Neuronal pathways for the propagation of herpes simplex virus type 1 from one retina to the other in a murine model

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Herpetic retinitis in humans is characterized by a high frequency of bilateral localization. In order to determine the possible mechanisms leading to bilateral retinitis, we studied the pathways by which herpes simplex virus type 1 (HSV-1) is propagated from one retina to the other after intravitreal injection in mice. HSV-1 strain SC16 (90 p.f.u.) was injected into the vitreous body of the left eye of BALB/c mice. Animals were sacrificed 1, 2, 3, 4 and 5 days post-inoculation (p.i.). Histological sections were studied by immunochemical staining. Primary retinitis in the inoculated eye (beginning 1 day p.i.) was followed by contralateral retinitis (in the un inoculated eye) starting at 3 days p.i. Infected neurons of central visual pathway nuclei (lateral geniculate nuclei, suprachiasmatic nuclei and pretectal areas) were detected at 4 days p.i. Iris and ciliary body infection was minimal early on, but became extensive thereafter and was accompanied by the infection of connected sympathetic and parasympathetic pathways. The pattern of virus propagation over time suggests that the onset of contralateral retinitis was mediated by local (non-synaptic) transfer in the optic chiasm from infected to uninfected axons of the optic nerves. Later, retinopetal transneuronal propagation of the virus from visual pathways may have contributed to increase the severity of contralateral retinitis.

Introduction

The most common features of ocular herpes simplex virus (HSV) infection are keratitis and anterior uveitis. Antiviral agents have reduced the severity of these diseases. However, herpetic retinitis still has a poor functional prognosis because it is often diagnosed late, and viral infection of the contralateral retina is usual. In acute retinal necrosis (ARN) syndrome, the most common form of herpetic retinitis in immunocompetent patients (Holland & Executive Committee of the American Uveitis Society, 1994; Duker & Blumenkranz, 1991; Culbertson & Atherton, 1993), the other eye is affected in some days to weeks after the first in 65% of cases if no antiviral drugs are prescribed (Palay et al., 1991). Strong iris and ciliary body inflammation rarely precedes by several days the onset of retinitis in the same eye (Culbertson & Atherton, 1993). Biological signs of inflammation are frequently detected on lumbar puncture and probably indicate an immune reaction to the presence of virus in the CNS. Of the two types of HSV, type 1 is the most frequent cause of ARN syndrome. Varicella-zoster virus is also frequently implicated (Duker & Blumenkranz, 1991; Culbertson & Atherton, 1993; Labetoulle et al., 1995).

Most experimental studies on herpetic retinitis have been based on anterior chamber inoculation of HSV-1 (Whittum et al., 1984; Shimeld et al., 1985; Whittum-Hudson & Pepose, 1987; Vann & Atherton, 1991), as in the von Szily model (von Szily, 1924). In these experiments, a necrotic viral retinitis develops almost exclusively in the uninoculated eye whereas the retina of the inoculated eye is uninfected or slightly infected (Whittum et al., 1984; Vann & Atherton, 1991; Dix et al., 1987; Pepose & Whittum-Hudson, 1987; Metzger & Whittum-Hudson, 1987). However, some studies showed a bilateral retinitis following anterior chamber inoculation. In
these experiments, mice were deeply immunosuppressed (Whittum-Hudson et al., 1985; Whittum-Hudson & Pepose, 1987; Atherton et al., 1989) or infected with a high dose of virus (up to \(2 \times 10^8\) p.f.u.) (Whittum-Hudson et al., 1987; Holland et al., 1987; Hemady et al., 1989; Liu et al., 1993) and exhibited severe infection of the iris and ciliary body in the inoculated eye.

We tried to replicate human bilateral herpetic retinitis as accurately as possible by studying the propagation of HSV-1 from one retina to the other in female BALB/c mice after intravitreal inoculation of a low dose of the highly virulent SC16 strain. Primary retinitis occurred in the inoculated eye without severe infection of iris and ciliary body early on; contralateral retinitis developed a few days later in the uninoculated eye.

