Possible latent infection with herpes simplex virus in the mouse eye

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Herpes simplex virus (HSV) was isolated from organ cultures of anterior segments of the eyes of mice inoculated with virus on the snout or directly onto the cornea at least 5 weeks previously. The frequency of isolation of the virus was not decreased by treatment of the animals with acyclovir, suggesting that the virus is latent by the criteria usually applied. Peroxidase-antiperoxidase staining of organ cultures that had shed virus showed that viral antigens were predominantly present in the anterior uvea. Inoculation of mouse eye anterior segments in vitro showed that this tissue was the most susceptible to productive infection. These results suggest the possibility that HSV can establish a latent infection in tissues of the anterior segment of the mouse eye.

Introduction

Herpes simplex virus (HSV) is known to produce a variety of clinical syndromes in the human eye (Easty, 1985; O'Day & Jones, 1985). The commonest consists of a corneal inflammation (keratitis) due to recrudescent disease. When limited to the superficial epithelium, the pathogenesis appears to be simple viral cytopathogenesis resulting in ulceration (O'Day & Jones, 1985; Collum et al., 1986). However, not infrequently, further episodes of disease result in the involvement of the deeper corneal tissue (stroma) producing a chronic stromal keratitis. This condition results in corneal scarring and neovascularization, with a corresponding reduction in visual acuity.

Histological studies of the infection in humans have been restricted to corneal buttons removed at the time of corneal grafting. Such surgery is usually delayed until the eye is not inflamed, so that only chronic inflammatory changes and scar tissue are seen with little evidence of specific pathogenic mechanisms. However, the presence of viral antigens (Metcalf & Kaufman, 1976; Meyers-Elliot et al., 1980) and complete or incomplete viral particles (Metcalf & Kaufman, 1976; Meyers-Elliot et al., 1980; Dawson et al., 1968; Jones et al., 1977; Ahonen et al., 1984) have been reported, and HSV has occasionally been isolated after organ culture of the cornea (Shimeld et al., 1982; Tullo et al., 1985; Coupes et al., 1986; Cook et al., 1986; Easty et al., 1987). Thus the lack of data available from humans has both hindered rational therapy and prompted studies in experimental animals.

In most experimental studies corneal inoculation, often with scarification (Metcalf et al., 1979) or intrastromal injection (Meyers & Pettit, 1983; Maudgal et al., 1984), has been used. However, inoculation on the snout allows virus to accede to the eye via neural pathways as a result of zosteriform spread (Blyth et al., 1984; Simmons & Nash, 1984; Anderson & Field, 1984; Claoué, 1986; Claoué et al., 1987a, b). This more closely resembles pathways thought to be important in recrudescence disease in humans (Falcon, 1985) and avoids trauma to the cornea during inoculation. We have previously reported preliminary observations that HSV can be isolated from eyes after organ culture long after inoculation (Claoué et al., 1987c).

Observations reported here, using either the zosteriform spread model or direct inoculation of the cornea with HSV, provide evidence that the virus may establish a latent infection in ocular tissue as well as the sensory ganglia of the mouse. This provides a further example that the virus may be able to establish latency in extraneural sites (reviewed in Hill, 1985).

Methods

Mice, inoculation procedures and preparation of tissues. All mice were 6 week old NIH inbred males from the colony in the Department of Microbiology. All were examined using the slit lamp before inoculation; any mouse with an ocular abnormality was excluded from experiments. All experiments were performed with HSV/SC16 (type 1) (Hill et al., 1975) which was assayed in Vero cells and stored at −70 °C. For zosteriform spread, the left-hand side of the tip of the snout was
shaved and inoculated with $10^9$ p.f.u. of virus by scarification (Claoué et al., 1987a). For corneal inoculation, a 10 μl drop of virus suspension containing $10^3$ p.f.u. was placed on the cornea which was then scarified 10 times using a 25-gauge needle.

Mice were killed by cervical dislocation except when the eyes were required for histology, when the animals were killed by an intraperitoneal (i.p.) injection of 0.25 ml of 60 mg/ml sodium pentobarbital because cervical dislocation was found occasionally to produce hyphaemas.

