Experimental Infection of Squirrel and Marmoset Monkeys with Attenuated Herpesvirus saimiri

Lawrence Falk, John Wright, Friedrich Deinhardt, Lauren Wolfe, Priscilla Schaffer, and Matilda Benyesh-Melnick

Departments of Microbiology, Rush-Presbyterian-St. Luke's and University of Illinois Medical Centers, Chicago, Illinois 60612 [L. F., J. W., F. D., L. F.] and Department of Virology and Epidemiology, Baylor College of Medicine, Houston, Texas 77025 [P. S., M. B-M.]

Summary

Herpesvirus saimiri (HVS) was propagated in vitro for 3 passages at 39°C and cloned 3 times at 34°C. This virus was inoculated into cotton-topped marmoset and squirrel monkeys; all inoculated monkeys became infected as HVS was reisolated after their circulating lymphocytes were cultured with vero cells and measurable levels of antiviral antibodies developed that were measured by immunofluorescence and/or neutralization tests. None of the inoculated monkeys developed any signs of overt disease and all inoculated monkeys have survived 9 to 14 months postinoculation. The attenuated virus appears to be genetically stable as virus isolated from an infected marmoset was passed 3 times in vitro and then inoculated into other marmosets, which became infected and remained clinically well. Marmosets latently infected with attenuated HVS were not protected when challenged with a large dose (770 plaque-forming units) of oncogenic HVS, although these marmosets survived about 3 times longer than did inoculated control marmosets.

Introduction

HVS, indigenous in squirrel monkeys, produces no recognizable disease in the natural hosts but induces malignant lymphoma and/or leukemia in closely related nonhuman primates; response of various species of nonhuman primates to experimental HVS infection has been reviewed (1). Marmoset monkeys (Saguinus sp.) have proven highly susceptible to experimental infection with HVS and, as we have shown in our own studies, as few as 2 to 3 infectious units of HVS will induce fatal malignant lymphomas in CT marmosets (Saguinus oedipus). In previous studies it was shown that, once infection of marmosets with HVS was demonstrated by either reisolation of the virus from their circulating lymphocytes or by the appearance of HVS-specific antibodies, the eventual outcome was malignant lymphoproliferative disease and death (8). With this sensitive animal model system it becomes possible, therefore, to evaluate chemical and/or biological agents that may possess potential therapeutic or preventive benefits. As has been reported at this symposium, Laufs and Steinke (5) have used the marmoset model system for evaluation of various HVS vaccine preparations and have shown that protection against challenge with about 100 PFU of HVS could be achieved using a killed HVS vaccine. We report the isolation of an attenuated strain of HVS and show that marmosets infected with the attenuated virus became latently infected with the virus and developed antiviral antibodies and that they have remained clinically well for over 14 months PI.

Isolation of Attenuated HVS

The isolation of attenuated HVS has been described in detail (7). HVS-11 [a strain isolated after cocultivation of circulating lymphocytes from a healthy squirrel monkey (2)] was serially propagated 3 times at 39°C in vero cells and cloned 3 times at 34°C in vero cells (isolation of plaques formed in infected vero cell monolayers overlaid with methylcellulose). The final virus stock preparation, designated HVS-39', had a titer of about 1 x 106 PFU when assayed in vero cell cultures at either 34 or 39°C.

Inoculation of Squirrel and Marmoset Monkeys with Attenuated HVS

Colony-reared, juvenile squirrel monkeys (Saimiri sciureus) (proven free of latent HVS infection) and wild-caught, adult CT marmoset monkeys (Saguinus oedipus) were inoculated with 6 x 106 or 1.3 x 107 PFU of HVS-39' (Table 1). Blood samples were collected from all monkeys at periodic intervals PI for hematological and serological studies and for virus isolation. Two squirrel and 2 CT marmoset monkeys became infected after inoculation with HVS-39' as shown by the recovery of HVS after cocultivation of their circulating lymphocytes with vero cells and/or by the development of antiviral antibodies. HVS was recovered from CT Marmosets 4322 and 5637 at 36 days PI and at subsequent intervals thereafter when blood samples were collected (Chart 1). Repeated attempts to recover HVS from the inoculated squirrel monkeys were unsuccessful. Despite the failure to recover HVS from the squirrel monkeys, both monkeys apparently became infected and developed anti-
Table 1