HSV-1 propagates within the nervous system by transneuronal (synaptic) transmission between connected neurons as well as local (non-synaptic) transfer from infected neurons to adjoining glial cells and neurons (Kristensson et al., 1982; Ugolini et al., 1987; Ugolini, 1992). After inoculation into the vitreous body, HSV-1 could theoretically invade the CNS by primary and secondary visual pathways, as well as sympathetic and parasympathetic routes [see, e.g., Bienfang (1994) and Nieuwenhuys et al. (1988) for anatomical details]. The results obtained in the present study suggest that invasion of the contralateral retina was initially mediated by local spread of the virus at the level of the optic chiasm between the axons of ganglion cells derived from the two retinae. Transneuronal transfer of the virus in visual pathways also occurred but could not explain the early time-course of contralateral retinitis.

Methods

- **Cells and viruses.** The virus used in this study was the wild-type SC16 strain of HSV-1 (Hill et al., 1975) cultured in BSR cells, derived from baby hamster kidney-21 cells. Viruses were concentrated before titration as previously described (Coulon et al., 1989) and kept frozen at \(-80\) °C. A further titration in African green monkey kidney (Vero) cells was performed (Coen et al., 1985) after thawing and dilution (i.e. immediately before use). Plaques were counted the following day.

- **Animal experiments.** All experiments were carried out in 6-week-old BALB/c female mice (Janvier Breeding, France). All surgical procedures were performed aseptically under general anaesthesia with equithesin (Babic et al., 1993).

  Prior to the injection, a small sclerotomy was performed using a fine needle (3/8c, 0-6 mm, Ethicon) inserted just behind the limbus of the cornea in the superior and temporal quadrant of the left eye to give access to the vitreous body without damaging the retina. The HSV-1 suspension (0-5 µl) was then slowly injected into the vitreous body using a glass micropipette (tip diameter approximately 50 µm) connected to a pressure delivery device (Ugolini, 1992). The micropipette was slowly withdrawn while the edges of the injection point were gripped with forceps to minimize leakage of virus in the periorcular area. All surgical procedures were performed under visual guidance using a surgical microscope (Universal 53, Zeiss).

In order to minimize the infection of extracocular tissues, a low dose of virus was used in most experiments. Eighteen mice were inoculated with 90 p.f.u. of the SC16 strain of HSV-1. They were killed at 1 (\(n = 3\)), 2 (\(n = 3\)), 3 (\(n = 6\)), 4 (\(n = 3\)) and 5 (\(n = 3\)) days post-inoculation (p.i.). Five additional animals were inoculated with 10° p.f.u. and were killed at 8, 12, 18, 24 and 48 h p.i. Control mice were killed 2 days after mock-infection. Mice were transcardially perfused, and spinal cord and superior cervical ganglia were prepared as previously described (Babic et al., 1994). The skull was decalcified in PBS containing 0-1 M EDTA for 7 days at 4 °C and then stored in PBS containing 20% sucrose for 24 h at 4 °C. Tissues were frozen and cut into 30 µm frontal sections on a cryostat (Bright). Three parallel series of sections were collected on gelatine-coated slides. The first and the second series were used for immunoperoxidase staining using the peroxidase–antiperoxidase (PAP) method. The third series was kept in reserve. The PAP method was the same as previously described (Ugolini, 1992), with the exception that peroxidase activity was detected by incubation in a metal-enhanced diaminobenzidine kit as substrate (Pierce). The sections were counterstained (Giemsa blue or neutral red), washed, dried and mounted with Entellan (Merck).