Eyes were enucleated after removing the globe and then opening the conjunctiva with scissors. The eyes were divided sagittally at the equator. The corneo-scleral button and attached anterior uvea constituted the anterior segment (AS).

**Isolation of virus.** Infectious virus was isolated after grinding tissues and freezing and thawing three times as described previously (Claoué et al., 1987c). For culture, AS and in one experiment posterior segments (PS) of eyes were placed in 1 ml of culture medium in bijou bottles and incubated at 35°C. All media contained 25% foetal calf serum and, when required, 1 μg/ml thymidine. The medium was changed completely every 48 h. Samples of medium (100 μl) were removed daily when required, 1 μg/ml thymidine. The medium was changed completely every 48 h. Samples of medium (100 μl) were removed daily for 17 days and placed on monolayers of Vero cells to test for virus growth.

Trigeminal ganglia (TG) were cultured singly for 7 days. On one occasion the ganglia were divided (Tullo et al., 1982) so that the ophthalmic division alone could be cultured. The tissues were then ground and frozen and thawed as above before suspensions were transferred on to Vero cells for virus assay.

**Histology.** Specimens for histology were fixed and prepared as described previously (Claoué et al., 1987a). Slides were stained for HSV antigens using a peroxidase-antiperoxidase (PAP) technique (Claoué et al., 1987a).

**Acyclovir treatment.** Mice were given acyclovir (ACV) 1 g/l in drinking water ad libitum for a 10 day period. On each of the 3rd, 4th and 5th days they also received two i.p. injections of 1.5 mg ACV in 0.1 ml phosphate-buffered saline (PBS). Placebo-treated mice received tap water to drink and injections of PBS instead of ACV.

**Acyclovir sensitivity of isolates.** Isolates of HSV and the parent HSV/SC16 were assayed in triplicate for sensitivity to ACV by the plaque reduction assay. Doubling concentrations of ACV from 0.5 to 4 μg/ml were used.

**Results**

**Isolation of HSV from TGs and AS of eyes from latently infected mice**

Mice were inoculated with virus either on the left cornea or on the skin of the snout. When primary disease had subsided, attempts were made to isolate virus from TGs and AS of eyes on the inoculated side. Details of numbers of mice, routes of inoculation and intervals between inoculation and tests on tissues are shown in Table 1. Latent infection was almost always established in the TG after inoculation on the cornea (Table 1, line 1) or on the skin of the snout (line 2) and virus was regularly isolated after culturing ganglia in vitro. After inoculation on the snout, latent infection was demonstrated in the ophthalmic division of the TG.

Attempts were then made to isolate virus from AS of eyes. The first mice tested had been inoculated on the snout and had scarred and vascularized corneas at the time when tissues were taken. Virus was not isolated from AS homogenized immediately after removal (line 3). However, when AS from clinically similar mice (but inoculated on the cornea) were cultured as explants, virus was isolated from three of 15 tissues (line 4).

The eyes from 12 mice, inoculated on the snout 286 days previously, which had developed scarred and vascularized corneas following primary disease were divided into AS and PS and both pieces were cultured as explants. HSV was isolated from only one PS (after 17 days in culture) whereas it was isolated from four of the AS (lines 6 and 7). The PS that shed virus came from an eye whose AS also yielded virus. Histological study of this PS showed no readily identifiable tissue but other samples which had not shed virus contained recognizable retinal architecture. By contrast, even after culture, no virus was isolated from AS of 35 animals that had not shown eye disease after inoculation on the snout (line 5).

The results of virus isolation from cultured AS were analysed to determine whether there was a relationship between the probability of virus isolation and the interval between inoculation and testing (Table 1, lines 8 and 9). Whereas HSV was isolated from 17.6% of AS cultured 122 to 368 days after inoculation, only 5.3% yielded HSV when culture was commenced 41 days after inoculation. Comparison of these incidences by χ² testing gave a P value of 0.03.

