Analysis by RNA–PCR of latency and reactivation of herpes simplex virus in multiple neuronal tissues

Shinji Tanaka, Hiroko Minagawa,* Yasushi Toh, Ying Liu and Ryoichi Mori

Department of Virology, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan

Following intracameral inoculation with herpes simplex virus type 1 (HSV-1), BALB/c mice develop acute necrotizing chorioretinitis and infectious virus is detected in the eyes, trigeminal ganglia, brain, spinal cord and adrenal glands during acute infection. In this study, we analysed the latent phase of this experimental animal system. In mice which survived the acute infection, latent HSV-1 was recovered from the trigeminal ganglia, brain and adrenal glands by co-cultivation with Vero cells. In these tissues, both the unspliced latency-associated transcript (LAT) and the spliced LAT were detected by RNA–PCR. Following in vivo administration of cyclophosphamide and dexamethasone to induce viral reactivation, ICP0 mRNA became detectable in the multiple neural tissues, and the spliced LAT disappeared whereas the unspliced LAT remained detectable by RNA–PCR. Sequence analysis of the RNA–PCR products revealed that the GC-AG splicing signal previously reported for LATs from trigeminal ganglia was also detected in LATs from the brain and adrenal glands, suggesting that the splicing of LATs might be associated with the maintenance of and/or reactivation from latency. The generalized latent infection of HSV-1 described in this study might serve as an experimental model of possible viral reactivation from organs that do not innervate the primary port of entry.

Introduction

Herpes simplex virus (HSV) causes both acute and persistent infections of humans. HSV, when inoculated on skin, mucosa or cornea, usually spreads through afferent nerves and establishes latent infection in the innervating ganglia (Whitley, 1990). Following reactivation from latently infected ganglia, HSV spreads through efferent nerves and induces recurrent infection at regions near the site of primary infection. In addition, HSV has been reported to cause primary infections of the central and/or peripheral nervous system including encephalitis, myelitis and adrenocorticitis (Bahrani et al., 1966; Blyth & Hill, 1984). Infection by HSV of the nervous systems, induced by viral recurrence from latency, can also cause severe and sometimes fatal diseases in clinical cases (Whitley, 1990). This viral recurrence will sometimes cause a generalized infection (Blyth & Hill, 1984; Craig & Nahmias, 1973; Corey & Spear, 1986). However, neither the distribution of latent HSV nor the dissemination of neural infection during latency and recurrence has been studied in any depth partly owing to the lack of an adequate experimental model (Stevens, 1989; Ho, 1992).

In this study, we analysed the latency and reactivation of HSV type 1 (HSV-1) in the various murine tissues following intracameral inoculation. In those mice, HSV-1 induces chorioretinitis (Whittum et al., 1984) and systemic spread of infectious virus was observed during the first 2 weeks post-inoculation (p.i.) (Liu et al., 1993). It has been reported that intracameral infection can cause latent infection in the trigeminal ganglia (Rodahl & Stevens, 1992; Minagawa et al., 1993). The latently infected mice post-intracameral inoculation could be useful for studying the mechanisms of latency and reactivation of HSV, in addition to those mice inoculated on the cornea (Spivack & Fraser, 1987; Stevens et al., 1987).

During latent infection with HSV, viral transcription appears to be limited to the latency-associated transcripts (LATs) (Rock et al., 1987; Spivack & Fraser, 1987; Stevens et al., 1987). LATs are at least in part antisense RNAs complementary to the 3' end of ICP0 mRNA, the first viral transcript of the replicating infection (Roizman & Sears, 1990). In this study we detected LATs and ICP0 mRNA using sensitive RNA–PCR during latency and reactivation. LATs of HSV-1 are composed mainly of two transcripts, the 2.0 kb LAT and the 1.5 kb LAT; the latter is derived from the former by alternative splicing (Wagner et al., 1988b; Wechsler et al., 1988). We identified the two types of LAT by RNA–PCR using primer pairs: one spans the spliced sites and the other...
week-old BALB/c mice (Kyushu University Animal Center) were inoculated into the bilateral ocular anterior chamber with 2 x 10^6 p.f.u. of HSV-1 (strain KOS) per eye, essentially as described (Liu et al., 1993). During the acute phase of infection, three mice were killed at each time point, 1, 3, 5, 7, 10 and 14 days p.i., and tissue samples including the eyes, trigeminal ganglia, brain, spinal cord, adrenal glands, liver, kidneys and spleen were collected for determination of infectious virus (Liu et al., 1993). The various tissues of mice were removed at days 1, 2 and 3 following dexamethasone administration, and were examined as described above.

