Recurrence Phenotypes and Establishment of Latency Following Rabbit Keratitis Produced by Multiple Herpes Simplex Virus Strains

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SUMMARY

Distinct high frequency recurrence (HFRc) or low frequency recurrence (LFRc) phenotypes were observed following rabbit keratitis with three type 1 and five type 2 herpes simplex virus strains. LFRc strains were found to have latently infected the animal but were detected very rarely, if at all, in the eye following the acute phase. The recurrence phenotypes defined in singly infected animals remained unchanged following bilateral infection of the same animal with strains of opposite phenotype. Co-cultivation of virus from bilaterally infected animals showed that both virus strains were capable of latently infecting the same animal. Restriction enzyme analysis of plaque-purified virus revealed that some ganglia were latently infected with both parental strains. Recombinants were also found. Some latently infected animals could be re-infected acutely. However, establishment of latency by the superinfecting strain was inhibited.

INTRODUCTION

The inoculation of herpes simplex virus (HSV) onto the rabbit cornea results in acute keratitis (Gruter, 1920; Williams et al., 1965). During the acute phase of the disease the virus spreads along the fifth cranial nerve to the ipsilateral trigeminal ganglion (Goodpasture & Teague, 1923). It subsequently resides there in a latent form recoverable by co-cultivation of the trigeminal ganglion but is not detectable in cell-free homogenates (Stevens et al., 1972; Stevens, 1975). Latently infected animals do not continuously shed detectable virus but may experience periodic recurrent infections during which reactivated virus can again be found in the eye (Stevens, 1975; Nesburn et al., 1967, 1972; Laibson & Kibrick, 1969).

The natural history of the human disease has been shown to be very similar to that observed during rabbit keratitis. The trigeminal ganglia of a significant proportion of human cadavers harbour latent virus (Baringer & Swoveland, 1973). In addition, recurrent infections have been shown to be due to re-expression of endogenous virus. The most rigorous evidence for this has been provided by restriction enzyme analysis, with which viruses isolated from different recurrent episodes of the same individual have been shown to be identical in their DNA fragment patterns, whereas viruses isolated from epidemiologically unrelated individuals invariably are different (Buchman et al., 1980; Lonsdale et al., 1980). The recurrence frequencies for different individuals can vary greatly. Some individuals apparently never experience a recurrence, while others have multiple recurrences. Despite the apparent ineffectiveness of the immune response to control re-expression of latent virus, re-infection by HSV of exogenous origin is rare and any given individual apparently retains only one latent virus that is genetically stable over many years (Buchman et al., 1980; Lonsdale et al. 1980).

Based on these observations, two fundamental questions arise regarding the HSV strategy that results in recurrent disease. First, what are the factors that control the frequency of

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reaction of latent HSV? Secondly, are individuals latently infected by only one strain of HSV as a result of immune mechanisms that prevent establishment of latency by another HSV strain?

The objective of this study was to address these questions utilizing the rabbit keratitis model. We demonstrate that recurrence frequency is at least partially dependent on the expression of virus-coded information and that latently infected animals inhibit the establishment of latent infection by another HSV strain while they remain susceptible to acute infection.

METHODS

Virus and cells. The various strains of HSV used in this study were obtained from the following sources. HSV-1: strain MacIntyre originally described by Dowdle et al. (1967) from J. G. Stevens, UCLA, Los Angeles, Ca.; strain McKrae (Williams et al., 1965) from A. B. Nesburn, USC, Los Angeles; strain 17 syn+ (Brown et al., 1973) from H. S. Marsden, Glasgow, U.K. HSV-2: strain 186 (Benyesh-Melnick et al., 1974) from P. A. Schaffer, Harvard Medical School, Cambridge, Mass.; strain CJ359 derived from a patient with recurrent keratitis from J. Chandler, Seattle, Wa.; strain HG52 (Timbury, 1971) from H. S. Marsden; strains 2461 and 2544 obtained by J. MacGregor, University of Colorado School of Medicine, Denver, Co., from recurrent genital lesions of two patients. All strains underwent at least one passage in rabbit skin (RS) cells (supplied by Dr R. Ward, Sandia Corp., Albuquerque, N.M., U.S.A.) before being used to inoculate rabbits. Virus was grown and titrated by methods previously described (Gerdes et al., 1979).

Rabbit infection. New Zealand white rabbits (1 to 3 kg) were infected by dropping 1 × 10^4 to 2 × 10^4 p.f.u. of virus in 50 to 100 μl of minimal essential medium (MEM) with 2% foetal calf serum (FCS) onto unscarified corneas. In the later experiments with strain 186, less virus (1 × 10^3 to 2 × 10^3 p.f.u./eye) was used, to decrease the mortality rate of the animals from acute encephalitis.

Detection of recurrent virus. Rabbit tear film samples were collected with a sterile polyester swab (on 3 to 5 days per week) placed in the lower fornix for 20 to 30 s, transferred to 0.5 ml of MEM + 10% FCS and stored at −70 °C. The samples were added to cultures of rabbit skin cells and followed for 10 days to detect infectious virus. The supernatant fluids and floating cells of positive cultures were collected and stored at −70 °C.