For three mice sacrificed at 3 and 4 days, the second series was used for an avidin–biotin complex (ABC) immunostaining method (Vector) in order to clearly differentiate the signal from background (see below). Sections were incubated in (i) 1% Triton (30 min at room temperature), (ii) rabbit polyclonal antibody anti-HSV-1 Macthryte (Dako) in PBS (1:200 dilution, overnight at 4 °C), (iii) goat serum (Dako) in PBS (1:50 dilution, 30 min at room temperature), (iv) goat biotinylated anti-rabbit antibody (Vector) in PBS (1:150 dilution, 30 min at room temperature), (v) avidin D in 0-1 M sodium bicarbonate + 0-15 M NaCl (10 µg/ml, 30 min at room temperature) and (vi) biotinylated β-galactosidase (Vector) in PBS + 1 mM MgCl₂ + 1 mg/ml BSA (1 U/ml, 30 min at room temperature). The sections were washed three times in PBS between each step, except between steps (v) and (vi) in which a 0-1 M sodium bicarbonate + 0-15 M NaCl solution was used. The β-galactosidase activity was detected by incubation for 3 h at 37 °C with β-galactosidase substrate (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) (Babic et al., 1994). Sections were then counterstained with neutral red and mounted as described above.

The distribution of HSV-1 labelled elements was studied in light microscopy. For each animal, around 400 sections were stained and examined. The amount of infection was assessed for each section in two independent sessions of examination. Moreover, the slides were randomly examined to limit the subjective part of the evaluation. In the early time of infection, infected cells were counted on each section. When infection progressed, they formed foci in which the number of labelled cells could not be accurately quantified. For these reasons, we summarized the results on a semi-quantitative scale: 1*, mild infection (few and well separated infected cells); 2*, moderate infection (numerous and adjacent infected cells, but unlabelled areas still present within the structure); 3*, severe infection (numerous foci of infection with tissue necrosis, covering all the structure); 4*, infection of the entire area of the fundus (concerning only the retina).

Results

Eighteen mice received 90 p.f.u. of the SC16 strain into the vitreous body of the left eye and were euthanized at various time after inoculation (1–5 days). Complete skulls, spinal cords and superior cervical ganglia were cut into 30 µm frozen sections. Two-thirds of the sections were immunolabelled for...
HSV-1 infection. The results of immunochemical staining studies are summarized in Figs 1 and 2 and in Table 1.

**Retinitis in the inoculated (left) and uninoculated (right) eyes**

HSV-1 immunostaining was first observed in retinal ganglion cells of the inoculated eye in two out of three mice at 1 day p.i. The following day, labelled ganglion cells were numerous in all mice (552, 261 and 52 cells respectively). Somata and dendrites were strongly stained, but axons (in the optic nerve fibre layer) were only weakly labelled. In two mice, the infection involved also the inner nuclear layer (546 and 243 cells), which contains the cell bodies of bipolar, amacrine, horizontal and Müller cells (Dacheux & Raviola, 1994). In one mouse, the somata of photoreceptor cells (outer nuclear layer) were infected. At 3 days p.i., the retinitis was multifocal. Infected cells in the ganglion cell and inner nuclear layers were no longer separated (Fig. 3A). Some staining consistent with infection of Müller cells was also detectable. Infected photoreceptor cells were numerous in three out of six mice. At 5 days p.i., the retinitis covered almost the entire area of the fundus. All retinal nuclear layers were strongly infected.

The uninoculated eye showed no sign of infection until 3 days p.i. At that time a faint signal was observed in the retina after immunoperoxidase staining. Since this signal was difficult to differentiate from background, we used an ABC immunostaining method (Vector) with β-galactosidase as chromogen on one-third of the sections from three of the six mice sacrificed at 3 days p.i. In our hands, this method was less sensitive but more specific than the immunoperoxidase method because it produced no tissue background staining. We observed two, three and four infected ganglion cells, respectively, in the uninoculated eye of the three mice (Fig. 3B). At 4 days p.i., several ganglion cells were labelled in all mice and the inner nuclear layer cells were infected in one of them (Fig. 3C). This indicated that a second viral cycle had already occurred in the uninoculated eye. At 5 days p.i., infected ganglion cells were randomly scattered over the entire surface of the retina. The inner nuclear layer was labelled, mostly below the infected ganglion cells. Some photoreceptor somata were infected in one mouse.