Methods

Infection of mice and preparation of tissue samples. Female 6- to 8-week-old BALB/c mice (Kyushu University Animal Center) were inoculated into the bilateral ocular anterior chamber with 2 x 10^6 p.f.u. of HSV-1 (strain KOS) per eye, essentially as described (Liu et al., 1993). During the acute phase of infection, three mice were killed at each time point, 1, 3, 5, 7, 10 and 14 days p.i., and tissue samples including the eyes, trigeminal ganglia, brain, spinal cord, adrenal glands, liver, kidneys and spleen were collected for determination of infectious virus (Liu et al., 1993).

At 8 or more weeks p.i., the tissues described above were collected aseptically, and were either explanted onto Vero cells for 28 days for reactivation of HSV-1. In vivo reactivation of HSV-1. In vivo reactivation of HSV-1 was performed as described by Shimeld et al. (1990). Latently infected mice were injected intraperitoneally with 250 mg of cyclophosphamide (Endoxan, Shionogi Pharmaceuticals) per kg followed by 20 mg of dexamethasone phosphate (Decadron, Banyu) per kg, 24 h later (Minagawa et al., 1993). The various tissues of mice were removed at days 1, 2 and 3 following dexamethasone administration, and were then examined as described above.

Preparation of RNA. Total RNA was extracted from murine tissues using the acid guanidinium thiocyanate–phenol–chloroform method as described by Chomczynski & Sacchi (1987). Briefly, samples were homogenized in a denaturing solution containing 4 M-guanidinium thiocyanate, 25 mm-sodium citrate pH 7.0, 0.5% sarcosyl and 0.1 M-2-mercaptoethanol. Following the addition of 0.2 M-sodium acetate pH 4.0, one volume of phenol (water-saturated), and a one-fifth volume of chloroform–isoamyl alcohol mixture (49:1), the suspension was centrifuged at 3000 r.p.m. for 40 min at 4°C. The aqueous phase was transferred into a fresh tube and precipitated with ethanol. The pellet was suspended with the denaturing solution, precipitated with ethanol again and dissolved with diethyl pyrocarbonate-treated water. The RNA concentration was measured as A260 spectrophotometrically.

Primers for RNA-PCR. Fig. 1 shows the design of RNA-PCR for detection of viral transcripts from extracted RNA (Lynas et al., 1989; Spiveack et al., 1991; Minagawa et al., 1993). We detected the 2.0 kb LAT (LAT_unspliced) using primers pLAT10 (5'-ATGGAGGCCAGAA-CCACAGTG-3') and pLAT15 (5'-CGCTCGTCCTCTCTGAC- TACTC-3') to amplify a 389 bp fragment (Wagner et al., 1988a). Using pLAT10 and pLAT13 (5'-GTGAGTTAGAAGACCCGTCTC-3') or a 389 bp fragment of LAT_unspliced or a 947 bp fragment of LAT_unspliced were amplified (Wagner et al., 1988a). ICP0 mRNA was detected using primers 3 (shown as P3 in Fig. 1) (5'-TTCCGTCCTCCGCTGAGGAT-3') and 5 (shown as P5 in Fig. 2) (5'-GACCCCTCCGCGCATACCA-3') to amplify a 157 bp fragment (Lynas et al., 1989). For a positive control, a 542 bp fragment of mouse β-actin mRNA was amplified using a primer pair (5'-GTCGGCGCTCTAGGACACATT-3' and 5'-CTCTTTGATGTCA-CGCAACGTTC-3') described by Schull et al. (1992). Using the numbering system of Perry & MacGeoch (1988) based on HSV-1 strain 17, the primers pLAT13, pLAT15, pLAT10, P3 and P5 corresponded to positions 119462 to 119482, 120112 to 120122, 120489 to 120408, 123212 to 123231 and 124114 to 124133, respectively.

