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A novel, cell-specific attenuation of a herpes simplex virus type 1 infection in vivo

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Abstract We have observed a cell-specific attenuation of herpes simplex virus type 1 strain 17syn+ in vivo that was dependent upon the cell type used to grow the virus. Direct corneal infection of rabbits with 17syn+ propagated in Vero cells caused 60% (6 of 10) to develop severe central nervous system (CNS) disease as evidenced by seizures and/or paralysis; all neurologically impaired rabbits died. In contrast, infection of rabbits with 17syn+ propagated in BHK-21 cells induced seizures and was fatal in 10% (1 of 10). The cell-specific attenuation of a 17syn+ occurred after one growth cycle in BHK-21 cells. To determine whether the decreased virulence of the BHK-21 cell-grown virus correlated with a less severe CNS inflammatory reaction, CNS tissues from rabbits infected with 17syn+ grown in Vero and BHK-21 cells were compared. Histopathological analyses revealed no differences in the location or severity of inflammatory lesions from rabbits infected with virus grown in either cell type.

Virus-induced corneal disease was less dependent upon the cell type used to propagate the virus as there were no significant differences in the type or severity of observed corneal lesions. Possible explanations based on differences between Vero and BHK-21 cells are discussed.

Keywords Herpes simplex virus type 1 · Attenuation · Tissue culture cells · Central nervous system disease

Introduction

Herpes simplex virus type 1 (HSV-1) is a member of the Herpesviridae family and is one of the eight human herpesviruses [37]. It is an enveloped, double-stranded DNA virus with a genome consisting of more than 152,000 base pairs and a GC content of 68% [37]. HSV-1 causes a number of acute and reactivated infections that are usually limited to the oropharynx [57]. The nuclei of the sensory ganglia innervating the site of initial infection become sites of latency in which the virus exists in a non-replicating state for various periods of time [9, 43, 55]. Reactivation occurs in response to a variety of unrelated stimuli including emotional stress, fever, menstruation, immune suppression, surgery, and exposure to UV light [18, 19, 32, 42, 43], although the frequency of reactivated lesions in infected individuals varies widely. In most cases, acute and reactivated infections are mild and self-limiting although, on rare occasions, HSV-1 can infect the central nervous system (CNS) and cause life-threatening encephalitis [57]. An explanation of what triggers these rare events is unknown. Given the close proximity of latent virus to the CNS and its propensity for reactivation, it is fortunate that the incidence of serious CNS disease is not more frequent. Unlike latent HSV-1 within the peripheral nervous system (PNS), which causes reactivated lesions at or near the site of the original infection, reactivation of latent HSV-1 within the CNS [2, 22, 23] can lead to herpes simplex encephalitis with such symptoms as confusion and behavioral changes, olfactory or gustatory hallucinations, as well as hemorrhagic or necrotic lesions within the cere-

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bral cortex [44]. Unfortunately, the mechanisms involved in triggering virus-host cell interactions including CNS reactivation are poorly understood.

In an attempt to elucidate the complex interactions between HSV-1 and a susceptible host, a number of laboratories have focused efforts on understanding the pathogenesis of HSV-1 infections and the host response to these infections. In doing so, several HSV-1 strains have been utilized that exhibit a wide range in their ability to cause disease including the avirulent strain –GC, the virulent strain +GC [47], the weakly virulent strain KOS-63, the more virulent strain KOS-79 [12, 45], and strain HFEM, which is nonvirulent in tree shrews [3, 39]. In addition, numerous studies have sought to define sequences of the viral genome that contribute to virulence. Such regions include the HSV-1 thymidine kinase gene [17], a region of the HSV-1 genome (0.40–0.44 map units) involved in DNA replication (containing oriL, DNA polymerase, and ICP 8) [11], as well as sequences located at 0.71–0.83 [53], 0.762–0.787 [3], 0.7615–0.796 [39], and 0.03–0.05, 0.73–0.78, and 0.78–0.80 [24] map units on the HSV-1 genome.