**Infection in visual pathways**

The left optic nerve was first labelled at 3 days p.i. in two out of six mice. In these animals, numerous glial cells were infected in the left optic nerve and also in the optic chiasm (Fig. 4A–D), showing that local transfer of the virus from infected axons to glial cells had already occurred (Ugolini, 1992). At this time, infection of central visual pathways was observed only in one among six mice. The right lateral geniculate nucleus (LGN) was mildly labelled (88 cells in the dorsal part and 18 cells in the intermediate leaflet) and some infected cells were found in the right pretectal and tectal areas (superior brachium of the colliculus, optic nerve layer of the superior colliculus and superficial grey layer of the colliculus). At 4 days p.i., brain infection was present in all mice. Both the suprachiasmatic (SCh) nuclei were symmetrically infected. In two among three animals, all subdivisions of the SCh were already labelled whereas only the medial parts were infected in the third animal. The LGN (dorsal, intermediate and ventral parts), the supraoptic nuclei and the superior colliculi and pretectal areas were also infected bilaterally, although much
Fig. 2. Distribution of HSV-1-positive neurons in the brain at different time-points. Infected areas are stippled. Numbers in parentheses indicate mean delay to detection of viral antigens (days p.i.). Black areas are brain ventricles. Abbreviations: AMPO, anteromedial preoptic nucleus; AP, anterior pretectal nucleus; Aq, Sylvius aqueduct; AVPO, anteroventral preoptic nucleus; bsc, brachium superior colliculus; DLG, dorsal lateral geniculate nucleus; EW, Edinger–Westphal nucleus; IGL, intergeniculate leaflet (lateral geniculate nucleus); LS, lateral septal nucleus; LV, lateral ventricle; MnPO, median preoptic nucleus; MPA, medial preoptic area; OC, optic chiasm; Op, optic nerve layer superior colliculus; OPT, olivary pretectal nucleus; opt, optic tract; OT, optic tract nucleus; OVC, occipital visual cortex; Pa, paraventricular hypothalamic nucleus; Pe, periventricular hypothalamic nucleus; PG, pituitary gland; SCh, suprachiasmatic nucleus; SO, supraoptic nucleus; Sp5, spinal trigeminal tract; SuG, superficial grey layer of the superior colliculus; TG, trigeminal ganglion; V3, third ventricle; V4, fourth ventricle; VLG, ventral lateral geniculate nucleus.

Infection of the iris, ciliary body and autonomic pathways

HSV-1 immunostaining in the iris and the ciliary body of the inoculated eye was restricted to a few cells in two out of three mice at 2 days p.i. At 3 days p.i., only two out of six mice showed a pronounced infection of these structures. In these animals, infection also occurred in the left ciliary ganglion (parasympathetic relay) but the left superior cervical ganglion (sympathetic relay) was infected only in one mouse. No infection occurred in central autonomic pathways [the parasympathetic Edinger–Westphal nuclei and the sympathetic intermediolateral cell group (IML) in the spinal cord]. At 4 and 5 days p.i., some iris and ciliary body infection occurred in all mice but was moderate in a half of them. Infection of central autonomic structures occurred only in mice in which iris and ciliary body were heavily infected.

Iris and ciliary bodies were not labelled in the uninoculated eye.

The picture of infection during the first 2 days p.i. was quite similar in the mice inoculated with $10^5$ p.f.u. except that the
Fig. 3. Distribution of labelled retinal cells. (A) 3 days p.i., inoculated eye; labelled retinal cells (immunoperoxidase staining) are clustered in triangular columns with a large base, suggesting that local transfer occurred between ganglion cells (arrow). The inner nuclear layer (INL) is infected. Magnification: 200×. (B) 3 days p.i., uninoculated eye; one ganglion cell (arrow and insert) labelled with the ABC-β-galactosidase method. The INL is not infected. Magnification: 500× (insert: 1250×). (C) 4 days p.i., uninoculated eye; ganglion cells (arrow) and INL cells are infected. Magnification: 200×.

infection in the iris and the ciliary body was readily detectable at 1 day p.i. and was heavy at 2 days p.i.