Co-cultivation of trigeminal ganglia. Trigeminal ganglia were removed from CO2-killed rabbits, minced and placed on monolayers of RS cells in MEM + 5% FCS. Cultures were maintained in MEM + 2% FCS for 5 to 8 weeks and monitored for expression of viral cytopathic effect (c.p.e.). Most of the c.p.e.-positive explants were detected 1 to 3 weeks after co-cultivation. Supernatants and cells from c.p.e.-positive explants were removed from the plates and stored at −70 °C.

Plaque purification of virus. Two methods for plaque purification of the virus were used. In the first, virus was diluted in MEM + 2% FCS to titres of less than 1 p.f.u./100 μl, then 100 μl was placed in each well of a cluster-24 plate containing monolayers of RS cells. Virus was allowed to adsorb 1 to 2 h at 37 °C, then removed and the cells overlaid with 0.75% Methocel in MEM + 5% FCS. The individual overlays were transferred to new monolayers of RS cells 3 or 4 days after initial infection and the original plates were stained with crystal violet (1% in 20% ethanol). The transferred virus that grew in wells corresponding to those wells which only contained one virus plaque were collected and stored at −70 °C. In the second method, virus plaques were also isolated under an agarose overlay (0.6% SeaKem agarose in MEM + 5% FCS) after infection of 60 mm plates with 5 to 20 p.f.u./plate. Pasteur pipettes were used to obtain virus from beneath the overlay for transfer to individual wells of cluster-24 plates of RS cells in MEM + 2% FCS. Virus was grown in these wells, the cell and supernatant collected and stored frozen at −70 °C.

DNA purification. Unlabelled HSV DNA was purified as follows. Approximately 5 × 10^8 rabbit skin cells grown in roller bottles were infected at low multiplicity (0.1) and harvested when cytopathic effect was complete. Cells were scraped from the plastic, collected by centrifugation and washed with cold (4 °C) phosphate-buffered saline. The DNA in this cell pellet was then extracted utilizing the Triton–NaCl method described by Pignatti et al. (1979), and treated with RNase (50 μg/ml, 2 h, 37 °C) and proteinase K (100 μg/ml in 1% SDS, 2 h, 37 °C). DNA was further clarified by two phenol–chloroform extractions followed by a chloroform extraction, ethanol precipitation and resuspension in 200 to 300 μl of 10 mM-Tris–HCl, 2 mM-EDTA, pH 7.5 (TE). DNA concentrations were then estimated relative to a calf thymus DNA standard by serial dilutions with 1 μg/ml ethidium bromide in TE and visualized by u.v. light. 32P-labelled HSV DNA was used in the later experiments and was prepared by a modification of the procedure described by Lonsdale (1979). Monolayers of rabbit skin cells in cluster-12 plates were infected with virus (multiplicities varied with source of virus isolate but were generally kept as high as feasible) which was allowed to adsorb for 1 to 2 h before it was removed. The cells were washed twice with 0.85 M-NaCl, then left in phosphate-free medium for 4 to 6 h after infection. This was replaced with 0.5 ml medium containing 20 to 40 μCi [32P]orthophosphate (Amersham). Virus was harvested 24 to 72 h later when c.p.e. was complete. SDS (0.5 ml of 5%) was added to the contents of each well which were then transferred
to tubes, treated with proteinase K (0.2 mg, 100 μl/tube) for 1 or 2 h at 37 °C then extracted once with phenol-
chloroform. The aqueous phase was removed, and 200 μl of 1 m-NaCl was added followed by 2 vol. ethanol
(−20 °C) to precipitate the DNA; the samples were stored at −20 °C overnight. The nucleic acids were then
pelleted, vacuum-desiccated until dry, then resuspended in 200 μl RNase solution (50 μg/ml in TE) for 2 h at 37 °C
with gentle shaking.

Restriction enzyme analysis. The restriction enzymes HpaI, BglII, KpnI, HindIII and EcoRI were obtained from
Bethesda Research Laboratories and Boehringer Mannheim. DNA (1 to 2 μg of unlabelled DNA, with variable
levels of ct/min with 32P-DNA) in 10 mM-Tris-HCl, 10 mM-MgCl2, 1 mM-dithiothreitol, 0-1 mg/ml gelatin (plus
50 mM-NaCl for HindIII or plus 100 mM-NaCl for EcoRI) was cleaved with a minimum of 6 units of the enzyme
indicated for 3 to 4 h. Reactions were stopped by addition of SDS, EDTA and glycerol. DNA fragments were
separated on 0.4, 0.5 or 0.6% agarose (SeaKem) gels in 89 mM-Tris base, 89 mM-boric acid, 2-5 mM-EDTA at 0.5 to
2-0 V/cm for 16 to 24 h. Unlabelled DNA bands were stained with ethidium bromide (1 μg/ml for 20 min, then in
H2O for 10 min) and photographed with u.v. light (Polaroid 665 film). 32P-labelled bands were detected by
autoradiography of dried gels with a Cronex Lightning-plus intensifier screen and Kodak XRP-5 or XAR-5 film at
−70 °C.

