# LATENT HERPES SIMPLEX VIRUS IN THE CENTRAL NERVOUS SYSTEM OF RABBITS AND MICE*

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The natural history of herpes simplex virus (HSV) has long been recognized to be unique and many faceted, with the nervous system playing a critical role. Of the syndromes induced in man, acute encephalitis is the most serious. The pathogenesis of this disease has not been defined, but at least some cases have been suggested to result from reactivation of a latent infection (1). Experimentally, encephalitis has been induced by immunologic and pharmacologic manipulation of rabbits previously inoculated with HSV, suggesting strongly that this disease resulted from activation of a latent or persistent infection in the brain (2–4). Recently, we showed that HSV can induce a latent infection of sensory ganglia in experimental animals (5, 6), a finding which has since been extended to natural infections in man (7, 8).

The concept that a latent infection could be reactivated to induce acute encephalitis would be strengthened if it could be shown directly that HSV does in fact induce a latent infection in the central nervous system. In addition, and perhaps of even greater significance, proof of such an association could be important in considerations of the etiology and pathogenesis of chronic degenerative diseases of the central nervous system. In this paper, we show that HSV does indeed induce a long-standing latent infection of the central nervous system. These infections were induced in the brain stems of rabbits after corneal inoculation of the virus, and in the spinal cords of mice after rear footpad infection.

## *Materials and Methods*

*Virus.*—The HSV type 1 (MacIntyre strain) used throughout these experiments has been described previously (9). Stock virus employed for mouse inoculation had been passaged 17–23 times in mouse brains, a typical pool possessing $4 \times 10^4$ $RK_{13}$ cell plaque-forming units (PFU) per milliliter (9). For rabbit corneal inoculation, "high titered" $RK_{13}$ cell grown stock virus with a titer of $10^8$ PFU/ml was employed.

*Mice.*—Inbred and outbred Swiss Webster mice 4 wk of age were used. No significant differences in response between these strains were noted in these experiments. Mice were infected by application of mouse brain passaged virus to scarified, inflamed rear footpads (9).

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*Rabbits.*—Male New Zealand white rabbits 6-wk old were used for corneal inoculation of virus. Here, one cornea was anesthetized, scarified, and then infected with two drops of undiluted stock virus ($\sim 10^7$ PFU) grown in $RK_{13}$ cells.

*Direct Virus Isolation from Tissues.*—Tissues were ground in a TenBroek homogenizer and centrifuged at 3,000 *g* for 5 min in a refrigerated centrifuge (9). The supernatant fluids were then assayed on monolayers of $RK_{13}$ cells which were incubated at 37°C and scored daily for cytopathic effects. Cultures which demonstrated no viral induced alterations by 1 wk after inoculation were discarded as negative.

*Cocultivation of Tissues.*—Tissues to be cocultivated were chopped into small pieces with razor blades and cocultivated with monolayer cultures of $RK_{13}$ cells. These cultures were maintained for 30 days, and fed intermittently with Eagles's minimal essential medium supplemented with 5–10% fetal calf serum. When viral cytopathic effects were suspected, a portion of the supernatant fluid was frozen at −70°C for further study.

*Serological Identification of Viral Isolates.*—Virus isolates to be identified were passed once in $RK_{13}$ cells and titrated. A neutralization mixture contaiining $10^5$ PFU of the virus isolate and a 1:1,000 dilution of heat inactivated (56°C, 30 min) anti-HSV rabbit serum was incubated 1 h at 37°C, diluted 1:100 and assayed on $RK_{13}$ cells. Under these conditions, 85–95% of the PFU of putative HSV isolates were neutralized. Stock HSV was neutralized 92% in a similar assay.

## RESULTS

*Rabbits.*—After corneal inoculation of 21 rabbits, all developed an acute encephalitis from which 14 recovered. These latter animals were used for studies of viral latency. They were killed at various intervals, and the trigeminal ganglion and half the brain ipsilateral to the eye inoculated were removed. The removed portions of the brain were then divided into four anatomic regions: (*a*) brain stem (that portion from the inferior coliculus to 1 cm caudal to the obex, including the pons), (*b*) cerebellum, (*c*) cerebrum (the region outside the lateral ventricles), and (*d*) diencephalon (the region from the superior coliculus forward and within the bounds of the lateral ventricles). Portions of each tissue were frozen for later assay; the remainder was cocultivated with $RK_{13}$ cells. The results of these experiments are presented in Table I. As can be seen, 5 of the 14 cultures of brain stem demonstrated cytopathic effects after 7–19 days of cocultivation, and infectious virus was recovered. Four of these five brain stems were assayed directly for virus; none could be isolated. In addition, each of the five rabbits harbored latent virus in the ipsilateral trigeminal ganglion. It should also be noted that virus could not be detected in other portions of the brain. Finally, all isolates were identified as HSV by neutralization tests employing specific, hyperimmune rabbit serum.

