The Weakly Virulent Herpes Simplex Virus Type 1 Strain KOS-63 Establishes Peripheral and Central Nervous System Latency Following Intranasal Infection of Rabbits, but Poorly Reactivates In Vivo

WILLIAM G. STROOP, PH.D., AND M. CAREENE BANKS, B.S.

Abstract. The virological, clinical and electrophysiological manifestations of acute and experimentally reactivated infections of the rabbit central nervous system (CNS) and trigeminal ganglia have been studied after intranasal infection with herpes simplex virus type 1 (strain KOS-63). All animals shed virus in nasal secretions during the acute phase of infection. Although no rabbits developed clinical signs during the acute phase of infection, mild electroencephalographic (EEG) abnormalities consistent with viral invasion of the CNS were seen. KOS-63 produced only occasional gross and histopathologic herpetic lesions of the CNS and was very rarely recovered from the brain. These results indicate that KOS-63 was poorly neuroinvasive and only mildly neurovirulent during the acute phase of infection. However, KOS-63 did establish latency within the CNS and trigeminal ganglia of infected rabbits as demonstrated by in situ hybridization and by recovery of virus from co-cultivation cultures, but not from cell-free homogenates of nervous tissue. Cyclophosphamide and dexamethasone injections were used to reactivate latent CNS and trigeminal ganglionic infections. Following injection of the drugs, no animal shed virus in nasal secretions or developed obvious clinical or EEG changes. However, KOS-63 was recovered from co-cultivation cultures of brain and trigeminal ganglia at greater frequency following drug injection than during latency. These results indicate that KOS-63 was only poorly susceptible to drug-induced reactivation. In vivo experiments confirmed that the apparent poor neuroinvasiveness and weak neurovirulence of KOS-63 was not due to viral temperature-sensitive defects, deficient production of viral thymidine kinase, or abnormal defects in viral DNA polymerase function.

Key Words: EEG; Electroencephalography; Encephalitis; Herpes simplex; Herpesvirus; HSV-1.

INTRODUCTION

In addition to causing acute or recurrent orofacial or ocular lesions (1–7), herpes simplex type 1 viruses (HSV-1) can also produce fatal encephalitis of human adults (8–10). Encephalitis begins with fever, headache, and confusion, and often progresses to produce clinical signs indicative of inferomedial temporal and frontal lobe involvement including seizures and electroencephalographic (EEG) changes (8). In untreated individuals, the disease frequently advances to coma and ends with death. The pathology is most commonly characterized by hemorrhagic or necrotic lesions of the inferior temporal lobes which may extend to the cingulate gyrus, insulae, and superior temporal lobes.

Comparative studies have indicated that HSV-1 strains differ with regard to their ability to produce encephalitis (11–15) or ocular disease (16–19) during acute or reactivated experimental infections, suggesting that viral genetic elements contribute significantly to the ultimate expression of the central nervous system (CNS) or ocular diseases. Rabbit models of acute (11, 12) or reactivated encephalitis (13) have been developed in which EEG abnormalities (13), seizures (11, 12, 15), and/or focal pathologic involvement of the temporal and frontal lobes (11–13) are prominent findings. These models involve inoculation of HSV-1 directly into the olfactory bulb or infection of the nasal mucosa; in both cases, virus spreads into the CNS along the olfactory pathway (7).

As a first step in developing an understanding of the viral genetic elements which may influence the manifestation of HSV-1-induced CNS disease following reactivation, we have focused our attention on identifying HSV-1 strains which mimic the stereotypic clinical, virologic and electrophysiologic features seen in the human adult. In the study reported here, we used the KOS-63 strain of HSV-1 since its ability to produce focal encephalitis during acute or experimentally reactivated infections of the rabbit has not been directly tested, and because its restriction map is known (20) which will facilitate subsequent correlations between disease phenotype and viral genotype.

There are two strains of KOS: KOS-63 and KOS-79 (14). In mice, KOS-79 is more neuroinvasive and neurovirulent than KOS-63 (14). Another strain of KOS, termed KOS(M), is biologically similar to KOS-63 (14, 21–23) and has been shown to be non-neuroinvasive following footpad inoculation of mice (21–23). The failure of KOS(M) to invade the CNS is unrelated to defects in
viral thymidine kinase gene activity (23) and glycoprotein C (24). Although these data indicate that KOS(M) and KOS-63 are likely to be weakly pathogenic, they must be interpreted with caution because murine neurovirulence does not always correlate with rabbit neurovirulence (23).

