Alphaherpesvirus Saimiri in Rabbits: A Model For Human Encephalitis?

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SUMMARY

One (KM91) of a series of isolates of alphaherpesvirus saimiri (aHVS) produced rapidly fatal encephalitis in rabbits following intradermal infection, whereas the others (KM180, KM322 and KM338) were non-lethal and produced ganglionitis and prolonged latency. Alphaherpesvirus saimiri KM91 initially produced ganglionitis but quickly ascended the spinal cord to the brain causing death 10 days post-infection. Prior infection with any of the three benign isolates or inoculation with β-propiolactone (βPL)-inactivated aHVS KM91 protected rabbits from lethal encephalitis when they were subsequently challenged with a lethal dose of aHVS KM91. Each of 20 rabbits co-inoculated in the same site with a lethal dose of aHVS KM91 and either aHVS KM322 (1.5 × 10³ to 1.5 × 10⁵ p.f.u.) or βPL-inactivated aHVS KM322 (1 × 10⁷ p.f.u. equivalents) survived. In contrast only half of those co-inoculated with aHVS KM91 and βPL-inactivated aHVS KM91 (1 × 10⁷ p.f.u. equivalents) survived. Co-inoculation of lethal aHVS KM91 (75 p.f.u.) and benign aHVS KM322 (1.5 × 10⁵ p.f.u.) into opposite flanks resulted in protection from encephalitis in one of four rabbits. Alphaherpesvirus saimiri KM91 was shown to have the capacity to become latent in dorsal root ganglia if the rabbit did not die.

INTRODUCTION

Several herpesviruses cause potentially fatal encephalitis in both man or other animals. Examples include herpes simplex virus (HSV) and varicella-zoster virus in man (Whitley et al., 1982; Weller, 1983), herpesvirus simiae in man (Nagler & Klotz, 1958) and pseudorabies virus in pigs (Hurst, 1933). Although herpesvirus encephalitis of animals can be studied in the natural host this option is not available for studying human encephalitis. Major advances in the study of herpesvirus neurovirulence have been made using various strains of HSV in either mice (Schröder et al., 1983) or rabbits (Stroop & Schaefer, 1986). However such studies can be difficult to interpret because of variation in neurovirulence between HSV strains.

Four closely related herpesviruses have been isolated from four temporally distinct outbreaks of fatal encephalitis in owl monkeys (Aotus trivirgatus) (K. McCarthy & M. J. Clarkson, unpublished data). These isolates have been designated KM91, KM180, KM322 and KM338 and have all been shown to be alphaherpesvirus saimiri (aHVS) (Leib et al., 1987), which was previously known as herpesvirus tamarinus (Melendez et al., 1966) (see below). All were found to be very similar to each other and to a reference strain (aHVS Boston) on the basis of restriction endonuclease digest patterns, polypeptide immunoprecipitation and growth characteristics (Leib et al., 1987). They are also closely related antigenically as demonstrated by kinetic neutralization studies (K. McCarthy, unpublished data). One of these isolates (KM322) has, however, been used in the rabbit as a model system for the study of the pathogenesis of shingles.

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It becomes established in prolonged (up to 6 years) latency without spontaneous reactivation in rabbit dorsal root ganglia (DRG) (Tosolini et al., 1981, 1982). Here we describe the neurovirulence of another of these isolates (KM91) which invariably causes fatal encephalitis in rabbits.

It should be noted that αHVS has also been named herpesvirus tamarinus, Herpes T, marmoset herpesvirus and Mar HV. Recently the Herpesvirus Study Group of the International Committee on Taxonomy of Viruses (Roizman et al., 1981) suggested that it be renamed herpesvirus saimiri I. The name alphaherpesvirus saimiri has been used in the present report to avoid confusion with the lymphotrophic gammaherpesvirus saimiri II (HVS II).

**METHODS**

**Viruses and cells.** The four isolates of αHVS originated from four separate outbreaks of lethal disease in monkeys between 1968 and 1971 (K. McCarthy & M. J. Clarkson, unpublished data) and were designated KM91, KM180, KM322 and KM338. Alphaherpesvirus saimiri London and αHVS Boston were obtained from Dr Hazel Ratcliffe of the Central Public Health Laboratory (Colindale, U.K.) and from Dr Luiz Melendez of the Massachusetts Primate Center (Boston, U.S.A.) (Melendez et al., 1981) respectively. Viruses were grown routinely in Vero cells in medium 199 (Gibco) containing 5% (v/v) foetal calf serum (FCS) and buffered with sodium bicarbonate (2% w/v).

**Experimental animals.** Adult male New Zealand White rabbits (Cheshire Rabbit Farm, U.K.) were used for all experiments.

