Recovery of Herpes Simplex Virus from the Corneas of Experimentally Infected Rabbits

By S. D. COOK,1, 2 S. K. BATRA1 and S. M. BROWN1*

1MRC Virology Unit, Institute of Virology, Church Street, Glasgow G11 5JR and
2Tennent Institute of Ophthalmology, University of Glasgow, Western Infirmary,
Glasgow G11 6NT, U.K.

(Accepted 15 April 1987)

SUMMARY

Rabbits were inoculated in the left cornea with one of three strains of herpes simplex virus (HSV) i.e. HSV-1 strain 17, HSV-1 strain McKrae, HSV-2 strain HG52, or with an HSV-1 McKrae/HSV-2 HG52 recombinant R40/2. Fifty-nine to 67 days after inoculation trigeminal ganglia and corneas were explanted and screened for release of infectious virus. Virus was isolated from all left trigeminal ganglia after organ culture irrespective of viral strain. Virus was isolated from three of 16 corneas of animals inoculated with HSV-1 strain McKrae and from one of four corneas from animals inoculated with the HSV-1/HSV-2 recombinant. The isolation of HSV from explanted corneas, after between 15 and 35 days in organ culture suggests that the cornea may be a site additional to dorsal root ganglia where latent HSV can reside in rabbits.

Evidence is accumulating that herpes simplex virus (HSV) may be capable of maintaining a latent infection in peripheral tissue in addition to dorsal root ganglia. HSV has been isolated following explantation and incubation from the clinically normal skin (Hill et al., 1983) and from the footpads (Al-Saadi et al., 1983) of latently infected mice. HSV has been obtained from guinea-pig skin when virus was not isolated from the corresponding ganglia but this was thought to be due to persistent infection (Scriba & Tatzber, 1981). In addition, virus has been isolated from posterior segment ocular explants from mice after long periods of co-cultivation (Openshaw, 1983).

Herpes simplex virus infection in man can affect the eye with recurrent disease leading to scarring of the cornea, visual impairment and eventually blindness. Until recently it was assumed that recurrent HSV infections of the eye were due to reactivation of the virus from dorsal root ganglia. However, the isolation of HSV from explanted human corneas after up to 11 days in tissue culture raises the question whether HSV can attain latency in some cells of the cornea. The human corneas from which HSV was recovered had been removed in the course of treatment for chronic stromal keratitis (Shimeld et al., 1982; Tullo et al., 1985).

Studies in experimental animals to detect latent HSV associated with eye infections have firmly established dorsal root ganglia as the site of latency (Stevens & Cook, 1971; Stevens et al., 1972). Until now the isolation of virus from cultured peripheral tissue has been uniformly unsuccessful (Nesburn et al., 1972, 1980).

Here we report the isolation of HSV from explanted corneas of latently infected outbred rabbits. These observations support the findings made in humans and lend weight to the hypothesis that HSV may be capable of maintaining a latent infection in corneal cells.

Outbred New Zealand white rabbits (weight 1-5 to 2 kg) were inoculated with either HSV-1 strain 17 (Brown et al., 1973), HSV-1 strain McKrae (from Dr J. M. Hill, Louisiana State University, New Orleans, La., U.S.A.), HSV-2 strain HG52 (Timbury, 1971) or a HSV-1

† Present address: Department of Ophthalmology, Bristol Eye Hospital, Lower Maudlin Street, Bristol, U.K.

0000-7672 © 1987 SGM
McKrae/HSV-2 HG52 recombinant (R40/2) whose genome structure is the same as that of the McKrae strain except for the sequence between 0.33 and 0.56 map units which originates from HG52 (S. K. Batra, unpublished results). The left eye was infected by placing 50 μl of a virus suspension in the lower cul-de-sac and massaging over the closed eye for approximately 1 min. The input titre varied depending on the infecting strain and was between $5 \times 10^5$ and $10^7$ p.f.u. per inoculum. Reactivation of virus was achieved using the iontophoresis of adrenalin technique (Kwon et al., 1981). Positive reactivation demonstrated that the animals had been successfully infected and that the virus could be recovered from the latent state.

When they were killed, the animals showed no clinical signs of infection and they were not shedding virus as detected by our assay system of screening eye washes on BHK21/C13 cells. Between 56 and 97 days post-inoculation, animals were killed and the trigeminal ganglia and corneas of each rabbit were explanted, subdivided and maintained in separate organ culture in Eagle’s medium (Glasgow modification) plus 25% calf serum (v/v) (EFC25) at 37 °C or 31 °C in the presence of CO₂.