**Effect of treatment with ACV on isolation of virus**

Because the explants that yielded virus were from clinically abnormal eyes, the possibility of chronic active infection existed. Therefore attempts were made to eliminate such infection by treating the animals with ACV. The mice used had been inoculated on the snout 142 to 156 days previously. Only animals with scarred and vascularized corneas were selected for the experiment. On the 10th day of treatment with ACV animals were killed and TG and AS were cultured in vitro. To determine whether ACV treatment diminished the likelihood of virus isolation by virtue of drug 'carry-over', 20 TG from treated animals were cultured in the presence of 1 μg/ml of thymidine (Table 2). All TG yielded virus, as did five of 45 AS (11%) from animals treated with ACV and three of 35 AS (8%) from those not given the drug.

**Sensitivity to ACV of isolates**

The virus isolates from the experiment where animals were treated with ACV were compared to HSV/SC16 for
### Table 1. Isolation of HSV from tissues of latently infected mice

<table>
<thead>
<tr>
<th>Line</th>
<th>Route of inoculation</th>
<th>Days from inoculation to explantation</th>
<th>Tissue explanted</th>
<th>Days in culture to virus isolation (mean)</th>
<th>Virus isolated/number tested (%)</th>
<th>Abnormal corneas at time of excision*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>Cornea</td>
<td>60</td>
<td>TG</td>
<td>4 to 8 (6)</td>
<td>15/15 (100)</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>Snout</td>
<td>286 to 722</td>
<td>TG</td>
<td>10 to 13 (11)</td>
<td>5/45 (11)</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>Snout</td>
<td>71</td>
<td>Ophthalmic division</td>
<td>Homogenized immediately for 15 days</td>
<td>0/54</td>
<td>No</td>
</tr>
<tr>
<td>4†</td>
<td>Cornea</td>
<td>60</td>
<td>AS</td>
<td>10 to 13 (11)</td>
<td>3/15 (20)</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>Snout</td>
<td>123 to 272</td>
<td>AS</td>
<td>6 to 14 (10-25)</td>
<td>4/12 (33)</td>
<td>Yes</td>
</tr>
<tr>
<td>6‡</td>
<td>Snout</td>
<td>286</td>
<td>AS</td>
<td>17</td>
<td>12/68 (17.6)</td>
<td>Yes</td>
</tr>
<tr>
<td>7‡</td>
<td>Snout</td>
<td>286</td>
<td>AS</td>
<td>ND§</td>
<td>2/38 (8-5)</td>
<td>Yes</td>
</tr>
<tr>
<td>8</td>
<td>Snout</td>
<td>122 to 368</td>
<td>AS</td>
<td>ND</td>
<td>0/54</td>
<td>No</td>
</tr>
<tr>
<td>9</td>
<td>Snout</td>
<td>41</td>
<td>AS</td>
<td>ND</td>
<td>0/20</td>
<td>No</td>
</tr>
</tbody>
</table>

* Abnormal corneas were all scarred and vascularized.
† Data in lines 1 and 4 are derived from the same animals.
‡ Data in lines 6 and 7 are derived from the same animals.
§ ND, Not determined.

### Table 2. Isolation of HSV from tissues of latently infected mice after treatment with ACV

<table>
<thead>
<tr>
<th>Treatment of mice</th>
<th>Tissue explanted</th>
<th>Days (mean) in culture to virus isolation*</th>
<th>Virus isolated/number tested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACV†</td>
<td>TG</td>
<td>7</td>
<td>40/40 (100)</td>
</tr>
<tr>
<td>ACV</td>
<td>AS</td>
<td>7 to 14 (8-6)</td>
<td>5/45 (1-1)</td>
</tr>
<tr>
<td>Placebo</td>
<td>TG‡</td>
<td>7</td>
<td>20/20 (100)</td>
</tr>
<tr>
<td>Placebo</td>
<td>AS</td>
<td>All on day 7</td>
<td>3/35 (8-6)</td>
</tr>
</tbody>
</table>

* Ganglia were cultured for 7 days then ground and assayed for infectious virus.
† ACV was administered as 1 g/l in the drinking water for 10 days, and 1.5 µg i.p. twice a day on days 3 to 5.
‡ Assayed in culture medium containing 1 µg/ml of thymidine.

Sensitivity to the drug, and by restriction enzyme analysis of their DNA. The ED50 values for the isolates from ACV-treated mice were 1.15, 1.40, 1.75, 2.10 and 2.35 µg/ml (mean 1.78); those for isolates from untreated mice were 0.50, 1.12 and 2.13 µg/ml (mean 1.25); and that for HSV/SC16 was 0.88 µg/ml.