RESULTS

Recurrence phenotypes

Not all HSV strains capable of inducing a latent infection cause recurrences with equal
frequencies in the rabbit keratitis model. In fact, distinct high frequency recurrence (HFRc) or
low frequency recurrence (LFRc) phenotypes became evident following infection of eyes with
different type 1 strains and five different type 2 strains as described in Table 1. Strain
McKrae, originally isolated from a patient with recurrent keratitis, recurred following the acute
phase in the rabbit eye in 85% of the animals; 5.3% of the tear-film samples collected from
infected eyes were positive. Similarly, strain 17 syn + recurred in 80% of the infected animals
with 2-4% of the samples positive. In contrast, strain MacIntyre, which was originally obtained
from a fatal case of encephalitis, was never observed to reactivate in any of the 12 animals
inoculated. As over 1000 samples were taken, this indicates the recurrence frequency is less than
0.1%. Although virus levels were not strictly titrated in either the eye or ganglia, there are several
observations that suggest that reduced replication by the LFRc strain did not occur. First, the
length of the acute phase was identical in most cases for eyes infected with HFRc or LFRc
strains. If replication in the eye had been restricted, the duration of the acute phase would have
decreased. Secondly, the proportion (67%) of infected animals confirmed to be latently infected,
as well as the length of co-cultivation time required to recover the virus, was similar for both the
HFRc and LFRc strain, suggesting that replication in the ganglia was not restricted. Therefore,
these results not only indicate a very real difference in the ability of HSV-1 strains to recur, i.e. at
least 20- to 50-fold differences in recurrence frequency, but also demonstrate that mechanisms
involved in recovery of latent virus by co-cultivation are not equivalent to those resulting in
recurrences in vivo.

All of the HSV-2 strains inoculated were obtained from patients with a history of recurrences.
However, in the rabbit keratitis model the type 2 strains tested appeared to be LFRc. The HSV-
2 strain most extensively investigated was strain 186, which had a recurrence frequency of less
than 0-05%. Of the 31 animals examined and over 2000 tear-film samples, only one was found to
be positive. The virus from this sample was shown to be strain 186 by restriction enzyme analysis
of 12 plaque-purified clones utilizing the five enzymes HpaI, BglII, EcoRI, HindIII and KpnI. To
learn whether this recurrent isolate was a mutant that had been selected in the animal, it was re-
inoculated into an additional group of 10 animals which were monitored for 4 months following
infection but did not show a single recurrence. HSV strain CJ359 (obtained from a recurrent eye
lesion) was also found to be LFRc, as no virus was found in over 1000 post-acute phase samples.
The recurrence phenotype of strain HG52 also appears to be negative. However, only 20% of the
inoculated animals were confirmed to be latently infected by co-cultivation. The phenotype of
this strain must therefore be considered unknown. The reason for the low recovery of latent virus
for this particular strain is unknown, but the observation suggests the possibility that certain
strains of HSV either establish latency with poor efficiency or are inefficiently recovered by
Table 1. Recurrence phenotype and confirmation of latency following infection of rabbit eyes with various HSV strains*

<table>
<thead>
<tr>
<th>Strain</th>
<th>HSV type</th>
<th>History†</th>
<th>Recurrence in animals/total infections</th>
<th>Total no. recurrent infections</th>
<th>No. positive cultures/total cultures</th>
<th>% Positive cultures</th>
<th>Acute crossover infection§</th>
<th>Latency¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>McKrae</td>
<td>1 R, Eye</td>
<td>28/33</td>
<td>69</td>
<td>126/2382</td>
<td>5.3</td>
<td>0/5</td>
<td>24/33</td>
<td>4/19</td>
</tr>
<tr>
<td>Maclntyre</td>
<td>1 Encephalitis</td>
<td>0/12</td>
<td>0</td>
<td>0/1039</td>
<td>&lt;0.1</td>
<td>0/5</td>
<td>8/12</td>
<td>2/12</td>
</tr>
<tr>
<td>17 syn⁺</td>
<td>1 R, Lip</td>
<td>8/10</td>
<td>18</td>
<td>24/1014</td>
<td>2.4</td>
<td>6/10</td>
<td>6/8 (9/10)</td>
<td>5/8</td>
</tr>
<tr>
<td>186</td>
<td>2 R, Genital</td>
<td>1/31</td>
<td>1</td>
<td>1/2112</td>
<td>0.05</td>
<td>6/24</td>
<td>29/29</td>
<td>20/29</td>
</tr>
<tr>
<td>HG52</td>
<td>2 R, Skin</td>
<td>1/10</td>
<td>1</td>
<td>1/660</td>
<td>0.15</td>
<td>0/10</td>
<td>2/10</td>
<td>0/10</td>
</tr>
<tr>
<td>CJ359</td>
<td>2 R, Eye</td>
<td>0/10</td>
<td>0</td>
<td>0/1090</td>
<td>&lt;0.09</td>
<td>0/10</td>
<td>10/10</td>
<td>8/10</td>
</tr>
<tr>
<td>2461</td>
<td>2 R, Genital</td>
<td>1/4</td>
<td>1</td>
<td>1/224</td>
<td>0.45</td>
<td>0/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td>2544</td>
<td>2 R, Genital</td>
<td>1/4</td>
<td>1</td>
<td>1/224</td>
<td>0.45</td>
<td>0/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
</tbody>
</table>