**Infection and tissue sampling.** Rabbits were infected by intradermal inoculation as described previously (Tosolini et al., 1982). Briefly, three intradermal injections of 0·2 ml each were made into the lateral aspect of the shaved left flank within three dermatomes (T12 to L5) on a paravertebral line joining the costal margin to the iliac crest, 10 to 12 cm from the midline. For the safety of the operators all injections were carried out with the rabbits briefly anaesthetized with ketamine hydrochloride (Parke-Davis). Members of this group of herpesviruses must be considered to be possible human pathogens and all procedures in the laboratory and animal house were developed and executed with this in mind. If left unrestrained the rabbits were often found after a short time to scratch and bite their skin lesions and they were therefore each fitted with a 30 cm diameter flexible 'Eton collar' of soft vinyl floor-covering 3 to 4 days post-infection. This protected the lesions from additional harm and prevented the establishment of an itching/scratching/biting cycle. The collars were well tolerated and allowed free access to food and water.

Tissue samples for isolation of free virus were taken and processed as soon as possible after death. Animals were killed at appropriate times by intravenous injection of sodium pentobarbitone. Samples from skin, dorsal root ganglia, spinal cord, brain stem and cerebral hemispheres were taken for culture and microscopy. The spinal cord was examined in five sections in dermatome levels S4 to S1 (section 1; SC1), L1 to L4 (section 2; SC2), L1 to T1 (section 3; SC3), T10 to T4 (section 4; SC4) and T4 to C1 (section 5; SC5). Samples were weighed so that virus obtained could be expressed as p.f.u./g of tissue. For culture, tissues were disrupted by shaking with glass beads in phosphate-buffered saline (PBS) A (5 ml) containing FCS (2% v/v) for 30 min at 4 °C. Tissue homogenates were then centrifuged at 2000 × g for 5 min to remove the larger debris and 0·1 ml of the supernatant was inoculated into each of two 15 cm × 16 mm tubes containing Vero cell monolayers. After adsorption for 1 h, the monolayers were washed thoroughly and incubated at 37 °C for 48 h, after which plaques were counted by dark ground microscopy at × 10 magnification.

For histology, tissues were fixed overnight in 5% formal saline and processed and stained using standard techniques (Culling, 1957).

**Co-infection.** Intradermal injection of live αHVS KM91 into the flank of rabbits invariably leads to fatal encephalitis. A dose of 25 p.f.u. of αHVS KM91 was co-inoculated intradermally into each of three sites on the rabbits’ left flanks together with either 1 × 10^7 β-propiolactone (βPL; Sigma)-inactivated αHVS KM91 particles, 1 × 10^2 βPL-inactivated αHVS KM322 particles, 1·5 × 10^3 p.f.u. αHVS KM322, 1·5 × 10^4 p.f.u. αHVS KM322 or 1·5 × 10^5 p.f.u. αHVS KM322.

Another group of animals was similarly injected three times with 25 p.f.u. αHVS KM91 into the left flank and 1·5 × 10^2 αHVS KM322 into the right. Control animals were infected with 25 p.f.u. αHVS KM91 into each of the three sites on the left flank. In all experiments where βPL-inactivated virus was mixed with live virus, part of the mixture was also inoculated in serial dilutions onto Vero cell monolayers to ensure that no residual βPL was present to inactivate the live virus.

**Superinfection.** Rabbits were injected intradermally in three sites on the left flank with a total of 1·5 × 10^3 p.f.u. of one of the following (relatively benign) viruses, αHVS KM180, αHVS KM322, αHVS KM338, αHVS Boston or αHVS London. Twenty-eight days later these animals were challenged with 25 p.f.u. of αHVS KM91 intradermally in each of three sites on the left flank. In addition each of the animals that had survived co-inoculation of live and inactivated αHVS KM91 was similarly re-challenged with a total of 75 p.f.u. of αHVS KM91.
Neurotropism of aHVS in rabbits

RESULTS

Clinical signs of KM91 infection

Following intradermal infection (75 p.f.u.) the rabbits developed erythematous papules 2 to 3 days post-infection at the three sites of inoculation. At this time the animals had necessarily been fitted with protective collars to prevent subsequent self-mutilation by biting at areas of intense irritation resulting from ganglionitis. The skin lesions had reached a maximum size by days 4 to 6 when they were approximately 10 to 12 mm in diameter. Some of these lesions then became blackened and necrotic. The skin lesions began to heal by days 7 to 9 but death always intervened before complete healing.

The first signs of central nervous system (CNS) involvement were seen on days 6 to 7 post-infection when the animals became lethargic and often swayed their heads from side to side. By days 8 to 9 the animals were neither eating nor drinking and showed flaccid paralysis of their hind limbs. This always led to coma and death 10 to 14 days post-infection. Infection of rabbits with doses ranging from as low as 3 p.f.u. to as high as 1·5 × 10^4 p.f.u. produced the same clinical results. Although paradoxically there was a tendency for rabbits infected with higher doses of KM91 to survive longer, infection was inevitably fatal.