**Detection of viral antigens in AS**

Serial sections of five whole eyes were examined after PAP staining immediately upon removal from animals 120 days after inoculation on the snout. None showed specific staining for viral antigens although care had to be taken not to misinterpret haemosiderin in macrophages as specific staining. The three AS from mice inoculated on the cornea which shed virus in culture (Table 1, line 4) were taken for histology immediately virus was found (two on day 13, one on day 14). Three from the same group which had not shed virus were taken on day 17 of culture. In addition the eight explant cultures of AS from snout-inoculated, ACV-treated animals which shed virus in culture, and two each from the treated group and placebo groups that had not shed virus, were taken either when virus was detected or after at least 17 days in culture. All tissues were examined histologically by haematoxylin and eosin and PAP staining.

All specimens contained the cornea with surrounding sclera and anterior uveal tissue which appeared healthy by morphological criteria. PAP staining revealed HSV antigens in all samples that had shed virus. Specific staining was always present in the anterior uvea (iris and ciliary body) and occasionally in limbal keratocytes (Fig. 1). It was never seen in specimens that had not shed virus.

**Inoculation of AS with HSV in vitro**

Anterior segments of eyes from uninfected 4 week old mice were cultured singly, each in 1 ml of medium containing 1 × 10^4 p.f.u. of HSV/SC16. Similar AS, cultured without virus, served as controls. Infectious virus in the cultures was assayed by transferring 0-1 ml of medium to Vero cell cultures. It was present in all of the
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Fig. 1. Diseased AS stained using PAP technique after organ culture. HSV antigens stain darkly and are present in the base of the ciliary body and part of the posterior leaflet of the iris. Bar marker represents 100 μm.

Fig. 2. Normal AS inoculated in vitro with HSV then incubated in organ culture for 2 days and stained using PAP technique. Viral antigens are present in the iris, predominantly in the posterior leaflet. Bar marker represents 100 μm.

Discussion

Ocular herpetic infection after direct corneal inoculation or following zosteriform spread produced a permanently damaged eye in which the neovascularization and corneal scarring resembled that seen in chronic stromal keratitis caused by HSV in humans (Easty, 1985). The histological evidence of inflammatory cells in the cornea (Clauè et al., 1987a) suggested that a possible source of the virus isolated after culture of AS of eyes might be a chronic replicative infection of the ocular tissues. However, this source is unlikely for a number of reasons. Eye washings taken immediately before culture of AS never yielded virus (data not shown) which suggests that the corneal surface had no active infection. Again, no virus was isolated when AS were ground and frozen and thawed (thus destroying all living cells) immediately after removal from the animal. Indeed, the time for which it was necessary to culture AS before infectious virus could be demonstrated was longer than that required for virus to become apparent from latently infected TG. This time interval alone is usually taken as satisfactory evidence that the tissue under investigation is latently rather than persistently infected. Thus the virus may have originated from a latent infection within the tissue of the eye as has been suggested in other experimental models (Shimeld et al., 1982; Cook & Brown, 1986, 1987; Cook et al., 1987; Abghari & Stulting, 1988). Furthermore, results were similar whether, during the primary disease, virus reached the eye via neural pathways or by direct corneal inoculation. The suggestion that HSV may establish latency in ocular tissue is further supported by the finding that HSV could be isolated from the AS of mice treated systemically with ACV. Such treatment also failed to eliminate latent infection with HSV-2 from the mouse footpad (Clements & Subak-Sharpe, 1988; Al-Saadi et al., 1988). The doses used in our study are, pro rata, known to produce therapeutic levels of intraocular ACV in humans (Hung et al., 1984) and similar ACV therapy had no effect on the recovery of HSV from latently infected TG (Field et al., 1979; Blyth et al., 1980). Finally, all the isolates of HSV from mice treated with ACV were shown to be fully sensitive to ACV in vitro, supporting the hypothesis that ACV therapy during latency does not produce a drug-resistant population of HSV as might occur in the presence of a low-grade chronic replicative infection.