RNA-PCR and sequencing analysis for viral transcripts. For synthesis of cdNA, 2 μg of total RNA from each tissue was denatured at 80°C for 5 min and incubated at 37°C for 1 h in a 20 μl solution containing 1 mm of each deoxynucleotide triphosphate, 5 μg of random hexamers pd(N)₆ (Pharmacia LKB Biotechnology), 100 U of Moloney murine leukemia virus reverse transcriptase (BRL) and 20 U of ribonuclease inhibitor (Toyobo). After boiling 10 μl of the synthesized cdNA for 15 min, enzymatic amplification with Taq DNA polymerase (Promega) was performed in 100 μl of solution (Tanaka et al., 1992). Forty cycles were carried out on a DNA thermal cycler (Perkin-Elmer Cetus) under the following conditions: 1 min at 94°C (denaturation), 2 min at 58°C (annealing), and 1.5 min at 72°C (polymerization). All the possible precautions were taken to avoid contamination. The amplified product was detected by electrophoresis on an agarose gel stained with ethidium bromide. Sequence analysis of the RNA-PCR products was performed essentially as described by Tanaka et al. (1993). After cloning the RNA-PCR products using the TA cloning kit (Invitrogen), the sequences of DNA from mixed recombinant colonies were determined by the dideoxynucleotide ribonucleotide chain termination method, using Sequenase version 2.0 (United States Biochemicals). It was ensured by PCR sequencing that the trigeminal ganglia RNA from
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Latently infected mice carried both spliced and unspliced LAT, for use as a positive control for PCR of LATs. RNA from Vero cells infected with HSV-1 KOS for 8 h was used as a positive control for ICP0, and each tissue of uninfected mice was used as a negative control.

Results

Acute phase infection in mice inoculated intracamerally with HSV-1

Following intracameral inoculation with $2 \times 10^5$ p.f.u. of HSV-1, infectious virus was detected in the eyes, trigeminal ganglia, brain, spinal cord and adrenal glands, but not in any samples of the liver, spleen or kidneys during the acute phase, i.e. by 14 days p.i. (Fig. 2, Table 1), a result consistent with a previous study (Liu et al., 1993). At 1 day p.i., infectious virus was detected in the eyes, brain, spinal cord and adrenal glands. Eyes were positive for infectious virus until 10 days p.i. The trigeminal ganglia harboured virus from 3 to 10 days p.i. The brain and adrenal gland were positive for infectious virus from 1 to 7 days p.i., and the spinal cord was positive at 3, 7 and 10 days p.i. Less than 10% of infected mice developed acute fatal encephalitis, and the surviving mice recovered, although they were afflicted by corneal opacity and/or ocular atrophy.

Establishment of latent infection in various neural tissues

The surviving mice were analysed for the establishment of viral latency by 8 weeks p.i. at the earliest (Table 1). Latent HSV-1 was recovered by co-cultivation with Vero cells of neural tissues including those of the trigeminal ganglia, brain and adrenal glands; these tissues were positive for infectious virus during acute infection. In contrast, infectious virus was not reactivated after explantation of tissues from the eyes, spinal cord, liver, kidneys or spleen.

By employing RNA–PCR of the murine tissue RNA, it was shown that LAT_{unspliced} was present in all the examined neural tissues including that of spinal cord whereas eyes, liver, kidneys and spleen did not contain detectable amounts of LAT (Table 1). Fig. 3 shows RNA–PCR data on LAT_{spliced}, LAT_{unspliced} and ICP0 of

Fig. 2. Virus growth in murine tissues including eyes (a), trigeminal ganglia (b), brain (c), spinal cord (d), and adrenal glands (e) during acute infection after intracameral inoculation with HSV-1. Three infected mice per time point were killed at 1, 3, 5, 7, 10 or 14 days p.i. and the tissue samples were collected for determination of the virus titres. Each point represents the geometric mean titres from three mice. The dashed lines indicate the limit of virus detection.
Table 1. Detection of infectious HSV-1 and LAT/ICP0 transcripts in various murine tissues at the acute or latent phase following intracameral inoculation

<table>
<thead>
<tr>
<th>Virus isolation</th>
<th>Eyes</th>
<th>Trigeminal ganglia</th>
<th>Brain</th>
<th>Spinal cord</th>
<th>Adrenal glands</th>
<th>Liver</th>
<th>Kidneys</th>
<th>Spleen</th>
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<tbody>
<tr>
<td>Acute*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Latent†</td>
<td>0/7</td>
<td>6/7</td>
<td>3/3</td>
<td>0/7</td>
<td>5/7</td>
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<td>Virus transcripts‡</td>
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<tr>
<td>LAT_unspliced</td>
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<tr>
<td>LAT_spliced</td>
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* To analyse acute phase infection, the infected mice were killed at 1 to 14 days after intracameral inoculation with HSV-1 and tissue samples were collected for determination of infectious virus with Vero cell monolayers (Liu et al., 1993).
† Number positive/number examined. At 8 or more weeks after infection, the tissues were removed from the mice for determination of latent HSV-1 by co-cultivation with Vero cell monolayers.
‡ Tissues of latently infected mice were analysed for the virus transcripts by RNA-PCR.

RNA from trigeminal ganglia, brain, spinal cord and adrenal glands. ICP0 mRNA was not detected in any samples examined (Fig. 3a to d, lanes 9). LAT_spliced was not detected in the spinal cord whereas LAT_unspliced was (Fig. 3c, lanes 1 and 5). Detection of LAT_spliced in tissues other than the innervating ganglia has not been reported to our knowledge (Ho, 1992).