*Mice.*—In these experiments, about 40% of the mice inoculated in one rear footpad became paralyzed in both hind legs within 10 days after infection with HSV. Two-thirds of these died with acute encephalitis, while the remainder either underwent a complete clinical recovery or were left with varying degrees of posterior paralysis. Since the leg contralateral to the foot infected became paralyzed, we reasoned that acute viral infection of the spinal cord was a certainty.

TABLE I

*Recovery of Infectious Herpes Simplex Virus after In Vitro Cultivation of Latently Infected Brain Stem from Rabbits*

| Rabbit number | Months post infection | HSV latent in trigeminal ganglia | HSV latent in brain stem |
|---|---|---|---|
| 1 | 3 | + | + |
| 2 | 3 | + | − |
| 3 | 3 | + | − |
| 4 | 1 | + | − |
| 5 | 1 | + | − |
| 6 | 1 | + | + |
| 7 | 1 | + | + |
| 8 | 2 | − | − |
| 9 | 2 | − | − |
| 10 | 12 | − | − |
| 11 | 5 | + | − |
| 12 | 12 | + | + |
| 13 | 9 | + | − |
| 14 | 9 | + | + |
| Totals | | 11/14 | 5/14 |

Rabbits were inoculated on one scarified cornea with HSV. Animals were then killed at intervals, and tissues were removed, divided, and cocultivated with $RK_{13}$ cells. Viral induced cytopathic effects in the $RK_{13}$ cells were scored at daily intervals and isolates were identified immunologically as HSV.

To determine whether latent HSV was present in the spinal cords of the mice which had recovered from the acute disease but still presented paralytic sequelae, the animals were killed, and their spinal cords were removed. In addition, four to seven sacrosciatic spinal ganglia contralateral to the side of inoculation were also collected. Both tissues were cocultivated with $RK_{13}$ cells. The results of several such experiments involving a total of 53 mice are presented in Table II. As can be seen four mice were found to harbor latent HSV in their spinal cords; viral specific cytopathic effects were produced in the $RK_{13}$ cells after 6–18 days of cocultivation, and infectious virus was recovered. Two of these spinal cords were ground in a TenBroek homogenizer and assayed for infectious virus directly; none was found. It is also of importance to note that latent virus was recovered from the corresponding contralateral sacrosciatic spinal ganglia in each of these mice. Again, the isolates were identified as HSV by neutralization studies.

## DISCUSSION

We have shown directly and unequivocally that HSV induces a latent infection in the central nervous system of mice and rabbits. These findings extend the observations of Good and Campbell (2, 3) and Schmidt and Rasmussen

TABLE II

*Recovery of Infectious Herpes Simplex Virus after In Vitro Cultivation of Latently Infected Lumbosacral Spinal Cords from Mice*

| Experiment number | Number of mice | Mice with right ganglia positive | Mice with spinal cords positive |
|---|---|---|---|
| | | Mice tested | Mice tested |
| 1 | 5 | 5/5 | 0/5 |
| 2 | 7 | 6/7 | 0/7 |
| 3 | 5 | 1/5 | 1/5 |
| 4 | 6 | 4/6 | 1/6 |
| 5 | 6 | 4/6 | 1/6 |
| 6 | 7 | 4/7 | 0/7 |
| 7 | 4 | 3/4 | 0/4 |
| 8 | 3 | 3/3 | 0/3 |
| 9 | 4 | 3/4 | 1/4 |
| 10 | 6 | 3/6 | 0/6 |
| Totals | 53 | 36/53 | 4/53 |

Mice were infected with HSV by inoculation of a rear footpad. After 1–8 mo, animals which had recovered from the acute neurologic disease were killed. Tissues were removed, chopped, and cocultivated with $RK_{13}$ cells. Viral induced cytopathic effects in the $RK_{13}$ cells were scored at daily intervals, and isolates were identified as HSV by immunologic methods.

(4). By inducing encephalitis in rabbits previously infected with HSV, they suggested that the disease resulted from reactivation of a latent infection in the brain.

It is now clear that HSV can induce latent infections in both the peripheral and central nervous systems of experimental animals. Thus, a critical, basic event required for support of the concept that the nervous system is the source of virus for recurrent cutaneous disease and some cases of encephalitis has been shown to occur. As stated earlier, application of these methods to human tissue has shown that latent infections occur in trigeminal ganglia; it now would be important to determine whether latent virus can also be detected in the central nervous system of man. Finally, the possible role of a latent viral infection in the genesis of chronic degenerative diseases of the central nervous system can now be seriously investigated in these experimental systems.

## SUMMARY

Herpes simplex virus (HSV) type 1 induces a long-standing latent infection in the central nervous system of mice and rabbits. The infection was established in the brain stems of rabbits after corneal inoculation of the virus, and in the spinal cords of mice after rear footpad infection. In these animals, infectious virus could not be recovered by direct isolation from tissues; it was detected only after the tissues were maintained as organ cultures in vitro.

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