The incubated cultures of corneas and ganglia were each screened regularly post-explantation by removing a standard amount (100 μl) of supernatant medium onto semi-confluent BHK21/C13 cells, incubating for 4 days at 31 °C and examining for plaques or c.p.e. after staining with Giemsa stain. Whenever reactivation occurred a representative number of viral plaques were picked from ganglial and corneal isolates and grown up to 50 mm plate stocks in BHK21/C13 cells. Isolates were compared to the original parental virus by restriction endonuclease digestion of ³²P-labelled viral DNA using the method of Lonsdale (1979).

The actual dose of virus chosen to infect the animals varied with the strain. HSV-2 strain HG52, being the least virulent, was used at $5 \times 10^6$ and $5 \times 10^7$ p.f.u./inoculum. At higher doses the majority of animals died. HSV-1 strain 17 was used at $5 \times 10^5$ and $5 \times 10^6$ p.f.u./inoculum and HSV-1 strain McKrae was used at $5 \times 10^5$, $5 \times 10^6$ and $5 \times 10^7$ p.f.u./inoculum. The R40/2 recombinant was used at a single infecting dose of $1 \times 10^6$ p.f.u./inoculum.

Virus was isolated from the left trigeminal ganglion of each infected animal after organ culture. Ganglia were subdivided into six. No explants yielded virus. The first detection of virus occurred between 4 and 24 days post-explantation. Time differences between first isolation of virus from different explants appeared to be independent of virus strain.

Virus was isolated from the corneas of four rabbits: three had been infected with HSV-1 strain McKrae and the fourth with the intertypic recombinant R40/2 (Table 1). In general, the corneas were a longer time in culture than ganglia before first detection of released virus (Table 2). When virus was isolated it was present in the supernatant medium for several days e.g. with R40/2 virus was recovered from the cornea between day 35 and day 49 post-explantation and from the ganglion of the same animal between day 24 and day 37 (Table 2). HSV was detected in only one of six corneal segments cultured from each of three animals and in two out of six segments cultured from the fourth animal (Table 1). In all three cases restriction endonuclease analysis of the DNA of the isolates with HindIII confirmed their structure to be the same as that of the parental McKrae strain virus and the isolate from the recombinant R40/2 infected rabbit also retained its characteristic parental HindIII profile (data not shown).

The isolation of HSV from explanted human corneas only after a prolonged period of time in organ culture raises three questions. Is the virus truly latent in the cells of the cornea, is the virus present as a chronic low grade infection which is undetectable clinically, i.e. persistent, and has the virus been recently transported to the corneas thereby requiring amplification before detection? The isolation of HSV from the corneas of latently infected rabbits could be attributed to any of the above possibilities.

The animals that shed virus from their corneas had been killed between 56 and 97 days after the initial eye infection. The presence of a latent viral infection in the animals was demonstrated by isolation of virus after iontophoresis of adrenalin and was substantiated by the isolation of virus from the left trigeminal ganglia after a period of incubation in organ culture. At the time of sacrifice the animals had not been shedding virus from their eyes for 14 days previous to death and their eyes were devoid of clinical signs of infection. The paucity of material precluded homogenization of corneal tissue immediately after explantation. The frequency of isolation was
Table 1. *Isolation of HSV from organ culture of left trigeminal ganglia and corneas*

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>M.o.i.*</th>
<th>Left trigeminal ganglia</th>
<th>Corneas</th>
</tr>
</thead>
<tbody>
<tr>
<td>HG52</td>
<td>5 × 10⁴</td>
<td>ND†</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>5 × 10⁶</td>
<td>3/3‡</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>5 × 10⁷</td>
<td>2/2, 3/3</td>
<td>0/2, 0/3</td>
</tr>
<tr>
<td>17</td>
<td>5 × 10⁵</td>
<td>2/2</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>5 × 10⁶</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>5 × 10⁷</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>McKrae</td>
<td>5 × 10⁵</td>
<td>4/4, 6/6, 3/3, 3/3</td>
<td>1/4, 0/6, 0/3, 2/3</td>
</tr>
<tr>
<td></td>
<td>5 × 10⁶</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>5 × 10⁷</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>R40/2</td>
<td>1 × 10⁶</td>
<td>4/4</td>
<td>1/4</td>
</tr>
</tbody>
</table>

* M.o.i., Multiplicity of infection used initially to infect the left eye.
† ND, Not done.
‡ Number of animals shedding virus per number in group infected.