Investigations into influence of host cells on virulence of other viral systems have led to observations that different cell types used to grow the virus can alter the outcome of infections of cultured cells. Poss et al. [36] determined that glycosylation differences in feline immunodeficiency virus glycoproteins affected its cytopathic nature. Similarly, differences in protein processing/glycosylation were shown to alter pathogenicity of Friend spleen focus-forming virus [16] and the host range of HIV [6]. In addition, cells found to secrete large amounts of proteoglycans have been shown to inhibit retrovirus infection *in vitro* [26]. It has been demonstrated that host cells are able to influence spread of some retroviruses by methylation of proviral DNA, thus inhibiting long terminal repeat-driven transcription [20]. Resistance of some cell types to viral infections has been attributed to the presence or absence of cellular receptors or other cell-specific factors [7, 13, 59, 52]. For example, CCR-5 and CXCR-4 are two chemokine receptors found to function as coreceptors for HIV in human CD4⁺ cells [25], reviewed in [4]. Likewise, herpesvirus entry mediator (HVEM) made cells resistant to virus entry susceptible to HSV-1 infection [28] and mediated cell-to-cell spread of the virus [38].

These apparent differences in virulence *in vitro* led us to ask if this is also observed *in vivo*. To test this, we grew one strain of HSV-1, 17syn+, in Vero and BHK-21 cells, two common laboratory cell types used to propagate HSV-1, and examined the ability of the virus to cause eye and CNS disease in an animal model. Ocular infection of rabbits with 17syn+ grown in Vero cells lead to the development of CNS disease as evidenced by seizures/paralysis in 60% of the animals. In addition, 60% of the rabbits died as a result of the infection. In contrast, one rabbit developed seizure activity and died when infected with the same strain of virus grown in BHK-21 cells. These results demonstrate that BHK-21 cells specifically attenuate an HSV-1 infection *in vivo*.

Methods

Cells and virus

HSV-1 strain 17syn+ was obtained from N. Fraser, Wistar Institute via J. Subak-Sharpe, and plaque-purified. We generated three separate virus stocks that were subsequently used to infect rabbits (see below). These stocks were generated in the following manner. 17syn+ was grown in Vero cells for one passage to generate the Vero cell-grown virus or for one passage through BHK-21 cells for the generation of the BHK-21 cell-grown virus. Vero cells and BHK-21 cells were maintained in minimum essential medium (MEM) with Earle's salts supplemented with 2 mM L-glutamine, 0.15% sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin, and either 2% or 8% heat-inactivated fetal bovine serum (PAA Laboratories, Newport Beach, Calif.). Cells were grown as monolayer cultures in 5% CO₂. At confluency, cells were infected with 17syn+ at a multiplicity of infection (MOI) of 0.01. After 1-h adsorption at 37°C, unbound virus was removed, fresh medium added, and incubation continued at 37°C until development of maximum cytopathic effect (cpe). The supernatant was clarified by low-speed centrifugation, aliquoted, and stored at –80°C. Virus titers were determined on Vero cells in 96-well plates as tissue culture infectious doses per ml (TCID₅₀/ml). As a check of virus titer, we titered our virus stocks on BHK-21 cells and found the titer of our virus stocks differed by no more than threefold as compared to titer determination on Vero cells. Similarly, when our virus stocks were titered on rabbit RK-13 cells, there was less than twofold difference between them. Based on these results, we used Vero cells for virus titration for the duration of the experiments. The Vero and BHK-21 cells were negative for mycoplasma. Rabbit corneal epithelial cells were aseptically obtained from the cornea of an uninfected rabbit. Initial epithelial cell growth was done in the presence of MEM supplemented with 20% serum and antibiotics. Thereafter, rabbit corneal epithelial cells were maintained in medium containing 10% serum. For these experiments, rabbit corneal epithelial cells were used between the fifth and tenth passages.

Animals

Specific pathogen-free New Zealand White rabbits (Myrtle Rabbitry, Thompson Station, Tenn.) were used in this study. Direct corneal inoculations were performed with 1×10⁵ TCID₅₀ per eye in a volume of 0.1 ml by dropping the virus into each eye followed by gentle massaging of the eyes.