The majority of data available on reactivated KOS infections pertains to the peripheral nervous system (PNS) and the eye. In studies of recurrent ocular infections of rabbits, Hill et al. (17) were unable to elicit KOS shedding in the eye following adrenergic induction, despite the fact that KOS established latent trigeminal ganglionic infection. However, since adrenergic stimulation exerts its primary effect on the ganglia, it is unclear whether KOS is inherently incapable of reactivation or whether a stronger systemic influence is needed to provoke a recurrence in vivo. Also, the relationship between in vivo reactivation in the PNS or CNS and the degree of neurovirulence possessed by a particular strain of HSV-1 is not well understood, and it is widely believed, but unproven, that HSV-1 strains of low virulence invade the CNS as a normal consequence of peripheral infection. The low virulent character of KOS-63 for PNS tissue provided an opportunity to determine whether this virus was capable of establishing CNS latency. In this report, we studied experimental rabbit infections to determine if KOS-63 established latency in CNS tissue and whether administration of systemic immunosuppressive therapy, previously shown to reactivate latent trigeminal ganglionic and CNS HSV-1 infections (13), would reactivate KOS-63.

MATERIALS AND METHODS

Viruses

KOS-63 is an isolate which originated from a clinical case of herpes labialis and has been passed numerous times in vitro (14, 22, 23). We obtained our stock of this virus from Dr. Robert R. Mc Kendall (Galveston, TX) and propagated it in either VERO or BHK-21 cells to final titers of between 10^4 and 10^6 50% tissue culture infectious doses (TCID50) per ml.

Viral Thymidine Kinase and DNA Polymerase Assays

The relative expression of HSV-1-encoded thymidine kinase in KOS-63 was determined using the ara-T test (26). Briefly, BHK-21 cells were infected with 1.0 TCID50 per cell and grown at 37°C in the presence or absence of 50 μg/ml of ara-T. At 48 hours, the clarified supernatants from these cultures were analyzed for virus content by TCID50 assay.

KOS-63 was tested for its ability to replicate in the presence of 250 μg/ml of phosphonoacetic acid (PAA) as described by Day et al. (27). However, because high titers of virus can break through PAA concentrations even as high as 500 μg/ml (Strop, unpublished observations), we diluted our virus stocks to contain a maximum of 10^4 through 10^5 TCID50 for this assay. The PAA-sensitive 186 HSV type 2 strain, graciously provided by Dr. Priscilla Schaefer (Boston, MA), and the PAA-resistant mutant of 186 (188), prepared as described by Day et al. (27), were used as controls.

Rabbit Infection and Reactivation

New Zealand White (NZW) rabbits were obtained from LIT Rabbity (Whitehall, MT). For infection, rabbits were tranquillized and a 0.1 ml volume of virus stock containing 5.5 x 10^5 TCID50 of infectivity was delivered to each nostril (13). Rabbits were treated with cyclophosphamide and dexamethasone to reactivate their latent infections as previously described (13). Briefly, on the 49th day post-infection (dpi), they were intravenously injected with 75 mg/kg of cyclophosphamide (Sigma Chemical Co., St. Louis, MO) followed 24 h later by an intravenous injection of 4 mg/kg of dexamethasone (Azium®; Schering Corp., Kenilworth, NJ). The 49th dpi on which cyclophosphamide was injected was defined as day 0 post-reactivation (dpr). The number of rabbits used for each experiment are indicated in the Results.

Daily swabs of nasal secretions were taken from rabbits to monitor virus shedding during acute and reactivated infections (13, 15, 16, 28). In the first experiment, swabs were taken from 12 rabbits from 1 through 12 dpi and from 45 to 4 dpi (13, 15). In the second experiment, swabs were taken from ten rabbits on 4 dpi to confirm infection, and then from 40 to 9 dpi (13, 15). All swabs were assayed for HSV-1 infectivity on VERO cells as described (13). All cultures were frozen intact at -70°C and subsequently thawed and repassed to confirm the presence or absence of HSV-1.