Table 2. *Time of release of HSV from organ culture of left trigeminal ganglia and corneas*

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>M.o.i.</th>
<th>Left trigeminal ganglia</th>
<th>Corneas</th>
</tr>
</thead>
<tbody>
<tr>
<td>HG52</td>
<td>5 × 10⁵</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>5 × 10⁶</td>
<td>8-18†</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5 × 10⁷</td>
<td>8, 7-22</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>5 × 10⁵</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5 × 10⁶</td>
<td>8-18</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5 × 10⁷</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>McKrae</td>
<td>5 × 10⁵</td>
<td>13-17, 4-7, 7-17, 8-13</td>
<td>18-21, 15-18, 28-31</td>
</tr>
<tr>
<td></td>
<td>5 × 10⁶</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>5 × 10⁷</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>R40/2</td>
<td>1 × 10⁶</td>
<td>24-37</td>
<td>35-49</td>
</tr>
</tbody>
</table>

* ND, Not determined.
† Time in days. Virus isolated between and including these days.

so low (one in 33 animals tested) and the number of positive segments so few, it was thought more important to maximize the chance of virus release by growing the whole cornea as explant tissue.

Virus was first detected from the four positive corneas on days 15, 18, 28 and 35 post-explantation. In each case, therefore, there was a considerable delay before virus isolation. The delay was similar to that found in isolation of virus from latently infected ganglia which in the case of the four positive animals was 12, 17, 8 and 24 days respectively. The long delay in release of virus from the corneas is not suggestive of a chronic low grade infection or of the interpretation that a small amount of virus had recently been transported from the dorsal root ganglion to the cornea and was then only amplified there. In either case amplification and therefore detection of infectious virus would probably have occurred within 2 to 3 days of explantation.

The isolation of virus from only one of six corneal segments in three animals and two of six in the fourth animal is in line with the results obtained in the human study where HSV was only released from clinically diseased segments of corneas. It could be that only certain parts of the rabbit corneas were initially infected.

Only corneas inoculated with the McKrae strain or the predominantly McKrae recombinant R40/2 released virus following explantation. Genetic variation between HSV strains is well
known. HSV-1 strains McKrae and 17 have a high frequency of recurrence (Gerdes & Smith, 1983). Because of the small sample number it would be unwise to extrapolate from the finding that only the McKrae strain was isolated from the corneas. The animals infected with $5 \times 10^6$ and $5 \times 10^7$ p.f.u. of the McKrae strain died soon after the primary infection and their ganglia and corneas were therefore not explanted. The frequency of isolation from the corneas of experimentally infected rabbits was found to be four out of 33 animals infected i.e. approximately 12%. The published human studies report an isolation frequency of two positives from three cases (Shimeld et al., 1982) and six positives from nine cases (Tullo et al., 1985).

The definition of HSV latency is functional at present and the precise state of the viral DNA unknown. The isolation of virus from rabbit corneas after a long period of time in organ culture taken together with the isolation from only a proportion of the corneas strongly suggests that HSV can become latent in some corneal cells by the functional definition. Support is given to the findings by studies in vitro showing that cultured rabbit corneal cells are capable of supporting a latent infection (Cook & Brown, 1986, 1987). It is less likely that the virus is there as a chronic low grade infection but the possibility cannot be entirely ruled out. The treatment of animals with a drug such as acyclovir prior to explantation should stop the spread of virus to the corneas and thereby rule out the possibility that virus has been recently transported from the ganglia. Similarly the addition of acyclovir to the explanted corneas would kill any replicating virus and therefore virus released after removal of the inhibitor would be classified as having been latent. However, as the frequency of isolation from corneas was so low (i.e. 12%) and given that not all segments released virus, the use of viral inhibitors has to be part of a long term study. The frequency of isolation from corneas compared to ganglia suggests that dorsal root ganglia are the preferred site for establishment of a latent infection following eye infection, but that some corneal cells are also able to support and maintain HSV in a latent state.

Our thanks are due to J. H. Subak-Sharpe for his interest in the work and critical reading of the manuscript. S.D.C. was supported by a MRC Training Fellowship. S.K.B. is a Commonwealth Scholar.

REFERENCES


Short communication


(Received 30 January 1987)