* New Zealand white rabbits were infected by dropping 10⁴ p.f.u./eye of the HSV strains indicated onto unscarified corneas.
† Clinical source of the original virus isolate. R = recurrent.
‡ Tear-film samples were collected, added to cultures of rabbit skin (RS) cells and followed for up to 10 days to detect infectious virus. Recurrence in animals/total = number of animals in which virus was detected following the end of the acute phase and the total number of animals observed. Total no. recurrent infections = cumulative number of recurrent episodes observed for all animals. No. positive cultures/total cultures = total number of eye cultures from which virus was recovered and the total number of eye specimens cultured. % Positive cultures = percentage of the total number of eye specimens cultured in which virus was detected.
§ Number of animals in which virus was detected in the opposite eye and the total infected during the acute phase of infection.
¶ Trigeminal ganglia were removed, minced and placed on monolayers of RS cells. Cultures were maintained for up to 8 weeks and monitored for expression of viral c.p.e. Numbers in parentheses refer to an estimate of latency for recurrence-positive strains which includes those animals in which virus recurred but from which virus was not recovered from the explanted ganglia.

standard co-cultivation methods. The only two possible exceptions to the negative trend for recurrence phenotypes of HSV-2 were strains 2461 and 2544, for which frequencies of 0.45% were observed. As this is considered to be borderline or intermediate and is based on a small sample size, additional studies would have to be done to determine whether these strains are HFRc or LFRc.

It was also observed that different strains of HSV differ in their ability to cross over to the opposite eye during the acute phase (Table 1). For example, 60% of the animals were observed to have virus in the opposite eye following infection with 17 syn⁺, whereas none of the animals infected with strains McKrae or Maclntyre was found to have had virus crossover during the acute phase. Certain strains apparently are capable of spreading either within the central nervous system or by means of a viraemia to the contralateral trigeminal ganglion without spreading to the opposite eye. For example, HSV-2 strains CJ359, 2461 and 2544 were found to latently infect contralateral trigeminal ganglia in 80 to 100% of the animals, despite the fact that none of the animals was observed to have virus in the contralateral eye at any time. HSV-1 strains were also able to latently infect contralateral ganglia, although the frequency was not as high. It was of interest to determine the recurrence frequency in contralateral eyes for recurrence-positive strains found to be latent in the contralateral trigeminal ganglia. The recurrence frequency for both strains McKrae and 17 syn⁺ from contralaterally infected trigeminal ganglia into the eyes innervated by those ganglia was only 0.3% (nine animals; two positive cultures of 566 total cultures). This observation suggests that recurrence may be less frequent when the virus spreads to the opposite trigeminal ganglion from within the central nervous system rather than from the eye.
Table 2. Recurrence phenotype and restriction enzyme analysis of recurrent and latent virus following bilateral infections

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Eye</th>
<th>Infecting virus*</th>
<th>No. of recurrences</th>
<th>Recurrences</th>
<th>Co-cultivation†</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>L</td>
<td>MacIntyre</td>
<td>0</td>
<td>–</td>
<td>Recomb. (5)</td>
</tr>
<tr>
<td>I</td>
<td>L</td>
<td>MacIntyre</td>
<td>0</td>
<td>–</td>
<td>McKrae McKrae</td>
</tr>
<tr>
<td>J</td>
<td>L</td>
<td>MacIntyre</td>
<td>0</td>
<td>–</td>
<td>NR NR</td>
</tr>
<tr>
<td>L</td>
<td>L</td>
<td>MacIntyre</td>
<td>0</td>
<td>–</td>
<td>NR NR</td>
</tr>
<tr>
<td>M</td>
<td>L</td>
<td>MacIntyre</td>
<td>0</td>
<td>–</td>
<td>McKrae McKrae</td>
</tr>
<tr>
<td>N</td>
<td>L</td>
<td>MacIntyre</td>
<td>0</td>
<td>–</td>
<td>NR NR</td>
</tr>
<tr>
<td>O</td>
<td>L</td>
<td>MacIntyre</td>
<td>1</td>
<td>McKrae</td>
<td>McKrae McKrae</td>
</tr>
<tr>
<td>R</td>
<td>L</td>
<td>MacIntyre</td>
<td>2</td>
<td>McKrae</td>
<td>McKrae McKrae</td>
</tr>
<tr>
<td>1</td>
<td>L</td>
<td>McKrae</td>
<td>5</td>
<td>McKrae</td>
<td>McKrae (4), 186 (5)</td>
</tr>
<tr>
<td>R</td>
<td>186</td>
<td>0</td>
<td>–</td>
<td>186 (7)</td>
<td>186 (5)</td>
</tr>
<tr>
<td>2</td>
<td>L</td>
<td>McKrae</td>
<td>1</td>
<td>McKrae</td>
<td>McKrae (2), 186 (3), recomb. (1)</td>
</tr>
<tr>
<td>R</td>
<td>186</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>186 (5)</td>
</tr>
<tr>
<td>3</td>
<td>L</td>
<td>McKrae</td>
<td>2</td>
<td>McKrae</td>
<td>McKrae (6)</td>
</tr>
<tr>
<td>R</td>
<td>186</td>
<td>0</td>
<td>–</td>
<td>McKrae (5), recomb. (1)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>L</td>
<td>McKrae</td>
<td>1</td>
<td>McKrae</td>
<td>McKrae (6)</td>
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<tr>
<td>R</td>
<td>186</td>
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<td>186 (6)</td>
<td>186 (6)</td>
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</tr>
<tr>
<td>7</td>
<td>L</td>
<td>McKrae</td>
<td>6</td>
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<tr>
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<td>186</td>
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<td>–</td>
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<tr>
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<td>L</td>
<td>McKrae</td>
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</tr>
<tr>
<td>R</td>
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<td>–</td>
<td>186 (5)</td>
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</tr>
<tr>
<td>9</td>
<td>L</td>
<td>McKrae</td>
<td>4</td>
<td>McKrae</td>
<td>186 (5)</td>
</tr>
<tr>
<td>R</td>
<td>186</td>
<td>0</td>
<td>–</td>
<td>186 (6)</td>
<td></td>
</tr>
</tbody>
</table>