Electroencephalography

Electroencephalography was performed using a circumferential montage as described (13, 15) on three infected animals in the first experiment at 3, 8, and 49 dpi, and on the same three animals at 0, 3, and 7 dpi. Baseline EEGs were obtained on each rabbit 1 day before infection so that each animal served as its own control. Animals were checked once or twice daily and all EEG recordings were made about the same time each day. Electroencephalograms were also obtained on five uninfected animals to derive normal EEG data for rabbits.

Virus Isolation

At selected dpi and dpr, rabbits were euthanized with sodium pentothal and immediately perfused with phosphate buffered saline. Two animals were studied at 6 dpi, six at 0 dpi, five at 3 dpi, two at 7 dpi, and two at 14 dpi, and their tissues were processed as follows. The left olfactory bulb, olfactory tract, frontal cortex, hippocampus-entorhinal cortex (inferior temporal lobe), and trigeminal ganglia were assayed in cell culture for the presence of cell-free virus or latent virus. One ganglion as well as portions of the CNS tissues from each rabbit were separately homogenized, frozen at -70°C, thawed, clarified of cell debris by centrifugation at 500 × g for 10 minutes, and the supernatant checked for viral infectivity on VERO cells. The other ganglion and the remainder of each CNS tissue were separately minced and co-cultivated with VERO indicator cells. All nervous system cultures were maintained and checked for development of virus-induced cytopathic effect. When viral cytopathic effect was observed, or after cultures had been maintained for 14-14 days, they were frozen at -70°C, thawed, and repassed onto fresh VERO cells to confirm virus positive or negative cultures. In some experiments, discussed in the Results, tissues were prepared in the same manner, except that the left
**TABLE 1**
Temperature Sensitivity of KOS-63

<table>
<thead>
<tr>
<th>Strain</th>
<th>Temp</th>
<th>Number of plaques at dilutions of stock*</th>
<th>Titer† (pfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10⁻²/ml 10⁻⁴/ml 10⁻⁶/ml</td>
<td></td>
</tr>
<tr>
<td>KOS-63</td>
<td>37°C</td>
<td>TMTC 167.5 13.0 1.7 x 10⁵</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40°C</td>
<td>TMTC 95.5 13.5 1.0 x 10⁴</td>
<td></td>
</tr>
<tr>
<td>-GC</td>
<td>37°C</td>
<td>TMTC 47.5 4.0 4.8 x 10²</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40°C</td>
<td>27 0 0 2.7 x 10⁴</td>
<td></td>
</tr>
</tbody>
</table>

* Plaques were counted in wells of plates initially inoculated with 10⁻⁴, 10⁻³, and 10⁻² dilutions of stock virus preparations. Plaque counts are shown as the average of two experiments run in parallel.
† Titters are expressed as the average number of plaque forming units (pfu) per ml of the original stock virus.
§ TMTC, too many plaques to count.

**TABLE 2**
Comparative Thymidine Kinase Activity of HSV-1 Strains

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Presence of ara-T</th>
<th>Mean titer*</th>
<th>Reduction in titer in presence of ara-T†</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOS-63</td>
<td>+</td>
<td>7.0 x 10²</td>
<td>4.9 x 10⁴ (99.99%)</td>
</tr>
<tr>
<td>HTZ§</td>
<td>+</td>
<td>5.5 x 10¹</td>
<td>6.4 x 10⁴ (99.99%)</td>
</tr>
<tr>
<td>-GC</td>
<td>-</td>
<td>3.5 x 10⁴</td>
<td></td>
</tr>
</tbody>
</table>

* The mean titer represents the results of three separate experiments run in parallel.
† The percent reduction in titer was calculated as the Δ% between the titer of virus present in cultures grown in the absence of ara-T and the titer of virus present in cultures grown in the presence of 50 µg of ara-T.
§ The HTZ (Heitzman) strain of HSV-1 was included as a positive control.

**Temperature Sensitivity**

Temperature-sensitive HSV-1 mutant strains in general do not replicate well at the normal temperature of the rabbit (39.5°C) and may as a result be non-virulent. We therefore compared the ability of KOS-63 to form plaques at 37°C and 40°C in VERO cells. KOS-63 was found to replicate equally well at both temperatures; the 37°C/40°C ratio for KOS-63 was 1.7, revealing no temperature sensitivity in this strain (Table 1). In comparison, the 37°C/40°C ratio for the temperature-sensitive -GC HSV-1 strain (15) was 171. Similar results were obtained when the experiment was repeated and plaques were counted at 33°C and 40°C.