<table>
<thead>
<tr>
<th>Monkey species</th>
<th>Inoculum</th>
<th>Passage in vivo</th>
<th>Titer of inoculum (PFU)</th>
<th>Animal</th>
<th>Virus isolation PI (days)</th>
<th>Survival days PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saimiri sciureus</td>
<td>HVS-39</td>
<td>1</td>
<td>$1.3 \times 10^{6}$</td>
<td>4057</td>
<td>Negative</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5195</td>
<td>Negative</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\geq 270$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\geq 270$</td>
</tr>
<tr>
<td>Seguinus oedipus</td>
<td>HVS-39</td>
<td>1</td>
<td>$6 \times 10^{2}$</td>
<td>4322</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5637</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\geq 420$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\geq 420$</td>
</tr>
</tbody>
</table>

viral antibodies that were first detected 12 and 19 days PI. CT Marmosets 4322 and 5637 developed antibodies to HVS-induced EA and LA; anti-LA antibody titers were first detected in serum from both marmosets 21 days PI, whereas antibodies to EA were detected 36 days PI. As illustrated in Chart 1, antibodies to both EA and LA remained at moderate levels for at least 26 weeks PI and, although the data are not shown, little change was noted in either EA or LA antibody titers at 56 weeks PI. During the entire observation period of 56 weeks, no overt signs of clinical disease were observed in Marmosets 4322 and 5637.

In order to evaluate the genetic stability of attenuated HVS-39, virus was isolated from Marmoset 4322 after cocultivation of circulating lymphocytes with vero cells and about $4 \times 10^{4}$ PFU of reisolated virus (passed in vero cell cultures 3 times) were inoculated into each of 2 CT marmosets, 994 and 4157 (Table 1). Both marmosets became infected as demonstrated by the reisolation of HVS from their circulating lymphocytes and the appearance of specific HVS antibodies, and both marmosets remained clinically well over 142 days PI when they were used in challenge experiments, described below.

**Challenge of Marmosets Inoculated with Attenuated HVS-39**

To determine whether or not marmosets infected latently with attenuated HVS would be resistant to infection with wild-type, oncogenic HVS, CT Marmosets 994 and 4157 were inoculated with about 770 PFU of oncogenic HVS-11, 142 days after initial inoculation with attenuated HVS (Table 2). At the time of challenge with oncogenic HVS both animals had antibodies to LA and Marmoset 4157 had antibodies to EA (Table 2); both marmosets also had neutralizing antibodies at titers of 1:32. Simultaneously, 2 normal CT marmosets were inoculated with the same dose of HVS-11; the latter 2 animals died from malignant lymphoma 22 and 23 days PI (Table 2), whereas Marmosets 994 and 4157 survived approximately 3 times longer and showed marked rises in antibody titers to both LA and EA.

**Serial Passage of Prototype HVS at 39°**

In order to determine whether or not the attenuation of HVS, as measured by loss of oncogenic potential for marmoset monkeys, was a phenomenon related to propagation of the virus at 39° and whether or not this was reproducible also with other HVS strains, the prototype strain of HVS (6) was serially passed 3 times in vero cells at 39° and then cloned 3 times at 34°. Pairs of marmosets were inoculated with these virus preparations (Table 3). As was expected, the 2 marmosets inoculated with virus grown at 37° died from HVS-induced lymphoma 52 and 65 days PI. Three serial passages of HSV at 39°, or serial passage at 39° and cloning at 34°, in this instance failed to produce attenuated virus as all marmosets inoculated with these virus preparations died 29 to 47 days PI.

**Discussion**

The exact origin of HVS-39° is unknown, but probably it arose by spontaneous mutation and/or selection during passage of HVS-11 at 39° and subsequent isolation during cloning at 34°. The results of our studies demonstrated, however, that the originally produced HVS-39° was no longer oncogenic when inoculated into marmosets and that this loss of oncogenicity was genetically stable as indicated by retention of the attenuated property through 2 serial passages of the virus in vivo. The failure to obtain attenuated HVS from the prototype strain of HVS after propagation at 39° and cloning at 34° suggests that growth of the virus at 39° does not induce attenuation of the entire virus.
Table 2

<table>
<thead>
<tr>
<th>Marmoset</th>
<th>Previous inoculation with HVS-39(^a)</th>
<th>Virus isolation(^a)</th>
<th>HVS antibody(^b)</th>
<th>Survival PI with HVS-11 (days)</th>
<th>Time of challenge</th>
<th>HVS antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>994</td>
<td>Yes</td>
<td>Yes</td>
<td>64–128</td>
<td>&lt;10</td>
<td>79</td>
<td>≥256</td>
</tr>
<tr>
<td>4157</td>
<td>Yes</td>
<td>Yes</td>
<td>126</td>
<td>64</td>
<td>74</td>
<td>≥256</td>
</tr>
<tr>
<td>4338</td>
<td>No</td>
<td>No</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>22</td>
<td>14</td>
</tr>
<tr>
<td>5656</td>
<td>No</td>
<td>No</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>23</td>
<td>14</td>
</tr>
</tbody>
</table>

\(^a\) Cocultivation of whole blood with vero cells.