The nucleotide sequences of LAT PCR products were then determined. Fig. 4 shows the sequence of LAT_spliced derived from the brain. By comparing the LAT_spliced sequences with the published sequences of LAT_unspliced and HSV-1 KOS DNA (Wagner et al., 1988a), the splicing donor signal was identified as GC and the acceptor signal as AG. These signals were previously reported for the sequences of LAT_spliced from trigeminal ganglia (Spivack et al., 1991), and did not fit Chambon’s rule (GT and AG, respectively). That the splicing donor signal was GC rather than GT is probably a unique signal for latency of HSV-1 in the neural tissues, as reported previously (Spivack & Fraser, 1987; Wagner et al., 1988b). The splicing junctions in LATs from the trigeminal ganglia, brain and adrenal glands were identical (data not shown).

In vivo reactivation of latently infected HSV-1 in various neuronal tissues of mice

We analysed reactivation in various murine tissues at days 1 to 3 (Fig. 3 and Table 2) after in vivo administration of cyclophosphamide and dexamethasone. Using RNA-PCR analysis, ICP0 mRNA was detected in the trigeminal ganglia and adrenal glands from day 1 (Fig. 3a, d, lanes 10) and in the brain from day 2 (Fig. 3b, lane 11). However ICP0 mRNA was undetectable in the spinal cord (Fig. 3c, lanes 10 to 12). LAT_unspliced was detected during days 1 to 3, whereas LAT_spliced disappeared from the brain and adrenal glands on day 2 (Fig. 3b, c, lanes 7) and the trigeminal ganglia on day 3 (Fig. 3a, lane 8). LATs or ICP0 mRNA were not detected from the eyes, liver, kidneys or spleen during days 1 to 3. Murine β-actin RNA was detected in all samples examined (data not shown).

Discussion

Generalized neural infection during HSV recurrence has been recognized in clinical cases occasionally (Blyth & Hill, 1984; Whitley, 1990). However the mechanisms of recurrent generalized infection as well as the systematic distribution of the latent virus remain to be clarified. We first analysed viral spread during acute infection in mice inoculated with HSV-1 intracameraly. During the first 2 weeks p.i., infectious virus was detected in the eyes, trigeminal ganglia, brain and adrenal glands, but not in the liver, spleen or kidneys (Table 1). The results shown in Fig. 2 are mostly consistent with a previous study (Liu et al., 1993) and suggested that HSV-1 had spread via neural routes. However the transient peak on 1 day p.i., reported by Atherton & Streilein (1987), might be caused by haematogenous spread. In the present study, we analysed HSV latency and compared its distribution with virus spread during the acute phase. Later than 8 weeks p.i., latent HSV-1 was detected by explantation of the various neural tissues where acute infection had been observed, as reported following intravenous inoculation (Cook & Stevens, 1976). Establishment of latency in the eyes following corneal inoculation has been reported (Cook et al., 1991; Abghari et al., 1992), but we could not detect latent virus or LATs in the eyes as late as 8 weeks after intracameral inoculation. It was shown by RNA-PCR that neural tissues positive for HSV-1 LATs...
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were negative for ICP0 mRNA. The failure of detecting ICP0 mRNA, the first transcript during replicating infection (Roizman & Sears, 1990) further reinforced the establishment of viral latency in these tissues (Deatly et al., 1988).

Using PCR analysis (Fig. 1), we could differentially detect LATspliced and LATunspliced (Fig. 3). LATunspliced has been observed even during viral replication in infected cell culture (Spivack & Fraser, 1987; Wagner et al., 1988b). In the current study, LATunspliced could be detected in the neural tissues, i.e. the trigeminal ganglia, brain, spinal cord and adrenal glands of latently infected mice. It is possible that RNA–PCR using primers pLAT10 and pLAT13, or pLAT10 and pLAT15, simultaneously amplified an unspliced minor LAT (Fraser et al., 1992). LATspliced was also observed in all the LATunspliced-positive neural tissues except the spinal cord, the only neural tissue from which HSV failed to reactivate. The coincidence of viral reactivation and detection of LATspliced suggested the possibility that LATspliced might play a role in the establishment and/or maintenance of viral latency. Following in vivo administration of cyclophosphamide and dexamethasone as a stimulus for virus reactivation (Shimeld et al., 1990; Minagawa et al., 1993), LATspliced eventually disappeared as the expression of ICP0 mRNA increased, whereas LATunspliced was always detectable.