**Thymidine Kinase Activity**

Next we determined the relative expression of KOS-63-encoded thymidine kinase. A thymidine kinase deficient HSV-1 strain is much less affected by the presence of ara-T than an HSV-1 strain.

**RESULTS**

**General Characteristics of KOS-63**

Although careful review of the literature indicated that KOS-63 and KOS(M) are probably identical, we felt it was important to confirm that KOS-63 shared the characteristics of KOS(M) which have been extensively studied in vivo and in vitro (21, 23). Because the weak invasive and virulent properties of KOS(M) are not related to viral temperature sensitivity and thymidine kinase expression, we tested our KOS-63 strain in vitro with regard to these parameters.

Fig. 1. Ten second circumferential EEG tracings from KOS-63-infected NZW rabbit No. 224 during acute and reactivated infections (time 0 at left). Contact electrodes A and B were placed over the olfactory bulbs; C and D were placed adjacent to the supraorbital crests; E and F were placed over the posterior cerebral cortex (see montage in the center of each recording). Tracings were recorded from contact electrodes B-A (channel 1), A-C (channel 2), C-E (channel 3), E-F (channel 4), F-D (channel 5), and D-B (channel 6). The electrocardiogram (EKG) is shown at the bottom of each recording. (A) 0 dpi immediately before infection. (B) 8 dpi. (C) 0 dpi immediately before injection of cyclophosphamide (49 dpi). (D) 7 dpi (56 dpi).

which expresses competent levels of the enzyme, and will therefore replicate nearly as well in the presence or absence of ara-T. KOS-63 and the thymidine kinase positive HTZ HSV-1 strain were both markedly inhibited by ara-T (Table 2), demonstrating that they both contained fully functional thymidine kinase. This result shows that KOS-63, like KOS(M), is not a thymidine kinase deficient virus.

DNA Polymerase Activity: We also determined the sensitivity of KOS-63 to PAA. We felt this was important because the HSV DNA polymerase gene has been implicated in neuroinvasion (23, 27), and because Field and Coen (30) demonstrated that PAA-resistant mutants of an unspecified KOS strain were markedly less virulent than wild type KOS after intracerebral inoculation of mice. Phosphonoacetic acid compromises HSV DNA polymerase function via a noncompetitive interaction with the enzyme pyrophosphate binding site. The replication of KOS-63 was completely inhibited by 250 μg of PAA when the input inoculum was equal to or less than 10^4 TCID (Table 3). When the input of KOS-63 was 10^5 TCID, breakthrough was observed. The PAA-resistant strain 186° replicated in PAA at all input titers (Table 3). This result indicates that KOS-63 is not inherently PAA-resistant.

Acute Phase Studies

Shedding of KOS-63 in Nasal Secretions: In the first of two experiments, 14 rabbits intranasally inoculated with KOS-63 were swabbed daily for 12 dpi to monitor viral shedding in nasal secretions. All rabbits shed virus in their nasal secretions for the first 5 dpi. Significantly fewer positive nasal swabs were obtained between 6 and 12 dpi (31/84) than between 1 and 5 dpi (59/60) (p < 0.001 by χ²), indicating that the acute phase of KOS-63 shedding began to decline by about 5 dpi.

Clinical Course and Electroencephalography: In the second experiment, 14 rabbits were intranasally inoculated with KOS-63. None developed clinical signs of infection at any dpi. Electroencephalography was performed on the same three rabbits at each of 3 and 8 dpi to detect subtle abnormalities that were not apparent clinically. Mild EEG changes were noted at 8 dpi consisting of 4-5
second, high voltage activity localized to the left portion of the brain (Fig. 1). These findings appeared to correlate with the histopathologic damage noted below.

**Virus Isolation from Nervous Tissue:** Based on our previous experience with a variety of HSV-1 strains in the rabbit model (13, 16, 25), KOS-63 should have been present between 3 and 9 dpi in an infectious, cell-free (non-latent) form within both the trigeminal ganglia and the CNS olfactory system. However, KOS-63 was only recovered from the homogenate of the olfactory tract from one of two rabbits killed at 6 dpi.

