Chronic Infections by Herpes Simplex Viruses and by the Horse and Cat Herpesviruses

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Rabbit eyes experimentally infected with either type 1 or type 2 herpes simplex viruses occasionally released virus spontaneously. Injection of adrenalin was not highly effective for stimulating virus release but did seem to have a slight and erratic activating capacity. No spontaneous virus releases were detected from the eyes of six cats infected with cat herpesvirus, but, when adrenalin was administered, an episode of virus release did ensue in one animal. Rabbit spinal cords could be chronically infected with either herpes simplex virus type 2 or equine herpesvirus type 2. The viruses could be reisolated over subsequent months from about half the animals without prior stimulation; the interval between inoculation of trypsinized spinal tissue into tissue cultures and the development of cytopathic effect was often long—more than 4 weeks in some cases.

Herpes simplex virus persists in human tissues and periodically produces skin lesions in some individuals. The form in which it persists during the clinically quiescent periods is not known; the virus may be slowly multiplying or may be in a nonreplicative state. There is similar uncertainty about varicella virus which is believed to persist in the dorsal ganglia, with occasional activation as zoster. How these viruses are clinically activated is not understood. There is no strong evidence one way or the other as to whether herpesviruses of animals persist in their hosts.

A number of experimental studies have been done of herpes simplex virus in rabbits. Chronic infections of the brains of rabbits by herpes simplex virus type 1 have been experimentally produced. Encephalitis developed spontaneously in a proportion of such rabbits, although anaphylactic shock (1) or inoculation of adrenalin (7) seemed to increase the likelihood of this. Other attempts to study the activating ability of adrenalin employed chronic infections of the rabbit eye by herpes simplex virus (4, 5); very few spontaneous releases of virus were observed during these experiments but intramuscular inoculation of adrenalin was reported to considerably increase the incidence of virus release. But Kaufman et al. (3) reported very frequent spontaneous releases of herpes simplex virus from the eyes of experimentally infected rabbits, such as would make exceedingly difficult the assessment of the activating potential of any stimulus.

We have studied several experimental herpes-virus-host relationships from the point of view of (i) ability of the virus to persist, (ii) detectability of the virus, and (iii) the ability of adrenalin to activate virus. The following virus-host relationships were investigated: herpes simplex viruses types 1 and 2 in the rabbit eye, the cat herpesvirus in the cat eye, herpes simplex virus type 2 in the lumbar spinal cord and dorsal ganglia of rabbits, and the horse herpesvirus type 2 in the lumbar spinal cord of rabbits.

MATERIALS AND METHODS

Viruses. The 01 isolate of herpes simplex virus type 1 was obtained from a mouth lesion; the Rodanuss strain was obtained from S. Kibrick. The MS isolate of type 2 virus was originally isolated from the central nervous system of a case of multiple sclerosis by Gudnadottir et al. (2). The cat herpesvirus (feline rhinotracheitis virus) was obtained from R. A. Crandell. The horse herpesvirus type 2 was the LK strain of Plummer and Waterson (6).

Neutralization tests. The doubling-dilution neutralization test was used to identify virus isolates, employing 50 plaque-forming units (PFU) of the virus isolate as challenge dose against dilutions of specific hyperimmune sera. The herpes simplex isolates were additionally typed by neutralization curves.

Inoculation of animals. The herpes simplex and horse herpes inocula were grown in primary rabbit kidney cultures; the cat herpesvirus was grown in primary cat kidney cultures. The inoculum for each animal, whether it be into the eye, femoral muscle, or lumbar spinal cord, was 5.0 log10 PFU. When inoculated into the eye, 0.2 ml of virus suspension was allowed to flow under the eyelids; neither the eye nor the eyelids were scarificated.
Isolation of viruses. Primary rabbit kidney cultures, maintained on 199 with 3% fetal bovine serum, were used for herpes simplex virus isolation from the rabbit eye. The swab was passed over the conjunctival surface and under the eyelids, and then immersed in the fluid of the tube culture for 0.5 hr at room temperature. Simultaneous swabbing over a period of several months into both rabbit and human amnion cultures indicated that the two culture systems were equally sensitive for primary isolations—nine isolates produced cytopathic effect in both rabbit and amnion, one in rabbit only, and one in amnion only. Cat herpesvirus isolates were made in primary cat kidney cultures. The method of isolating herpesvirus from the central nervous system is described below.

Adrenalin inoculations. Adrenalin (Parke, Davis & Co., Detroit, Mich.; 1 mg/ml) was given to rabbits as five injections over a period of 2 days, each consisting of 0.5 ml subcutaneously and 0.2 ml intramuscularly in the front legs, and to the cats as five injections, each of 0.7 ml intramuscularly, over a period of 2 days.