On development of severe seizures (characterized by frequent seizure episodes lasting several minutes that also included hindlimb paralysis on occasion), the rabbits were killed. Only those rabbits that exhibited seizures from which they were deemed unlikely to recover were killed before the end of the experiment. All animal use protocols complied with the guidelines set forth in the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Virus shedding

Eye swabs were obtained daily from 1 to 10 days post infection using wetted cotton-tipped applicators. Swabs were placed in sterile eye swab medium (1× MEM supplemented with twice the amount of penicillin and streptomycin) and frozen until titer determination on Vero cells.

One-step growth curves

Triplicate cultures of confluent monolayers of Vero or BHK-21, or rabbit corneal epithelial cells in 25-cm² flasks were infected as already described except that the MOI was 10. For the growth curves, Vero cells were infected with virus propagated in Vero

cells, BHK-21 cells were infected with virus grown in BHK-21 cells, and rabbit corneal epithelial cells were infected with virus propagated in Vero or BHK-21 cells. Flasks were frozen at 0, 1, 2, 5.5, 7.5, 12, 24, 48, and 72 h post infection. Virus titer determination for the growth curves was done on Vero cells.

Statistical analyses

All statistical analyses were performed with SigmaStat and SigmaPlot (SPSS, Chicago, Ill.). Chi-square and Fisher Exact tests were utilized to determine if differences between CNS disease (i.e., seizures and/or hindlimb paralysis), eye disease, and mortality in the two groups of rabbits were statistically significant.

Histological analyses

After euthanasia, using 100 mg/kg sodium nembutal and transcardial perfusion with PBS, brains were removed and fixed in formalin or 1 mM sodium *m*-periodate, 75 mM lysine, 2% paraformaldehyde in 37 mM phosphate buffer, pH 7.4 for 24 h [46, 48, 49, 50]. The brains were then cut into 11–12 blocks, processed for paraffin embedding, and sectioned [48]. At least two sections from each block were stained with hematoxylin and eosin for histological examinations [49]. Severity of inflammation was graded as: 1+, mild inflammation; 2+, more severe inflammation with moderate perivascular cuffing; 3+, more severe inflammation with mild necrosis; and 4+, very severe inflammation with pronounced necrosis [47].

Corneal assessment

Immediately prior to euthanasia, corneas were stained with 1% lissamine green B to visualize herpetic lesions [8]. Photographs of the eyes were used to classify corneal epithelial keratitis as punctate, dendritic, or geographic.

Results

A single strain of HSV-1 was propagated in Vero and BHK-21 cells, and its virulence and ability to cause eye disease was tested. The rationale behind this experiment was to determine if standard cell types, used to generate laboratory virus stocks, influenced the outcome of an infection in vivo. Per eye, 1×10^5 TCID were used for an ocular infection of 4–5 lb New Zealand White rabbits. Eye swabs were collected over a 10-day period and rabbits were observed daily for development of CNS disease and death. At the end of the experiment (19 days post infection), all surviving rabbits were killed.

Attenuated HSV-1 infection in vivo

Strain 17syn+ from Vero cells caused seizures and/or paralysis in six of ten (60%) of the rabbits that also subsequently died, while the same virus, when grown in BHK-21 cells, caused seizures and was fatal in only one of ten rabbits (Tables 1 and 2). The seizures induced by the Vero cell-grown virus and the single seizure induced by the BHK-21 cell-grown virus were the same, consisting of bilateral facial tonic-clonic movements, with the rabbits assuming an unnatural sitting position with turning of the

Table 1 Disease due to 17syn+ grown in Vero cells (+ signs of disease, – lack of disease signs, × died, *N.D.* not done)