**Histopathology and in situ Hybridization:** Histologic examination of the brain and trigeminal ganglia was performed on the two animals sacrificed at 6 dpi to assess the extent of viral-induced CNS damage. A gross lesion was seen on the brain stem of one rabbit at the trigeminal nerve root entry zone (not shown) which was histologically characterized by infiltration of the fifth cranial nerve tract by mononuclear cells (Fig. 2A). Both rabbits had inflammatory infiltrates in the trigeminal meningeal nucleus (Fig. 2B). These findings indicate that KOS-63 invaded the brain during the same period it was being shed from the nose.

The trigeminal ganglia from these animals were histologically normal; however, in situ hybridization revealed the presence of KOS-63-specific RNA (Fig. 3C) which was restricted to the neuronal nuclei. Although the hybridization was performed on DNAse-treated tissues, the probe used was not specific for latency associated transcript (LAT) RNA. The RNA detected, therefore, could reflect either an acutely infected ganglion cell or a latently infected one. However, the facts that the animal which was probed was not shedding virus in nasal secretions at 6 dpi, and that cell-free virus was not recovered from the contralateral ganglia of either of the two rabbits sacrificed at 6 dpi, argue that the hybridization signal represented latent virus. Also characteristic of HSV-1 latency was the observation that the autoradiographic grains over ganglionic neurons containing KOS-63 were restricted to the nucleus and were few in number (28, 29). Although we did not attempt to quantitate the number of positive cells, they were rare; only about two to three positive cells were found per section of trigeminal ganglia.

**Latency Phase Studies**

We performed two studies to determine whether KOS-63 established latency. First, we explanted trigeminal ganglia and portions of the olfactory bulbs, olfactory tracts, frontal cortices, and inferior temporal lobes to provoke virus reactivation in vitro. Second, we used in situ hybridization to visualize cells latently infected with KOS-63.

**Histopathology and Virus Isolation from Nervous Tissue:** Six KOS-63-infected rabbits (Nos. 232, 233, 254-257) were swabbed from 40-48 dpi to check for sponta-

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**Fig. 2.** Histopathology of acute (A and B) and reactivated (C and D) KOS-63 infection. (A) Mild leptomenigitis and infiltration of the fifth cranial nerve tract in rabbit 221 by mononuclear cells at 6 dpi. (B) Inflammation of the trigeminal mesencephalic nucleus in rabbit 226 at 6 dpi. The nucleus is adjacent to the fourth ventricle (upper left) and the locus ceruleus (center-right). (C) Meningitis with parenchymal infiltration in the entorhinal cerebral cortex in rabbit 228 by mononuclear cells at 7 dpR. Note the karyolitic neurons (arrow). (D) Mild inflammation of the trigeminal ganglion of rabbit 229 at 7 dpR. Bars = 100 μm.
neous reactivation. Virus was not recovered from any swab culture. Immediately after swabbing the rabbits at 48 dpi, all six animals were killed and processed for analysis (Table 4). Histopathologically, the ganglia and brain tissues from all rabbits appeared normal. Virus was not recovered from either cell-free homogenates or from cocultivation cultures of any CNS region assayed (Table 4). However, KOS-63 was recovered from explanted trigeminal ganglia from two of the six rabbits (Table 4).

In situ Hybridization: In situ hybridization was performed on DNase-pretreated sections of CNS and ganglionic tissues taken from the same six rabbits that were analyzed virologically. Nuclear-restricted, HSV latency-specific RNA was detected in CNS neurons of three of the six KOS-63-infected rabbits examined (Fig. 3). The trigeminal ganglia from these animals could not be examined since they were used to prepare co-cultivation cultures and cell-free homogenates. Within the CNS, positive cells were found in the trigeminal principal sensory nucleus in the medulla in one rabbit (Fig. 3A) and in the anterior cerebral cortex adjacent to the entorhinal fissure in another rabbit (Fig. 3B). Interestingly, although KOS-63 RNA positive cells were detected in the entorhinal cortex removed from rabbit 255 (Fig. 3B), virus was not recovered from the contralateral entorhinal cortex by co-cultivation techniques.

Reactivation Phase Studies

Since KOS-63 was recovered at 48 dpi from co-cultivation cultures of ganglia, and since viral RNA was observed in the nuclei of CNS neurons, KOS-63 established both ganglionic and neuronal latency. However, because KOS-63 was recovered from co-cultivation cultures of the ganglia, but not from the CNS, it appeared that KOS-63 latency was less common in the CNS than in the ganglia. It was, therefore, of interest to determine whether KOS-63 could be reactivated in ganglia or brain in vivo. Based on the data obtained with other HSV-1 strains in this model (13, 28), KOS-63 was expected to replicate titers sufficient to be recovered in cell-free homogenates and to spread centrifugally back to the periphery following drug-induced reactivation.