\(^b\) Determined by indirect immunofluorescence tests; values represent reciprocal of highest dilution of plasma staining HVS-infected cells.

Table 3

<table>
<thead>
<tr>
<th>Growth temperature of virus(^a)</th>
<th>Marmoset species</th>
<th>No. developing lymphoma/No. inoculated</th>
<th>HVS antibodies(^b)</th>
<th>Virus isolation(^c)</th>
<th>Survival days PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>37(^\circ)</td>
<td>WL(^d)</td>
<td>2/2</td>
<td>2/2 (31)</td>
<td>2/2 (31)</td>
<td>52, 65</td>
</tr>
<tr>
<td>39(^\circ)</td>
<td>CT</td>
<td>2/2</td>
<td>2/2 (31, 24)</td>
<td>2/2 (17, 31)</td>
<td>33, 35</td>
</tr>
<tr>
<td>34(^\circ)</td>
<td>CT</td>
<td>2/2</td>
<td>2/2 (24, 38)</td>
<td>2/2 (24, 31)</td>
<td>29, 47</td>
</tr>
</tbody>
</table>

\(^a\) 39\(^\circ\); HVS passed serially 3 times in vero cells incubated at 39\(^\circ\); 34\(^\circ\), HVS cloned 3 times in vero cell cultures incubated at 34\(^\circ\).

\(^b\) Determined by indirect immunofluorescence tests; numbers given in parentheses are days PI antibodies first detected.

\(^c\) Cocultivation of whole blood with vero cells; numbers given in parentheses represent days PI virus first recovered from circulating lymphocytes.

\(^d\) WL, white lipped; CT, cotton topped.

The ability to obtain attenuated HVS after propagation of the virus at 39\(^\circ\) may be a property associated with HVS-11 and not with the prototype strain; this possibility is currently being investigated.

The sequence of events following infection of marmosets with either HVS-39\(^\circ\) or oncogenic HVS is probably similar: the virus infects and transforms a population of T- (thymus-derived) lymphocytes and becomes repressed in these cells. At approximately the same time or shortly thereafter, antibodies to HVS-induced LA and/or EA appear in the serum of the infected monkeys. Usually, about 7 to 14 days after the appearance of antibodies, measurable numbers of lymphocytes are present in the circulation that harbor the HVS genome in a repressed state. Activation of the viral genome generally occurs when these lymphocytes are cultured alone in vitro or cultured with permissive monolayer cells, i.e., vero cells. In marmosets infected with oncogenic HVS, the number of HVS-genome positive lymphocytes usually increase until death concomitantly with the onset of lymphocytic leukemia, and in terminal stages of the disease 1 of 3 lymphocytes has been shown to yield HVS when cultured in vitro. In contrast, in marmosets infected with attenuated HVS the number of virus-yielding lymphocytes was only about 1 of 5 \times 10^5 – 1 \times 10^6 lymphocytes; this value remained relatively constant over 14 months' observation, during which time the marmosets remained clinically healthy.

Despite the fact that a persistent latent infection of marmosets with HVS-39\(^\circ\) failed to protect against challenge with a rather large dose of oncogenic HSV [770 PFU (375 LD\(_{50}\))], the disease course was significantly prolonged compared with the typical shorter disease course in the inoculated control marmosets. Successful vaccination of marmosets with a killed preparation of HVS has recently been demonstrated (3-5), although only the vaccinated marmosets that were challenged with low doses of virulent HVS (about 100 PFU) survived the challenge, whereas vaccinated marmosets challenged with larger doses of virus (about 1000 PFU) survived longer but ultimately also died from malignant lymphoma.

Two questions arise from these studies: (a) what mechanisms repress proliferation of virus-infected lymphocytes in marmosets inoculated with HVS-39\(^\circ\); and (b) are these mechanisms similar to those that regulate the interaction of HVS with lymphocytes in the natural hosts, squirrel monkeys? In contrast to marmosets infected with oncogenic HVS, infection of marmosets with HVS-39\(^\circ\) resulted in a virus-cell interaction that did not lead to uncontrolled lymphoproliferation; such a virus-cell interaction occurs in squirrel monkeys after primary infection with HVS and apparently this virus-cell relationship persists for life in squirrel monkeys.

Although vaccination of man with a live attenuated tumor virus vaccine may pose insurmountable problems, studies of oncogenic versus attenuated HVS will provide valuable additional basic information on the pathogenesis of HVS-induced disease, the genetics of oncogenic versus nonon-...
cogenic herpesviruses, and latent versus malignant infections by these viruses.

**Acknowledgments**

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**References**

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