Rabbit	Eye disease			CNS disease		Death
	Punctate	Dendrite	Geographic	Seizures	Paralysis	
407	+	+	–	+	–	× ^a
408	–	+	+	–	–	–
409	–	–	–	+	–	× ^a
475	+	+	+	–	–	–
476	–	–	–	+	–	× ^b
478	+	+	–	–	–	–
479	–	–	–	+	–	× ^a
516	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	+	–	× ^b
517	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	+	+	× ^b
518	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	–	–	–

^aRabbits that died from seizures

^bRabbits killed due to severe seizures

Table 2 Disease due to 17syn+ grown in BHK-21 cells

Rabbit	Eye disease			CNS disease		Death
	Punctate	Dendrite	Geographic	Seizures	Paralysis	
440	–	–	–	+	–	× ^a
441	–	+	–	–	–	–
442	–	–	+	–	–	–
480	–	–	–	–	–	–
481	+	–	–	–	–	–
482	–	–	–	–	–	–
484	–	+	+	–	–	–
519	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	–	–	–
520	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	–	–	–
521	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	–	–	–

^aRabbits killed due to severe seizures

head to one side. Many of these seizures appeared to be of the Jacksonian type previously described for HSV-1 strain +GC-infected rabbits [47]. The rabbits also developed a patterned constellation of signs including wetness about the mouth, chin, forepaws as well as grinding of teeth and/or lethargy at time of sacrifice. There was no evidence that any animal became comatose. In addition, we observed one rabbit infected with 17syn+ grown in Vero cells that developed hindlimb paralysis. The observed differences in CNS disease using a single strain of HSV-1 were quite surprising and demonstrated a dramatic attenuation of a herpesvirus infection in vivo that was cell-type specific. Statistical analyses of the CNS disease and death caused by strain 17syn+ grown in Vero cells compared to when it is grown in BHK-21 cells was significant (CNS disease: $P=0.0198$, death: $P=0.0198$, Fisher Exact test).

Interestingly, rabbits infected with virus grown in either cell type developed equivalent type and severity of corneal disease as assessed by staining of the corneas with lissamine green. An example of the corneal lesion induced

Fig. 1 Herpetic corneal lesions induced by 17syn+ grown in Vero cells and visualized using 1% lissamine green at 10 dpi. A large geographic lesion (dark blue) is present over most of the cornea and extends to the upper right quadrant of the cornea. Smaller dendritic and punctate lesions adjacent to the geographic lesion can also be seen

by HSV-1 strain 17syn+ grown in Vero cells is shown in Fig. 1. Also when the remainder of ocular disease was compared between Vero cell-grown and BHK-21 cell-grown virus (Tables 1 and 2), there was no statistically significant difference ($P=0.7025$, Chi-square test). Since the only difference in this experiment was the cell type used to grow the virus, we concluded that the attenuation of CNS disease and mortality is due to propagation of 17syn+ in BHK-21 cells. While it is difficult to extrapolate these observations to the outcome of human HSV-1 infections, this is a novel finding that could have important implications for future experiments, especially those involved in vaccine development or using HSV-1 for therapeutic purposes in humans.

Virus shedding

We monitored virus shedding from the eyes over a 10-day period by performing eye swabs. As shown in Fig. 2, shedding of virus over this time period was similar, regardless of the cell type used to propagate the virus. These results suggest that the lack of progression to a diseased state using BHK-21 cell-grown virus was not due to slower growth in corneal epithelial cells in vivo as compared to Vero cell-grown virus. Additionally, one-step growth curves on Vero cells (using 17syn+ grown in Vero cells) or on BHK-21 cells (using 17syn+ grown in BHK-21 cells) (Fig. 3) also demonstrated that virus grown in BHK-21 cells was able to replicate to high titers. As a final check on the ability of the virus to replicate, we repeated our