* Strains MacIntyre (LFRc) and McKrae (HFRc) are HSV-1; strain 186 (recurrence-negative) is HSV-2.
† Abbreviations: recomb., recombinant; NR, none recovered. The numbers in parentheses are the numbers of virus clones found to have the designated restriction fragment pattern.

Frequency of recurrence and latency following simultaneous bilateral infection

To confirm the recurrence phenotypes with a minimum of variation contributed by the host, animals were infected simultaneously in both eyes. In an early experiment, nine rabbits were inoculated in both eyes with the HFRc strain, McKrae. This experiment was designed to determine whether latency could be established with equal frequency in both trigeminal ganglia and whether recurrences in the two eyes are independent. During the 4 months following infection, five of the seven surviving animals were observed to have left eye recurrences while six of seven had right eye recurrences. There was a total of nine left eye recurrences and 11 right eye recurrences, indicating that the animals were latently infected in both trigeminal ganglia and that recurrences could occur in either eye. In addition, only once was virus found recurring in both eyes at the same time. If a circulating factor is involved in the induction of the virus, then re-expression of virus in both eyes at the same time should have been common.

Next, strains with opposite recurrence phenotypes were inoculated into the same animals (Table 2). In the first experiment, two HSV-1 strains were used. The HFRc strain McKrae was inoculated into the right eye and the LFRc strain MacIntyre was inoculated into the left eye. In the second experiment (Table 2), rabbits were bilaterally infected with the HSV-1 HFRc strain...
McKrae and the HSV-2 LFRc strain 186. In each experiment, virus was shed for similar periods of time (11 to 15 days) by both strains during the acute phase of the infection. In the rabbits infected with the two HSV-1 strains, all seven had one or more recurrences in the eyes infected with McKrae strain. One apparent recurrence (O left) was observed in the eyes infected with Maclntyre strain. However, restriction enzyme analysis of the virus obtained indicates that it was in fact the McKrae strain (data not shown). Similar results were found in the other experiment, in which all of the eyes infected with strain McKrae were found to have had a recurrence while in only one of the eyes infected with strain 186 was a recurrence observed. For the first experiment, there was a total of 19 McKrae strain recurrences and no recurrences by strain Maclntyre, while in the other experiment there was a total of 21 McKrae strain recurrences and one strain 186 recurrence. The possibility cannot be excluded that it was the presence of strain McKrae in the rabbit which allowed the single recurrence of strain 186. As noted earlier, analysis of 12 plaque-purified clones of the virus from this recurrence did not show any change from the restriction enzyme patterns of the parent strain (data not shown) and the phenotype remained LFRc when a clone of the isolate was re-inoculated into 10 additional rabbits. Therefore, it is unlikely that the recurrence event was due to mutation or recombination with strain McKrae.

These observations prove that differences seen in the recurrence phenotype are maintained under identical conditions of immunity, that is, in the same animal. The experiment also determined that the recurrence phenotype defined in a singly infected animal remains unaltered during simultaneous infection with another HSV strain of opposite phenotype. Therefore, viral information must be involved in determining the ability of strains to recur and this is apparently not mediated in any way that can affect other strains present.

**Restriction enzyme analysis of virus recovered from double infections**

In all experiments where two virus strains were inoculated into the same rabbit, virus recovered both from recurrences and from co-cultivation of trigeminal ganglion explants was analysed by restriction enzyme cleavage patterns. This was done not only to determine which strains were being recovered but also to check the genetic stability of the virus in vivo.

