p.i.) are presented in Fig. 3. The ratio of PV-1/Mahoney plus- to minus-strand RNA was 40% lower in IMR-32/PV-cured cells than in IMR-32 cells. This seems to indicate that cellular factors are involved in restriction of viral RNA synthesis in persistently infected IMR-32 cells. This result does not exclude the possibility that viral factors also contribute to restricting replication of PV in IMR-32 cells.

The virus yields were determined in parallel with the analysis of viral RNA synthesis. The maximal virus titre was obtained at 12 h.p.i. The restriction in viral RNA synthesis observed in IMR-32/PV-cured cells was associated with a lower virus yield (Fig. 3). This suggests that the restriction of RNA replication may contribute to the decrease in virus yield.

Host cell components regulate the amount of plus- and minus-strand PV RNA synthesized according to the host cell type (Lopez-Guerrero et al., 1991). However, the mechanisms of the differential control of viral RNA synthesis leading to highly divergent amounts of plus- and minus-strand RNA are poorly understood but probably involve strand-specific initiation processes (for review, see Xiang et al., 1997). Efficient selection in viral RNA replication is mediated by ribonucleoprotein complexes at the 5’ and 3’ ends, involving not only the RNA polymerase 3Dpol and a number of other viral proteins but also cellular factors including the poly(rC) binding protein and the poly(A) binding protein 1 (Xiang et al., 1997; Herold & Andino, 2001). It is therefore plausible that modification of one or several of these components during persistent infection could lead to the selective restriction of the synthesis of one strand (Andino et al., 1990; Giachetti & Semler, 1991).
culture are going across different steps of the complete cell lifecycle, in contrast to post-mitotic neurons in vivo, it would be of interest to examine the contribution of cell lifecycle to the control of PV RNA synthesis in IMR-32 cells.

In conclusion, the restriction of PV plus-strand RNA production may limit virus replication and thereby contribute to the persistence of PV in the CNS of paralysed mice. Although the mechanism involved in this restriction may be different in vitro, the IMR-32 cell model highlights the relevance of a global approach, using oligonucleotide arrays, to identify the cellular factors involved in the inhibition of PV RNA synthesis. Identification and characterization of these factors will improve our understanding of PV RNA replication and PV persistence.

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References


kinetics in coxsackievirus B3-induced murine myocarditis: biphasic pattern of clearance following acute infection, with persistence of residual viral RNA throughout and beyond the inflammatory phase of disease. 


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