CNS spread of KM91 in rabbits

The spread of KM91 following intradermal inoculation of 75 p.f.u. into three adjacent sites is summarized in Fig. 1. The virus multiplied locally in the skin, reaching maximum titres (10^3 p.f.u./g) on day 5 at which time the virus first appeared in the left (ipsilateral) DRG. Virus titres in the left DRG reached a maximum on day 6 by which time the virus was detectable in SC2 and SC3 which corresponded to the inoculation dermatomes. Virus appeared in SC1 on day 8 when it was also isolated from the right (contralateral uninoculated) DRG. The virus titres in SC2 and
SC3 increased until day 9 at which time SC4 and SC5 became virus-positive. By day 10, the brain stem, cerebellum and cerebral hemispheres were all infected. At this time titres in the skin and SC3 were considerably diminished and virus was no longer detectable in SC1, SC4 and SC5. High titres of virus were however still present in the left DRGs.

Histology of infected tissues

Tissue from rabbits that had died from KM91 encephalitis were sectioned and stained with haematoxylin and eosin. Sections of infected skin showed infiltration with inflammatory cells particularly in the dermis and around hair follicles. Infected dorsal root ganglia showed marked congestion with inflammatory cells and neuronal destruction. These findings were common to the virulent and the non-virulent strains. Examination of SC3 (the spinal cord level adjacent to the DRGs serving some of the dermatomes of infection) infected with strain KM91 revealed gross tissue damage with areas of necrosis, inflammatory cell infiltration (Fig. 2) and perivascular cuffing. Microscopical evidence of cerebral damage was not abundant but careful inspection of sections of the cerebral hemispheres always revealed occasional focal lesions with associated microgliosis and perivascular cuffing (Fig. 3).

Co-inoculation studies

Four rabbits were infected intradermally with a mixture of a normally lethal dose (75 p.f.u.) of live KM91 and βPL-inactivated KM91 (virulent) (initial titre 10^7 p.f.u.). Only two of the four animals infected in this way died (Table 1). The two survivors were killed at a later date and latent KM91 was recovered from explant cultures of their DRGs. In contrast co-inoculation of live KM91 together with βPL-inactivated KM322 (initial titre 10^7 p.f.u.) resulted in the survival of all four rabbits. Similarly, following co-inoculation of KM91 with a variety of doses (1.5 × 10^3
to $1.5 \times 10^5$ p.f.u.) of non-lethal KM322, none of six infected rabbits developed encephalitis or died. However, following inoculation of KM91 and KM322 into opposite flanks three of four rabbits developed encephalitis and died.

**Challenge of previously infected rabbits (superinfection)**

Groups of five rabbits that had been previously infected with the non-lethal strains of αHVS (KM180, KM322, KM338, Boston or London) were challenged 28 days later with a lethal dose (75 p.f.u.) of KM91. None of the 25 rabbits died or even showed signs of infection. Control experiments had previously demonstrated that non-lethal latent virus was present in DRG from one rabbit in each of the groups.

One of the rabbits that had initially been co-inoculated with a mixture of live and βPL-inactivated KM91 (see Table 1) was later challenged with a lethal dose of KM91 alone; again no signs of infection developed. Rabbits inoculated with βPL-inactivated KM91 ($1 \times 10^7$ particles) and subsequently challenged with a lethal dose of KM91 (75 p.f.u.) did not develop encephalitis.

**DISCUSSION**

Alphaherpesvirus saimiri is an alphaherpesvirus that was originally isolated from the white-lipped tamarin (*Tamarinus nigricollis*) (Melnick et al., 1964). Its natural host was later found to be the squirrel monkey (*Saimiri sciureus*) (Melendez et al., 1968) in which it caused only mild illness. However in tamarins, marmosets and owl monkeys, αHVS produces lethal infection with neurological involvement and the four isolates KM91, KM180, KM322 and KM338 were obtained from four separate but identical outbreaks of lethal infection in owl monkeys. These four isolates have been found to be closely related to each other and to a reference strain of αHVS (Boston) using a variety of genetic, immunological and biological techniques (Leib et al., 1987).
Table 1. Survival of groups of rabbits co-injected with a lethal dose of αHVS KM91* and other viruses