Shedding of KOS-63 in Nasal Secretions: At 49 and 50 dpi, a total of 12 rabbits (Nos. 223, 225, 226, 228–231, 258–260, 262, 263) were intravenously injected sequentially with cyclophosphamide and dexamethasone to reactivate latent CNS and trigeminal ganglionic infections. These drugs were used together because neither drug alone is capable of reactivating latent HSV infection in the rabbit (16). Rabbits were swabbed for 9 days prior to drug-induced reactivation to determine if any rabbit had spontaneously reactivated its latent infection before the drugs were administered. No rabbit shed virus in nasal secretions prior to drug administration. A total of 16 rabbits (Nos. 222–231, 258–263) were monitored by daily swabbing assays to isolate KOS-63 from nasal secretions during the reactivation period. Twelve rabbits were swabbed through 3 dpR, seven were swabbed through 6 dpR, five through 7 dpR, and four through 14 dpR. KOS-63 was not recovered from the nasal secretions of any rabbit.

Histopathology: Histologic examination of the brain and trigeminal ganglia was performed on five rabbits at 3 dpR (52 dpi; Nos. 230, 231, 258–260), and two at each of 6 dpR (55 dpi; Nos. 262, 263), 7 dpR (56 dpi; Nos. 228, 229), and 14 dpR (63 dpi; Nos. 223, 225). The brains of the five rabbits sacrificed at 3 dpR were normal. However, by 6 dpR rabbit 262 had a small inflammatory focus in the principal trigeminal sensory nucleus in the pons, and rabbit 263 had moderate necrosis in the anterior.
pyriform cerebral cortex (not shown). At 7 dpi, rabbit 228 had a gross hemorrhagic lesion in the entorhinal cortex of the inferior temporal lobe (not shown) which was characterized histologically by inflammation and necrosis (Fig. 2C). The other rabbit killed at 7 dpi (No. 229) had minor inflammation of the trigeminal ganglion (Fig. 2D) and fifth cranial nerve tract in the pons. The brains of both rabbits killed at 14 dpi (Nos. 223, 225) were normal. Together, these results indicate that KOS-63 is reactivatable in vivo. However, since only the brains of animals killed between 6 and 7 dpi contained histopathologic lesions, the reactivation of KOS-63 was either infrequent, or the reactivated virus was rapidly cleared from the CNS.

Virus Isolation from Nervous Tissue: To confirm that KOS-63 had reactivated in vivo, we measured the amount of virus present in the brains and trigeminal ganglia of two rabbits at each of 3 (Nos. 230, 231) and 7 dpi (Nos. 228, 229). So that we could detect only cell-free virus rather than both cell-free and latent virus, we prepared homogenates of portions of the olfactory bulb, olfactory tract, anterior cerebral entorhinal cortex, hippocampus and adjacent entorhinal cortex, and one trigeminal ganglion. In this experiment, we were unable to isolate KOS-63 from any nervous tissue sample. However, because the lower limit of sensitivity of the cell-free virus assay ranged between 4 x 10^4 and 2 x 10^5 TCID/gm depending on the mass of the tissue sample prepared, it was possible that very low levels of cell-free virus present in a given sample were missed by the assay. We, therefore, repeated this experiment with another group of five animals (Nos. 258-260, 262, 263); however, this time we assayed the CNS and ganglionic tissues by both co-cultivation and cell-free assays. Although there was variability among rabbits, we were able to recover virus by co-cultivation techniques from all nervous system tissues examined from all animals studied (Table 4). It is of interest to note that although virus was recovered from several areas of the olfactory pathway at 3 and 7 dpi, only the 7 dpi animals were noted to have histologic evidence of CNS infection.

The fact that the virus was not recovered from latently infected rabbits, but was isolated from co-cultivation cultures of CNS tissues from all animals subjected to drug-induced stimulation (Table 4), corroborates the histologic impression that KOS-63 reactivated in vivo.