In an initial experiment, recurrent virus isolates obtained from animals infected only with strain McKrae appeared to be genetically stable. Virus from six recurrences in the same rabbit was analysed with the four enzymes *KpnI*, *HindIII*, *HpaI* and *EcoRI*. However, minor variations in the size of certain bands from the repetitive hinge region, such as *HpaI* fragment R or *EcoRI* fragment K, were common. Such hinge region variability has previously been reported for virus grown in vitro (Buchman et al., 1980; Lonsdale et al., 1980). As was observed for singly infected animals, recurrent virus obtained from bilaterally infected animals also appeared to be genetically stable. The 19 recurrences from the strains Maclntyre and McKrae virus-infected animals were analysed using enzymes *HpaI*, *HindIII* and *KpnI*, and found to be identical to the parental strains.

The 22 recurrences from strains McKrae and 186 virus-infected animals were also compared with those enzymes and with *BglII* and *EcoRI* as well. Here, viruses from two recurrences were found to be significantly different from the parental strains. In the *HpaI* restriction pattern from one of these recurrences (lanes 3 and 4, Fig. 1), bands K and U disappeared and a single higher molecular weight band below fragment I appeared. This was the only change seen in the unique regions of the HSV genome (near map position 2) among over 40 recurrences analysed. Since only the *HpaI* pattern was altered, the change must be quite small, possibly only a single base change. In the other recurrence (lane 8, Fig. 1), bands M, R and V disappeared and a new band was present below F. Changes in this virus isolate were also seen with the other four enzymes, but, as in this case, the change, although substantial, appears to be in the hinge region of the genome. Neither of these altered DNAs contains characteristics of strain 186 that would allow them to be classified as recombinants. Although the recurrence that contained the unique region change was the only recurrence seen in that animal (2 left eye), the other isolate is the first of four recurrences from a single eye. The virus from none of the later recurrences contained the changes noted here.
Fig. 1. HpaI restriction enzyme cleavage of DNA obtained from recurrences following bilateral infection of the eyes of rabbits with HFRc HSV-1 strain McKrae and LFRc HSV-2 strain 186 as described in Table 2. The samples shown are as follows: lane 1, McKrae; lane 2, rabbit no. 1, 171 days post-inoculation; lane 3, rabbit 2, 49 days; lane 4, rabbit 2, 50 days (this is a later sample from the same recurrence shown in lane 3); lane 5, rabbit 5, 46 days; lane 6, rabbit 7, 91 days; lane 7, rabbit 7, 129 days; lane 8, rabbit 9, 25 days; lane 9, rabbit 9, 136 days; lane 10, 186. The HpaI cleavage pattern of strain McKrae is essentially identical to that published by Preston et al. (1978) for HSV strain 17 syn+ and its map is shown at the bottom of the figure.

Restriction enzyme cleavage patterns of the DNA of parental virus strains were also compared with those recovered from co-cultivations of trigeminal ganglia. In the first experiment where the HSV-1 strains MacIntyre and McKrae were used to infect left and right eyes, respectively, four of the seven co-cultivations from the left trigeminal ganglia were positive, whereas all seven from the right trigeminal ganglia were positive. Restriction analysis
showed only the pattern of strain McKrae in the virus isolated from the right ganglia. However, the analysis of virus from the left ganglia was more complex (Fig. 2, Table 2). From only one rabbit (M) was MacIntyre obtained without any trace of the other strain. Strain McKrae rather than MacIntyre was found in the left trigeminal ganglion of rabbit O and, as noted earlier, had been able to recur from there. The restriction patterns of the viral DNA from the remaining left trigeminal ganglia (G and I) suggested that either both parents and/or recombinants of them were present. (Note bands BX and B, M and M in Fig. 2.) Virus from these last two ganglia was plaque-purified twice and five clones of each were analysed with HpaI, KpnI and HindIII (Fig. 3). All appeared to be recombinants. The map positions of four cleavage sites that differ between McKrae and MacIntyre strains were deduced from maps published for strains KOS (Morse et al., 1977), 17 syn+ (Preston et al., 1978), J and F (Locker & Frenkel, 1979). The patterns of recombination which could be analysed among these clones are summarized in Fig. 4. Two classes with different crossover sites were found among the clones from the left trigeminal ganglion of rabbit G (G1, two clones; G2, three clones). One group (three clones) from rabbit I apparently contains a double crossover.
Fig. 3. (a) HpaI, (b) KpnI and (c) HindIII analysis of twice plaque-purified clones of virus obtained from trigeminal explants of rabbits G and I (Table 2) suspected to contain recombinants. Clones representative of each recombinant class (G1, G2, I2) are shown with the parental strains. ▲, Fragment characteristic of parental strain Maclntyre (MacI); letter designation refers to map position (Fig. 4). ▼, Fragment characteristic of parental strain McKrae (McK); letter designation refers to map position (Fig. 4). ○, Fragments that map in areas of the HSV genome known to demonstrate variability in subclones (Locker & Frenkel, 1979; Lonsdale et al., 1980). ▐, Unmapped fragment found in parental strain Maclntyre. □, Unmapped fragment found in parental strain McKrae. *, New fragment not previously observed in either strain McKrae or Maclntyre. ●, Unmapped variable fragment occasionally seen in Maclntyre.