<table>
<thead>
<tr>
<th>Co-inoculant</th>
<th>Survival</th>
</tr>
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<tbody>
<tr>
<td>βPL-inactivated αHVS KM91 (1 × 10^7 p.f.u.)†</td>
<td>2/4</td>
</tr>
<tr>
<td>βPL-inactivated αHVS KM322 (1 × 10^7 p.f.u.)†</td>
<td>4/4</td>
</tr>
<tr>
<td>αHVS KM322 (1-5 × 10^3 p.f.u.)†</td>
<td>2/2</td>
</tr>
<tr>
<td>αHVS KM322 (1-5 × 10^4 p.f.u.)†</td>
<td>2/2</td>
</tr>
<tr>
<td>αHVS KM322 (1-5 × 10^5 p.f.u.)†</td>
<td>2/2</td>
</tr>
<tr>
<td>αHVS KM322 (1-5 × 10^6 p.f.u.)§</td>
<td>1/4</td>
</tr>
<tr>
<td>Saline</td>
<td>0/4</td>
</tr>
</tbody>
</table>

* The lethal dose of αHVS used was 75 p.f.u.
† The same site was used for inoculation of αHVS and the co-inoculant.
‡ Latent virus was recovered from one of these surviving rabbits by explant culture of infected dorsal root ganglia.
§ Inoculation of the two strains was in opposite flanks.

After intradermal inoculation of αHVS KM322 into rabbits' flanks, ganglionitis followed by prolonged latency was observed. Spread of αHVS KM322 beyond the spinal cord or ganglia of the dermatome was never observed (Tosolini et al., 1982). In contrast αHVS KM91, after an initial ganglionitis spread beyond the dermatome up the spinal cord to the brain causing encephalitis and death 10 days after inoculation. This was seen regularly with a dose as low as 75 p.f.u. (and even with 3 p.f.u. in the few experiments in which this low dose was used). The extreme neurovirulence of αHVS KM91 following intradermal inoculation is a phenomenon which has not been described for any other strain of αHVS. In spite of its high neurovirulence it appears that αHVS KM91 has the capacity to become latent under conditions allowing survival of the host, and it can later be recovered easily by explant culture of sensory ganglion fragments.

The fragments produced by digestion of αHVS KM91 and αHVS KM322 DNA with the restriction endonucleases BgⅡ, EcoRI, KpnⅠ and BamHI were identical but some differences were observed when HindⅢ and especially XhoⅠ were used (Leib, 1986; Leib et al., 1987). Whether these differences between the DNAs of KM91 and KM322 could account for the difference in neurovirulence remains a matter for further study.

The differences in neurovirulence could not be explained in terms of growth kinetics or thymidine kinase phenotype since the two viruses were identical in these respects (Leib, 1986; Leib et al., 1987). However the lethal nature of KM91 infection in rabbits even at low titres does provide a well-defined model for the study of neurovirulence. It is noteworthy that αHVS is a potential cause of encephalitis in humans (Melendez, 1968). However the strain which presumably caused the case reported by Melendez was not isolated and its neurovirulence in rabbits is unknown.

Our rabbits that had developed latent infections with αHVS KM322 were completely protected against the lethal effects of αHVS KM91. This is not entirely surprising since the two viruses are serologically closely related (Leib et al., 1987). The finding that co-inoculation in one syringe of KM91 and KM322 did not result in encephalitis was more unexpected although a similar phenomenon has been reported for HSV-1 in mice (Schröder et al., 1983). The protection induced by co-inoculation of αHVS KM322 operates in the absence of viral replication since inactivated KM322 also induced protection and it even occurred when the viruses were inoculated into opposite flanks. This implies that there is a factor induced by KM322 which can rapidly produce a protective response in the host to prevent the development of encephalitis even when the neurovirulent KM91 strain is inoculated at the same time. Inactivated KM91 also protected against lethal encephalitis but apparently less well than a similar inoculum of inactivated KM322. Although under these circumstances the ability of KM91 to ascend the spinal cord and cause encephalitis was limited, it was still able to reach the dorsal root ganglia where it became latent. It should be noted that the relatively benign KM322 strain is able to kill rabbits when directly introduced into the brain.
Neurotropism of $\alpha$HVS in rabbits

Furthermore all four strains were apparently of similar (100%) lethality in the owl monkeys from which they were derived. It appears that all four KM isolates are potentially neurotropic; these studies reveal differences among the four in their ability to gain access to the CNS which become manifest in rabbit infection but not in the owl monkey. There seems to be a factor in the makeup of strain KM322 which restrains its inherent rabbit neurovirulence and which can also restrain co-inoculated neuroinvasive KM91. Although short specific HSV DNA sequences have recently been shown to control neurovirulence in mice and tree shrews (Rosen et al., 1986) the nature and function of the gene(s) involved are still unknown.

Further studies are now in progress to identify the genes responsible for the neurovirulence of $\alpha$HVS KM91 and for the virulence restraint operated on it by strain KM322 for rabbits and to examine the immunobiology of $\alpha$HVS in general. Such investigations may reveal common mechanisms controlling the neuropathogenicity of alphaherpesviruses.

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