Clinical Course and Electroencephalography: Following administration of cyclophosphamide and dexamethasone on days 49 and 50 dpi, respectively, none of 12 rabbits displayed signs indicative of neurologic disease; however, one rabbit (No. 226) died of cyclophosphamide-induced liver toxicity (13, 16). Immediately prior to the cyclophosphamide injection at 49 dpi (0 dpi), EEGs were obtained from the same three rabbits which had had EEGs during the acute phase of infection. The tracings from these animals at 3 dpi (52 dpi) and 7 dpi (56 dpi) were essentially unchanged from the 0 dpi recordings (Fig. 1). These results indicate that KOS-63 was capable of producing mild EEG changes only during the acute phase of infection.

Challenge of KOS-63-Infected Rabbits with Neurovirulent HSV-1 Strain +GC

We investigated the possibility that the non-lethal nature of KOS-63 might protect rabbits against a subsequent challenge by a lethal strain of HSV-1. For this experiment, we used four rabbits that had been given cyclophosphamide and dexamethasone at 49 and 50 dpi, respectively. Because the drugs are immunosuppressive, we waited an additional 50 days for the immune status of the animals to recover. This is three times longer than the time re-
quired for the immune response of mice to recover after injection of 200 mg/kg of cyclophosphamide (Stroop, personal observations). At 100 dpi (51 dpR), we intranasally inoculated these four rabbits with 10^6 TCID of HSV-1 strain +GC. +GC has previously been shown to cause death in 85-100% of rabbits (15). Two rabbits died as a result of viral challenge; one at 4 days and one at 9 days after challenge. The other two rabbits remained healthy. These results demonstrate that preinfection with KOS-63 provides only partial protection against a lethal challenge with +GC.

**DISCUSSION**

We undertook studies with KOS-63 as part of our ongoing work to define the pathogenic characteristics of HSV-1 infection in the intranasally infected rabbit. The rabbit model is more suitable than murine models for the study of the viral and host factors which contribute to encephalitis because it more closely approximates many of the pathological and electrophysiological features of the disease as seen in the human adult (7).

The following data indicate that KOS-63 was capable of invading the rabbit CNS as well as the PNS: (i) virus was recovered from the olfactory tract during the acute phase of infection; (ii) virus was recovered from latently infected trigeminal ganglia; (iii) cells harboring latent virus were detected in the ganglia and the CNS. By identifying latent KOS-63 infection within the CNS, our data document that this low virulent HSV-1 strain also establishes CNS latency in rabbits.

However, KOS-63 was not very virulent once it reached the CNS. This conclusion is supported by the observation that virus was isolated from only one CNS sample taken from the olfactory pathway from one of two rabbits at 6 dpi. However, since KOS-63 was isolated from only one sample and because the histopathologic damage elicited by KOS-63 at 6 dpi was relatively minor, KOS-63 would appear to only rarely cause a significant brain infection. The *in vitro* virologic data demonstrate that this lack of virulence was not due to deficient viral thymidine kinase activity, grossly abnormal viral DNA polymerase function, or viral temperature sensitivity.

The fact that KOS-63 caused only minimal EEG changes during the acute phase of infection also supports the concept that KOS-63 is weakly virulent following intranasal infection. Electroencephalographic abnormalities have previously been shown to occur following intranasal infection of neuroinvasive HSV-1 strains in the rabbit (13, 15). Following H129 infection, slow waves were seen at 3 dpi, high voltage slow waves at 7 dpi, and high voltage phase reversals with repetitive spikes and sharp discharges were seen at 9 dpi (13). +GC caused similar EEG abnormalities which were also associated with motor seizures and post-ictal slow waves (15).

Analysis of KOS-63 latency and reactivation was significantly hampered by the low virulence of this virus. In previous studies which employed cyclophosphamide and dexamethasone to reactivate the highly virulent H129 HSV-1 strain in rabbits, cell-free virus was easily detected in brain and ganglionic homogenates, and virus was recovered from ocular and nasal secretions (13, 16). In addition, H129 was shown to re-express HSV RNA and DNA by *in situ* hybridization and to spread to adjacent CNS centers by electron microscopy (28). In contrast, KOS-63 was not recoverable in cell-free homogenates at any time after reactivation and did not reappear at the periphery. KOS-63 was difficult to recover even from latently infected trigeminal ganglia; virus was recovered in co-cultivation cultures in only two of six rabbits studied (Table 4). Since KOS-63 was not isolated in any form during latency, but was recovered from co-cultivation cultures of the brain and the ganglia of the five rabbits subjected to drug-induced reactivation (Table 4), it is possible to conclude that KOS-63 reactivated but was unable to replicate to titers sufficient to be recovered in cell-free homogenates. This conclusion is supported by both the histopathologic and EEG data obtained during drug-induced reactivation. Histopathologic damage was only noted in three animals between 6 and 7 dpR; animals examined at 3 and 14 dpR were normal. The EEG tracings obtained from three rabbits subjected to the drug protocol also failed to show any of the abnormalities previously seen during experimentally induced CNS reactivation (13).