RESULTS

Herpes simplex types 1 and 2 in the rabbit eye. Two strains, 01 and Rodanus, of type 1 herpes simplex virus and one strain, MS, of type 2 virus were inoculated into the left eyes of adult rabbits. A few of the animals died of encephalitis during the ensuing 3 weeks. Virus was isolated from the infected eye of each surviving animal for a period of about 2 weeks immediately after virus inoculation. All of them showed some degree of conjunctivitis; the eyes of the 01 and Rodanus rabbits soon became clinically normal, but the inoculated eye of about 70% of the MS rabbits retained a cloudy abnormality of the cornea.

During subsequent months, the inoculated eye of each rabbit was swabbed every other day, and an attempt was made to isolate virus in rabbit kidney tissue cultures. The results show that spontaneous releases of virus were detected (Table 1), but their frequency was not high, particularly where type 2 virus was concerned. Attempts to increase the release of virus by intramuscular inoculation of adenalin were not markedly successful. Despite 15 episodes, of virus release among the 01 rabbits, none of these episodes closely followed any of the 22 inoculations of adenalin. If isolation of virus during the 2-week period immediately following inoculation of adenalin is regarded as adenalin-stimulated, then in the Rodanus and MS rabbits, the adenalin did seem to slightly increase the frequency of virus release (see Table) 1—5-fold in the case of the Rodanus virus and 13-fold in the case of MS virus.

All seven animals inoculated with the Rodanus strain showed virus release at one time or another during either “non-adrenalin weeks” or “adrenalin weeks”; but not all the 01 and MS rabbits

<table>
<thead>
<tr>
<th>Virus</th>
<th>Rabbis showing virus release</th>
<th>Episodes of release during &quot;non-adrenalin weeks&quot;</th>
<th>Episodes of release during &quot;adrenalin weeks&quot;</th>
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<tbody>
<tr>
<td>Type 1 virus</td>
<td></td>
<td></td>
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<tr>
<td>Strain 01</td>
<td>13/23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15/683&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0/44</td>
</tr>
<tr>
<td>Strain Rodanus</td>
<td>7/7</td>
<td>4/116</td>
<td>4/24</td>
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<tr>
<td>Type 2 virus</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Strain MS</td>
<td>6/22</td>
<td>3/157</td>
<td>3/39</td>
</tr>
</tbody>
</table>

<sup>a</sup> Episodes of virus release do not include the initial virus release during the first 2 to 3 weeks after inoculation of virus into the animals. An “adrenalin week” is any week falling within the 2-week period immediately after the inoculation of adenalin.

<sup>b</sup> Expressed as number of animals showing virus release over number of animals infected.

<sup>c</sup> Expressed as total number of episodes from all the rabbits of a given group over the total obtained by adding together the weeks during which the individual members of the group were swabbed.

As did show virus release (except, of course, during the initial 2 weeks). The duration of each episode of virus release varied from 1 to 12 days, average 6 days, for the 01 rabbits; and 1 to 6 days, average 4 days, for the Rodanus rabbits; each of the six episodes of release of MS virus was detected on 1 day only. Virus isolates were serologically identified.

Cat herpesvirus in the cat eye. Six 3-month-old kittens were each inoculated with cat herpesvirus into the left eye. Virus was isolated in cat kidney cultures for a period of about 2 weeks from the inoculated eye of each animal. All the animals developed conjunctivitis and rhinotracheitis. During subsequent months, the inoculated eye was swabbed every other day. Apart from the initial release of virus, no virus was detected over a period of 3 months. Each of the six cats was then given adrenalin intramuscularly during a 2-day period. At 6 days later, cat herpesvirus was isolated from the left eye of one cat; the swabs taken from this animal during the following 10 days were all positive. No further isolates were made from this cat during the subsequent 2 months. No virus was isolated from the other cats at any time (except for the initial 2 weeks).

Herpes simplex in the rabbit spine. Earlier experiments had shown that inoculation of the MS strain of type 2 herpes simplex virus into the

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*Table 1. Virus isolations from the eyes of rabbits inoculated with strains of type 1 and type 2 herpes simplex viruses*
femoral muscle of the hind leg of the rabbit resulted in invasion of, and inflammation of, the dorsal ganglia and dorsal horn of the lumbar spinal cord; permanent spastic paralysis of the limb was often the outcome. Inoculation of adrenalin during subsequent months seemed to result in an increase in paralysis of some of them, although virus could not be isolated from the homogenized ganglia and spinal cords when the animals were killed at the time of the increase.

An experiment was therefore done in which 43 2-month-old rabbits were inoculated with MS virus into the femoral muscle of the left back leg. All developed some degree of paralysis of the limb. Between 6 and 11 months later, some of the animals were dosed with adrenalin and observed for a change in the degree of paralysis. Any that showed an increase in paralysis were killed, and the lumbar spinal cords were examined for inflammation and for infective virus. The rabbits which did not show an increase in paralysis and the rabbits which were not dosed with adrenalin were also killed at various times, and their lumbar spinal cords were examined (Fig. 1). (Only 38 rabbits are shown because 5 of the original animals were either killed because of the severity of their paralysis or died of some other cause.)