The work of Subbuga et al. (1988) demonstrates that HSV nucleic acid can persist in the ocular tissue of experimental animals when the primary infection has subsided and this could represent HSV in a latent state. Our findings that the isolates of HSV from cultured AS were identical to the strain used for the original inoculation as defined by restriction endonuclease

20 inoculated cultures assayed on day 2 and in the three assayed on day 7. It was not found in samples from uninoculated cultures (seven on day 2, one on day 7). On each day of culture (from days 1 to 7) at least two inoculated AS and one uninoculated sample were taken for histological examination. All samples contained corneo-scleral and anterior uveal tissues which, by morphology, appeared relatively normal except for thinning of the corneal epithelium to a monolayer by day 5. No specific staining for HSV antigens was seen by PAP stain of control cultures. Inoculated AS showed viral antigens in corneal endothelial and anterior uveal cells at 24 h after infection, with limbal keratocytes also staining after 72 h (Fig. 2). Staining was never seen in the corneal epithelium.
analysis (data not shown) are in agreement with those of Darville et al. (1987) who studied virus strains isolated from recrudescent lesions in the skin. They also agree with those of Caudill et al. (1986), although in the latter experiments (where HSV shedding was activated by iontophoresis) the source of virus could equally have been from the latently infected TG. Such latent infection was also a potential source of virus in the experiments reported here because all animals carried the virus in the TG. However, no specific stimuli were given to induce reactivation of infection and spontaneous reactivation is known to be very rare in mice (Hill et al., 1981; Sekizawa et al., 1980), so this source can be discounted.

No obvious explanation is known for the finding that HSV was isolated more frequently from eyes assayed more than 122 days after inoculation than from those tested at earlier times. If latent infection in the eye is established during primary infection, this observation may suggest a weakening of the control of latency with time so that virus is more easily detected. Alternatively, if latency in the eye becomes established after the occasional reactivation of virus in the TG, an increasing incidence of isolation with time would be expected.

We were unable to repeat the finding of Openshaw (1983) that HSV antigens were seen in the retina after culture of eyes. We isolated HSV far more frequently from AS than from PS and histology showed viral antigens most frequently in uveal tissue. Furthermore, in the one case where HSV was isolated from PS tissue, we cannot exclude contamination with tissue from the AS (which also showed viral antigens). Although our findings are similar to those of Abghari & Stulting (1988), these workers inoculated their mice directly onto the cornea and cultured only this tissue. Thus, in their experiments, the viral genome must have been present in the cornea. Nevertheless the proportion of samples from which virus was recovered is very similar to our results.

Immunohistochemical examination of cultured AS which yielded HSV suggests that virus is present most often in the anterior uveal tissue. This shows an interesting similarity with the acute infection, where HSV replicates predominantly in this tissue (Claoué et al., 1987a). However, the fact that the same ocular tissue appears to be the most susceptible to viral replication when AS are inoculated in vitro raises the possibility that virus is latent in some other tissue in the AS and merely infects the anterior uvea when latent virus is reactivated.

Although HSV is widely considered to establish latency only in neural tissue, evidence for extraneural latency in skin and footpad tissue of laboratory animals has been accumulating (reviewed by Hill, 1985; Clements & Subak-Sharpe, 1988; Al-Saadi et al., 1988). The isolation of HSV from AS of diseased mouse eyes after culture supports results of other workers using mice (Openshaw, 1983; Shimeld et al., 1982) and rabbits (Cook et al., 1987; Cook & Brown, 1986, 1987; O'Brien & Taylor, 1989). Moreover, the observations are not confined to experimental infections as is made clear from studies on human corneal buttons removed at the time of penetrating keratoplasty for stromal herpetic keratitis (Shimeld et al., 1982; Tullo et al., 1985; Coupes et al., 1986; Cook et al., 1986). Indeed, the number and variety of such reports on apparent latency in peripheral sites may require the abandonment of the thesis that the nervous system provides the sole site of latent HSV infection.

If, as results from the present experiments suggest, HSV is able to establish a latent infection in ocular tissues, the infection may reactivate as happens in ganglia. In the eye, such reactivation could be involved in the initiation and perpetuation of chronic herpetic eye disease.

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References


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