There was no HSV-1 labelling in mock-infected mice.
### Table 1. Quantification of immunochemical labelling in infected areas (observation on serial sections)

Semi-quantitative scale: 1*, mild infection (few and well separated infected cells); 2*, moderate infection (numerous and adjacent infected cells, but unlabelled areas still present within the structure); 3*, severe infection (numerous foci of infection with tissue necrosis, covering all the structure); 4*, infection of the entire area of the fundus (only concerning the retina).

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<th>Time after infection (days)</th>
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<td>Time after infection (days)</td>
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Discussion

We studied the propagation of HSV-1 strain SC16 in mice after injection into the vitreous body of the left eye. Histological sections were studied by immunochemical staining. BALB/c mice were chosen because of their high susceptibility to HSV-1 infection (Lopez, 1975; Pepose & Whittum-Hudson, 1987) and in order to enable comparison with previous studies on experimental herpetic retinitis (Whittum et al., 1984; Atherton et al., 1987; Metzger & Whittum-Hudson, 1987; Vann & Atherton, 1991). The use of inbred mice was also aimed at minimizing individual variability of the results (Lopez, 1975). The high neurovirulence of the SC16 strain (Hill et al., 1975; Shimeld et al., 1985; Ugolini et al., 1987) made it possible to obtain a bilateral retinitis in all mice. When a less neurovirent strain is used, like the KOS strain (Thompson et al., 1986), bilateral retinitis does not always occur even after inoculation of much higher doses of virus (Atherton et al., 1987; Pepose & Whittum-Hudson, 1987; Metzger & Whittum-Hudson, 1987). Similar differences between the SC16 and KOS strains were observed following anterior chamber inoculation of high doses of virus (Whittum-Hudson et al., 1987).

Our inoculation procedure, based on the use of a glass micropipette connected to an adjustable pressure delivery device (Ugolini, 1992), made it possible to inject small volumes (0.5 µl) and minimize leakage from the vitreous body. The low titre of the inoculum (90 p.f.u.) was aimed at reducing the risk of non-retinal tissue infection. Despite these measures, a slight infection of the iris and ciliary body occurred in 50% of the animals sacrificed before 3 days p.i. but such infection was not pronounced until 5 days p.i., i.e. several days after contralateral retinitis had consistently developed in all mice. Experiments using 10^3 p.f.u. in 0.5 µl induced a heavier infection of iris and ciliary body in the inoculated eye, but no more labelled retinal ganglion cells at 1 day p.i. in comparison with inoculation of 90 p.f.u. Thus, the use of a high titre of the inoculum reduced the specificity of intravitreal inoculation by facilitating the diffusion of the virus from the vitreous body to the anterior chamber without increasing the initial involvement of retinal cells.

Retinal ganglion cells were the first site of virus replication. The virus propagated through ganglion cell dendrites reaching first the inner nuclear layer (2 days p.i.) and then the outer nuclear layer (3 days p.i.). Therefore, three successive cycles of infection occurred during this period of time, indicating that a complete viral cycle including transneuronal transfer in the retina took about 24 h. The distance between retinal cells is very short. In case of more distant neurons, the complete viral cycle probably takes longer due to the time required for axonal transport of the virus.

The anterograde (centrifugal) propagation of viral material in ganglion cell axons was directly detectable by labelling of the optic nerve fibres and the connected nuclei of visual pathways in the brain. Infection of the LGN, tectal and pretectal areas was consistently more extensive on the right side (Fig. 5B). This is in keeping with the predominantly crossed retinal input to these structures in rodents (Fig. 4A–C) (Pickard, 1982). Such a decussation is particularly pronounced in albinism (as in BALB/c mice) (Apkarian et al., 1984). The symmetrical labelling of the SC1 is explained by their symmetrical connections to both retinae (Hendrickson et al., 1972; Moore & Lenn, 1972; Pickard, 1982, 1985). This can also explain the bilateral infection of other hypothalamic nuclei which are connected with the SC1 (Pickard, 1982; Youngstrom et al., 1987). As in other studies of the propagation of HSV-1 or pseudorabies virus (PrV) from the vitreous body, the labelling of the occipital cortex was not reproducible (Norgren et al., 1992; Card et al., 1991). This result is probably due to an impairment of anterograde transport and transneuronal transfer of HSV-1 over long distances related to virus-induced degeneration of the transporting infected neurons, as shown for the SC16 strain in other models (Ugolini et al., 1989; Ugolini, 1995).