A similar but more detailed analysis was done for the virus recovered from trigeminal ganglia of the McKrae (left eye) and 186 (right eye) virus-infected rabbits. Plaque-purified clones from each positive co-cultivation (five to nine clones per ganglion) were compared with the parental strains. As an example of this type of analysis, the BglIII cleavage patterns of 12 clones isolated from rabbit 2 are shown in Fig. 5. Here it is obvious that clone 6 is a recombinant. At least six of the seven rabbits were latently infected with both McKrae and 186, if one includes those animals where McKrae recurred but was not detected in the co-cultivated virus (rabbits 7 and 9, Table 2). In four cases, both strains were apparently latent in the same trigeminal ganglion without any substantial change in the recurrence frequency of either strain. Two recombinants were found among these clones obtained from the co-cultivated virus (Table 2). They were mapped relative to the parent strains using restriction enzymes KpnI, HindIII, BglII, HpaI and EcoR1 (data not shown) and the resulting maps are shown in Fig. 6.

Re-infection of latently infected animals

The experiments just discussed confirmed that, following simultaneous bilateral infection, more than one strain of HSV can establish latent infection in the same ganglion. However, examination of virus obtained from different ganglia of the same human cadaver has invariably found only one virus strain latent (Lonsdale et al., 1980). Can latency be established by an HSV strain re-infecting an animal that has already been latently infected? Table 3 summarizes the establishment of acute or latent infection following either simultaneous or delayed bilateral infection. In animals simultaneously infected by two different strains (see exp. 1 and 3, Table 3), both infecting strains had similar acute phases and established latent infections. In experiment 2, one eye was initially infected with HFRc strain 17 syn+, then both eyes inoculated with HFRc strain McKrae (10^4 p.f.u./eye) 140 days after the original infection. On the day following re-inoculation, strain McKrae was found in half of the eyes inoculated. In one eye (previously unoinoculated), virus was still acutely present 1 week after re-inoculation.
Fig. 4. Summary of the genome structures of the recombinants identified following co-cultivation of trigeminal ganglia of rabbits G and I (Table 2). Four cleavage sites that distinguish the DNA of MacIntyre and McKrae strains of HSV located relative to maps previously published (Morse et al., 1977; Preston et al., 1978; Locker & Frenkel, 1979) are shown at the top of the figure. The dash ending in a circle indicates the presence of a restriction enzyme cleavage site characteristic of the parental DNA while the dash without a circle indicates the absence of a cleavage site. G1, G2 and I2 designate the recombinant classes described in Fig. 3. The heavy lines indicate the DNA sequences presumed to be present in each recombinant. The general topology of the HSV genome as described by Sheldrick & Berthelot (1974) is shown at the bottom of the figure.

Table 3. Acute or latent infection following simultaneous or delayed bilateral infection

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Virus strain</th>
<th>HSV type</th>
<th>Day infected</th>
<th>First acute phase (range in days)</th>
<th>Second acute phase (range in days)</th>
<th>Latency in trigeminal ganglia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MacIntyre</td>
<td>1</td>
<td>0</td>
<td>8-18</td>
<td>Previous infected eye Previous uninoculated eye</td>
<td>Ipsilateral Contralateral</td>
</tr>
<tr>
<td>2</td>
<td>McKrae</td>
<td>1</td>
<td>0</td>
<td>8-18</td>
<td>1 day 1-7</td>
<td>3/7 0/7</td>
</tr>
<tr>
<td>2</td>
<td>17 syn*</td>
<td>1</td>
<td>0</td>
<td>8-14</td>
<td>3/3</td>
<td>1/3</td>
</tr>
<tr>
<td>3</td>
<td>McKrae</td>
<td>1</td>
<td>0</td>
<td>8-18</td>
<td>5/7</td>
<td>1/7</td>
</tr>
<tr>
<td>4</td>
<td>CJ359</td>
<td>2</td>
<td>0</td>
<td>7-16</td>
<td>6/6</td>
<td>5/6</td>
</tr>
<tr>
<td>4</td>
<td>McKrae</td>
<td>1</td>
<td>134</td>
<td>0-3</td>
<td>0/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

Re-infected animals were observed for an additional 2 months, during which time none of the five recurrences observed was due to strain McKrae as determined by restriction enzyme analysis. At the termination of the experiment, only strain 17 syn* was recovered from co-cultivations of the trigeminal ganglia.

In experiment 4 (Table 3), the animals were infected with the HSV-2 LFRc strain CJ359 in one eye and re-inoculated 134 days later with HSV-1 strain McKrae in both eyes.
HSV recurrence phenotypes and latency

Fig. 5. *BglII* restriction enzyme cleavage of DNA from virus clones isolated by co-cultivation of the left and right trigeminal ganglia of rabbit 2 (Table 2). Left eyes were infected with HSV-1 strain McKrae (McK) and the right eye with HSV-2 strain 186. The patterns of six clones obtained from each ganglion are shown. ▼, Fragment of clone 6 left ganglion characteristic of strain McKrae; <, fragments characteristic of strain 186 that are missing in this clone.