The role of LATS in latency or replication of HSV is still unclear (Fraser et al., 1992). Because the LATS overlapping the 3’ terminus of ICP0 mRNA are transcribed from the complementary DNA strand, LATS could function as antisense inhibitors of ICP0 transcription (Farrell et al., 1991). Some workers have proposed that the LAT gene might encode more than one species of putative proteins although LATS are not polyadenylated (Wechsler et al., 1988). Our results suggest that LATspliced of HSV-1, rather than LATunspliced, may have important roles in the maintenance of latency, or in the induction of reactivation. However the splicing of LATS studied here does not affect the potential

ethidium bromide. Template RNA samples were isolated from the murine trigeminal ganglia (a), brain (b), spinal cord (c), and adrenal glands (d) at latent phase (lanes 1, 5 and 9) and during reactivation phase on day 1 (lanes 2, 6 and 10), day 2 (lanes 3, 7 and 11) or day 3 (lanes 4, 8 and 12) following cyclophosphamide and dexamethasone administration. PCR amplification was performed to detect 297 bp of LATspliced using primers pLAT10 and pLAT13 (lanes 1 to 4), either 947 bp of LATunspliced or 389 bp of LATspliced using primers pLAT13 and pLAT10 (lanes 5 to 8), and 157 bp of ICP0 mRNA using primers P3 and P5 (lanes 9 to 12). These data are summarized in Table 2. Lane M represents HpaII-digested pUC18 as a size marker, indicating the positions of 501 and 489 bp, 404 bp, 331 bp, 242 bp, 190 bp, 147 bp, 111 and 110 bp, 67 bp and 34 bp, respectively.

Fig. 3. Detection of HSV-1 transcripts in various tissues of latently infected mice following in vivo drug administration. RNA–PCR products were electrophoresed on an agarose gel and stained with
transcripts (encoding functional proteins) overlapping
the complementary ICP0 gene or putative proteins
encoded by LATs (Stevens et al., 1987; Wechsler et al.,
1988; Spivack et al., 1991). In addition, LAT\textsubscript{spliced} was
not recognized during latent infection of HSV-2 (Mitchell
et al., 1990). Therefore LAT\textsubscript{spliced} might be merely a by-
product derived from alternative splicing of HSV-1
LAT\textsubscript{unspliced}.

We also detected the unusual splicing signal of LATs,
which had been reported for LATs from trigeminal
ganglia (Spivack et al., 1991), brain and adrenal glands.
The donor signal was identified as GC and the acceptor
signal as AG, different from the GT–AG consensus
(Jackson, 1991). The GC signal at the splicing donor site
rather than a GT signal might be recognized specifically
during the latency of HSV-1, not only in the trigeminal
ganglia (Spivack et al., 1991) but also in other multiple
tissues in the nervous systems where viral reactivation
was recognized. A GC donor splicing signal has been
reported in several other animal genes (Jackson, 1991).
The alternative GC splicing of LAT might be related to
regulatory functions in the latently infected neurons.

The selection of alternative splicing pathways
represents an important step in the genetic expression for
its regulation. In the neuronal cells, splicing patterns of
various neuropeptides are altered at different points of
development and potential (Latchman, 1990). Neuron-
specific alternative splicing was recognized in several
genes including a proto-oncogene c-src (Martinez et al.,
1987) and a clathrin light chain B gene (Stamm et al.,
1992). Recently, protein factors which control cell type-
specific alternative splicing have been identified
(Latchman, 1990; Ge & Manley, 1990; Ge et al., 1991).
The establishment of latency might be associated with
the expression of the specific splicing factors. In the
spinal cord where the splicing signal of LAT\textsubscript{unspliced} was
undetectable, viral latency could not be detected by
explantation. The possible factors involved in the splicing
signal of LATs should be analysed to understand the
molecular mechanisms of establishment and/or main-
tenance of latency.

In summary, we have shown the establishment of

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<th>Table 2. Detection of LATs and ICP0 mRNA using RNA–PCR in various murine tissues following the in vivo administration of cyclophosphamide and dexamethasone</th>
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<tbody>
<tr>
<td>Eyes</td>
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<tr>
<td>LAT\textsubscript{unspliced} Day 1–3</td>
</tr>
<tr>
<td>LAT\textsubscript{spliced} Day 1</td>
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<td>Day 2</td>
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<tr>
<td>ICP0 RNA Day 1</td>
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latency and reactivation of HSV-1 in multiple neural tissues where acute infection had occurred. The splicing signal of LATs might be conserved to act specifically during latency in these various neural tissues and might be turned off once reactivation occurred. This animal model should be useful in studying the molecular mechanisms of latency and reactivation of HSV-1.

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