As can be seen, a few of the rabbits did indeed show a marked increase in paralysis shortly after inoculation of adrenalin, but the results do not conclusively demonstrate whether the adrenalin is actually increasing the multiplication and activity of the virus. Inflammation of the lumbar dorsal ganglia and horn was certainly as great among the controls as among the adrenalin-treated animals.

The method used in this experiment for the isolation of virus was much more efficient than the homogenization of the spinal tissue used in earlier experiments. The cells of the lumbar ganglia and spinal cord not actually used for histology were separated by the conventional trypsinization technique by using 0.25% trypsin, and then inoculated into tube cultures of primary rabbit kidney cells, 12 tubes per specimen. The cultures were maintained for 7 weeks. The medium was changed every 6 days; the cell-inoculum still floating in the medium of each tube was centrifuged down and resuspended in 2 ml of fresh medium, which was then added back to the tube. By this method, a number of the rabbits yielded virus. But the time lapse between inoculation of the cultures with the ganglion and cord cells and the first development of cytopathic effect (CPE) was often long, varying between 8 and 43 days; in addition, there was no case in which all the 12 inoculated tubes developed CPE. Fig. 2 shows the development of CPE as a function of time, in the case of 13 of the rabbits which yielded virus.

Uninoculated control cultures were included in these experiments, and were medium changed along with the inoculated tubes; none of the control cultures developed CPE, indicating that

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**Fig. 1.** Fate of 38 rabbits which had been inoculated with type 2 herpes simplex virus into the femoral muscle of the left back leg. All had developed some degree of paralysis as a result of this; some had retained a permanent spastic paralysis, whereas others seemed to recover. CNS, central nervous system.
herpesvirus type 2 would multiply in the central nervous system of young rabbits. The following experiment was done to determine whether the virus would persist in the central nervous system and, if so, how readily it could be reisolated in rabbit kidney cultures. Virus was inoculated directly into the brain or lumbar spinal cord of 6-week-old rabbits. The animals were killed after various intervals. As can be seen from Table 2, virus was reisolated, by the method described above, from 9 of 18 animals killed between 2 and 34 weeks after the original injection of virus. Once again, the time interval between inoculation of the trypsinized cord cells into the kidney cultures and the first appearance of CPE was often long—up to 64 days. Isolates were serologically identified. None of the rabbits showed any clinical symptoms during the experiment.

**DISCUSSION**

Neither our experiments nor those of other workers answer the question of whether herpesviruses are actually multiplying during their clinically quiescent periods. Inability to isolate virus might only reflect a technical difficulty in detecting small amounts of infective virus. Frequent isolation of virus, on the other hand, does not necessarily mean that the virus is in a replicative form; only occasional spontaneous release from individual cells would be necessary to render virus detectable. The considerable time lapse between inoculation of ganglion and cord cells into tissue cultures and the first appearance of CPE was surprising, especially for type 2 herpes simplex virus which has an eclipse period of less than 10 hr. In addition, different tube cultures receiving similar inocula of the same suspension of spinal cells would often develop CPE at widely different times. These results suggest that either a viral inhibitor is present in the inoculum, or the virus is in a cell associated, non-replicative form.

Injection of adrenalin was not highly effective for activating herpesvirus in the eye, although it did perhaps have slight activating capacity. Objective assessment of increase in paralysis of an already paralyzed limb was difficult to achieve, but, in spite of this, adrenalin did seem to markedly increase paralysis in some of the rabbits whose spinal cords were chronically infected with herpes simplex virus type 2, which is of course in harmony with the precipitation by adrenalin of encephalitis in rabbits chronically infected with herpes simplex virus (7). Prior stimulation was not however mandatory in our experiments for the subsequent isolation of either herpes simplex virus or the horse virus from the spinal cord.

The complete absence of observable clinical symptoms adds to the difficulties of interpretation of results present in preliminary experiments. The problem of cross-contamination did not contribute to our results.

**Horse herpesvirus in the rabbit spine.** Preliminary experiments had shown that equine herpesvirus type 2 would multiply in the central nervous system of young rabbits. The following experiment was done to determine whether the virus would persist in the central nervous system and, if so, how readily it could be reisolated in rabbit kidney cultures. Virus was inoculated directly into the brain or lumbar spinal cord of 6-week-old rabbits. The animals were killed after various intervals. As can be seen from Table 2, virus was reisolated, by the method described above, from 9 of 18 animals killed between 2 and 34 weeks after the original injection of virus. Once again, the time interval between inoculation of the trypsinized cord cells into the kidney cultures and the first appearance of CPE was often long—up to 64 days. Isolates were serologically identified. None of the rabbits showed any clinical symptoms during the experiment.

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effects in the rabbits whose spinal cords were chronically infected with the horse herpesvirus raises interesting questions about the central nervous system as a site for persistence of herpesviruses in their natural hosts.

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LITERATURE CITED