Fig. 6. Summary of the genome structures of the recombinants identified following co-cultivation of trigeminal ganglia of rabbits 2 and 3 (Table 2). The solid areas represent the DNA sequences presumed to be present in each recombinant.
A more significant acute phase was found in this experiment with virus detected in 10 of 12 eyes one or more days after re-infection (0 to 3 days for previously infected eyes; 4 to 9 days for previously uninoculated eyes). As before, the animals were followed for an additional 2 months, during which time no recurrences were detected. At the termination of the experiment, once again only the initial infecting strain could be shown to have established a latent infection. The absence in both experiments of either recurrences of the highly recurrence-positive strain McKrae or of any trace of this strain in the co-cultivations strongly suggests that it was not able to establish latency. Therefore, although latently infected animals can be re-infected acutely, the prior infection appears to inhibit a second re-infecting virus from establishing its own latent infection. This "immunity" is not type-specific, since latent HSV-2 apparently blocked the establishment of latency by a re-infecting type 1 strain.

**DISCUSSION**

What regulates HSV reactivation? Serological investigations suggest that from 80 to 90% of the human population has been exposed to HSV, whereas only about 30% experience recurrent infections (Jones, 1976). Among those who have recurrent HSV infections, the frequency of recurrence varies greatly. The factors that control the frequency of recurrence of HSV are at present not understood. Recurrence in man has been reported after such diverse stimuli as fever, u.v. light, emotional disturbances or stress, a variety of skin diseases, menstruation, and trigeminal root section (Rapp & Jerkofsky, 1973). Although there is frequent speculation that the regulation of recurrence involves the immune response, the critical immunological factors have not been defined. Although it is generally concluded that recurrence must be influenced by the immune response, our results suggest that the frequency for a given individual may also be regulated by the strain of HSV latent in his or her ganglia.

The hypothesis that recurrence frequency for rabbit keratitis might be dependent in part on virus expression has been previously suggested by Martin *et al.* (1977) for HSV strain RE. However, although none of their rabbits had recurrences, these authors were able to confirm that latency had been established in only one animal. The type 1 HSV strain McIntyre and type 2 strains 186 and CJ359, are clearly capable of latently infecting the ganglia of rabbits. The latent viruses are recoverable by co-cultivation methods, but are not capable of recurrent infection in the eye, indicating that co-cultivation is not equivalent to the reactivation that results in recurrent infection in the eye. Recurrence of latent virus in the rabbit keratitis model is therefore HSV strain-dependent. The specific viral functions required must include additional expression beyond that required for the establishment and maintenance of latency. The identification of viral information critical to recurrence should be possible by the analysis of recombinants between HFRc and LFRc strains.

Regarding genetic stability and diversity of recurrent HSV, investigations by Buchman *et al.* (1980) and by Lonsdale *et al.* (1980) have clearly shown that epidemiologically unrelated virus isolates are readily distinguishable by their restriction enzyme cleavage patterns. The remarkable variability of HSV strains does not seem to be due to a high mutation rate of individual nucleotide sequences, since virus strains that have undergone serial passage in cell culture in the laboratory are genetically stable. For the limited number of human cases investigated, each individual apparently retains only one latent virus that is genetically stable and undergoes very little mutational change despite multiple recurrences. Our experiments reported here with infected animals suggest that HSV strains are also stable during recurrent infection of the rabbit.

Although recombinants were detected upon co-cultivation of ganglia obtained from multiply infected animals, it is unclear whether the recombination event occurred in the animal or during co-cultivation. An observation that would have confirmed that the recombination event did indeed occur *in vivo* would have been the detection of a recombinant shed during a recurrence in the eye. Restriction analysis of the virus DNA in two of the 41 recurrences did reveal changes in one or more cleavage sites relative to the recurrence-positive parent. However, the DNAs of these isolates were not sufficiently altered to distinguish whether the change was due to recombination or mutation.
Are latently infected individuals immune to subsequent acute or latent infection? Restriction enzyme analysis of viruses obtained from humans suggests that re-infection from an exogenous source is not common (Buchman et al., 1979; Gerdes et al., 1981), and that any given individual retains only one strain of HSV latent in his or her ganglia (Buchman et al., 1980; Lonsdale et al., 1980). Despite this observation, it is clear that the immune response does not prohibit re-infection by the latent virus of an individual, since it can be re-inoculated at other anatomical sites and recur there (Goldman, 1961; Blank & Haines, 1973). Our results utilizing the rabbit keratitis model suggest that acute re-infection is possible in latently infected animals. However, the establishment of latency by the re-infecting strain is inhibited. Similar observations were recently reported by Centifanto-Fitzgerald et al. (1982). We confirm their results and further suggest that the immunity is intertypic.

Our observations may have significant implications with regard to the strategy for immunization against recurrent disease in humans. For example, it might be feasible to utilize an attenuated, recurrence-negative HSV strain to latently infect individuals who would then be immune to subsequent latent infections by more virulent recurrence-positive strains. However, it should be emphasized that the recurrence phenotypes observed in rabbits, in particular for HSV-2 strains, did not necessarily correlate with the clinical history of the original isolate. Furthermore, very little is known of the potential complications resulting from a virus latent in the nervous system. In addition, the oncogenic potential of HSV must always be considered a potential danger of any HSV vaccine.

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