Virus propagation was also observed in non-visual structures. The infection of autonomic pathways occurred only after heavy infection of the iris and ciliary body, which is consistent with results obtained following anterior chamber inoculation of HSV-1, PrV and rabies virus (Vann & Atherton, 1991;
Labelling of the locus coeruleus at 5 days p.i. is probably related to its multiple connections to infected structures, including the LGN (Kromer & Moore, 1980). Pituitary gland infection may result from its connections with the hypothalamus (Kelly & Svanson, 1980; Kiss et al., 1984).

As the infection progressed, infected glial cells appeared around positive neurons. Such local transfer from neurons to glial cells has been reported for alphaherpesviruses in several models [see, e.g., Card et al. (1991); Norgren et al. (1992); Ugolini et al. (1987); Ugolini (1992)]. As in other studies (Holland et al., 1987), we observed staining of retinal Müller cells. Infection of these cells, vital to retinal metabolism (Newman, 1994), together with virus replication in neuronal cells is probably responsible for the dramatic retinal necrosis observed after HSV-1 infection. Infected glial cells were also detected in the optic chiasm as early as 3 days p.i. This may have important implications, as discussed below.

Previous findings following inoculation of HSV-1 strain KOS into the anterior chamber led to the conclusion that the SCh nucleus had a key role in events leading to contralateral retinitis (Vann & Atherton, 1991). In such experiments, inoculation resulted in iris and ciliary body infection in the absence of ipsilateral retinitis, followed by labelling first of the ipsilateral Edinger–Westphal nucleus (3 days p.i.), and later of the ipsilateral SCh (5 days p.i.). Contralateral retinitis developed 2 days later, at the same time as infection of the LGN and hypothalamic nuclei (other than the ipsilateral SCh).

In the present study, intravitreous inoculation of the SC16 strain resulted in ipsilateral retinitis at 1 day p.i. and contralateral retinitis starting at 3 days p.i. The delay in virus propagation from the inoculated eye to connected central visual structures (at least 2 days) and the fact that these structures were consistently infected only at 4 days p.i. when two cycles of virus replication had already occurred in the contralateral retina (Fig. 3 C) argue against the possibility that retinoepital propagation from central visual structures (including the SCh nucleus) may be responsible for the onset of such retinitis. The same applies to autonomic pathways, in view of delay in the start of their infection. A more likely explanation for the onset of contralateral retinitis is the occurrence of local transfer of virus in the optic chiasm or optic tracts, i.e. from the axons of ganglion cells of the inoculated eye to axons of ganglion cells of the other eye. Indeed, local transfer of HSV-1 from transporting axons can cause the infection of adjoining (not synaptically connected) neuronal elements (Ugolini et al., 1987; Ugolini, 1995), and viral material was detected in axons and glial cells in the left optic nerve and the optic chiasm at 3 days p.i. (Fig. 4 D).

In conclusion, the timing of virus propagation suggests that contralateral retinitis in our model was initiated by local transfer of the virus between ganglion cells axons at the optic chiasm. Later, virus propagation from central visual pathways could have contributed to the dramatic increase in the infection of the uninoculated eye. Together with previous studies (Whittum et al., 1984; Vann & Atherton, 1991; Margolis et al., 1989), these results help to improve understanding of the pathogenesis of ARN syndrome. In addition, the present study provides a highly reproducible animal model which could serve to test the efficacy of antiviral drugs or to determine the function of specific viral genes, as has been done in other models for HSV-1 and PrV (Card et al., 1992; Babic et al., 1993, 1994; Liu et al., 1993; Enquist et al., 1994; Balan et al., 1994; Dingwell et al., 1994, 1995; Sun et al., 1996). We plan to use this murine model to test the propagation of HSV-1 mutants carrying deletions of genes that may be involved in virus propagation in the CNS.

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