Since neurons latently infected with KOS-63 were relatively rare by *in situ* hybridization and because KOS-63 did not replicate to measurable titers during the reactivation period, we did not attempt to identify viral nucleic acids or viral antigens in tissues obtained from rabbits subjected to the drug protocol.

The poor neuroinvasiveness of KOS-63 and its inability to replicate to sufficient titers within the ganglia and CNS probably account for the apparent rarity of latently infected cells *in vivo*. Without sufficient replication, KOS-63 would be unable to effectively spread to and establish latency within a large number of trans-synaptically connected neurons *in vivo*. The inability to induce significant KOS-63 reactivation *in vivo* may, therefore, be related to the low number of latently infected cells.

However, it is uncertain as to whether KOS-63 is genetically inherently incapable of high yield reactivation or whether this virus is rapidly cleared from nervous tissue by immune mechanisms when it does reactivate. If KOS-63 was genetically capable of high yield reactivation (i.e. to a level where virus could be recovered from cell-free homogenates) but was rapidly cleared from the CNS by immune mechanisms, it would be expected that there would have been more histologic evidence of inflammation during drug-induced reactivation, such as that previously reported during reactivated H129 infection (13, 16, 28). The infrequent and relatively minor lesions
produced by KOS-63 infection or reactivation suggest that this virus is not highly immunogenic in the rabbit. The virus challenge experiments support this opinion, in that +GC challenge of KOS-63-infected rabbits provided only 50% protection. In contrast, preinfection of rabbits with the equally non-virulent −GC strain afforded 90% protection against subsequent +GC challenge (15). Although antigenic dissimilarity could explain the failure of KOS-63 to effectively protect rabbits, two lines of evidence suggest this is not likely the case. First, strains of HSV-1 are antigenically similar and induce type-common and type-specific crossreactive immunity which is protective in vivo—in some cases, even against antigenically dissimilar strains of HSV type 2 (31). Second, the gB glycoprotein of KOS contains epitopes which can protect mice against challenge by a different HSV-1 strain (32). Therefore, it is more likely that the failure to recover KOS-63 during reactivation was due to its inherent (genetic) inability to be effectively reactivated coupled with the relative rarity of latently infected cells in vivo.

Following administration of the drugs used to reactivate latent CNS and ganglionic infections, no rabbits shed virus in nasal secretions. This is in marked contrast to the H129 and F strains which can easily be recovered from these secretions for up to 12 dpR (13). Hill et al (17) used adrenergic stimulation to provoke recurrent KOS herpetic eye disease. In this study, rabbits were subjected to 6-hydroxydopamine iontophoresis followed by topical administration of 2% epinephrine. Although this procedure successfully provoked reactivation of latent infections induced by several other HSV-1 strains, no rabbit latently infected with KOS re-expressed virus in tear film. However, since both our study and the study by Hill et al demonstrated that KOS could establish latency, and because direct adrenergic stimulation and systemic administration of cyclophosphamide plus dexamethasone both failed to produce shedding at the periphery, it would appear that KOS reactivation is separate from the establishment and maintenance of latency. These data also imply that the control over these phenomena are determined by viral genes whose functions are clearly different in KOS than in other HSV-1 strains that can establish latency and be relatively easily reactivated in vivo. Support for this concept comes from studies by Wagner et al (33) in which the expression of HSV-1 LAT was measured in two species of animals inoculated with different HSV-1 strains. Rabbits latently infected with KOS(M) expressed an unusually high amount of the smaller LAT RNA (1.5 kb) as opposed to mice. This study suggests that host factors as well as viral factors influence the molecular events associated with the establishment and maintenance of latency. We are presently determining whether the high expression of this transcript is related to the low neurovirulence of KOS-63 and/or its poor ability to be exogenously reactivated in the rabbit.

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