Fig. 2 Representative graph (eight rabbits – four from each group) showing virus shedding from eyes after ocular infection of rabbits with 1×10^5 TCID per eye with HSV-1 strain 17syn+ grown in Vero cells (circles) or BHK-21 cells (squares). Values on the abscissa indicate days post infection that swabbing was performed. 17syn+Vero indicates HSV-1 strain 17syn+ grown in Vero cells and 17syn+BHK-21 indicates strain 17syn+ grown in BHK-21 cells

one-step growth curves on rabbit corneal epithelial cells, again using 17syn+ grown in Vero or BHK-21 cells. The results from the growth curve on rabbit corneal epithelial cells are presented in Fig. 4 and, again, show that 17syn+ from BHK-21 cells was able to replicate to high titers. Indeed, the one-step growth curves demonstrated that 17syn+ in either BHK-21 or rabbit corneal epithelial cells replicated to higher titers than in Vero cells at many points during these assays (Figs. 3, 4). These results suggest that the attenuation observed in vivo is not due to a growth defect of the virus, but rather is due to a feature(s) unique to BHK-21 cells. Use of 17syn+ grown in Vero or BHK-21 cells to determine the LD₅₀ following intracranial (ic) and ocular infection of BALB/c mice yielded identical LD₅₀ values (LD₅₀ ≤ 1 by ic inoculation and $10^{5.9}$ by ocular inoculation), thus providing additional support for the idea that the observed attenuation was not due to an inability of the virus to replicate (data not shown). These latter results

Fig. 3 One-step growth curves of HSV-1 strain 17syn+ grown in Vero cells (circles) used to infect Vero cells or strain 17syn+ grown in BHK-21 (squares) used to infect BHK-21 cells; MOI=10. Equivalent numbers of cells were seeded per flask to determine titer of virus. Each time point (hours post infection) represents triplicate cultures (MOI multiplicity of infection)

also imply that the attenuation observed *in vivo* is, in addition to being cell-type specific, animal species-specific.

During the course of the one-step assay in rabbit corneal epithelial cells (Fig. 4), we observed that the BHK-21 cell-derived virus grew more slowly than the Vero cell-derived virus between the 10- and 30-h time points. One possibility is that this slower growth might result in an attenuated infection *in vivo*, perhaps by giving the host immune system a chance to respond before the virus can replicate widely within the CNS. However, based upon our data the slower growth does not provide an adequate explanation for the attenuation observed *in vivo* for the following reasons. The first is that the time of appearance and severity of eye lesions observed in both groups of rabbits was not significantly different (Tables 1 and 2). *In vivo*, it appears that the BHK-21 cell-grown virus was able to replicate similarly over the same time frame as the Vero cell-grown virus (see Fig. 2). Second, the time of appearance, severity, and location of brain lesions were also similar throughout the brains in both sets of rabbits (see below and Fig. 5).

Fig. 4 One-step growth curves of HSV-1 strain 17syn+ in low passage rabbit corneal epithelial cells. 17syn+ was grown in Vero cells (circles) or BHK-21 cells (squares) and used to infect confluent monolayers of rabbit corneal epithelial cells at an MOI of 10

Back-passage through Vero cells

We next asked the question of what happened to neurovirulence when the BHK-21 cell-grown virus was now passaged one time back through Vero cells. We wanted to determine whether the virus was as virulent as the original Vero cell-grown virus or whether it was attenuated like the original BHK-21 cell-grown virus. Strain 17syn+, that had been propagated one time in BHK-21 cells, was used to infect Vero cells to generate a virus stock for this experiment. After determining the virus titer we infected 20 rabbits by the ocular route as described. The rabbits were monitored daily for evidence of CNS disease or death. Our results are presented in Table 3 and show intermediate results in that 7 of 20 rabbits exhibited CNS disease with the same animals succumbing to the infection. While this virus was more neurovirulent than when grown in BHK-21 cells (compare Tables 3 and 2), the difference was not statistically significant (CNS disease: $P=0.21$, death: $P=0.06$, Fisher Exact test). Likewise, a comparison of the neurovirulence of this virus with virus grown only in Vero cells (compare Tables 3 and 1) re-

Fig. 5 Representative histopathology of two regions of brains of rabbits infected with 1×10^5 TCID per eye of HSV-1 strain 17syn+ grown in Vero cells (A, C) and BHK-21 cells (B, D). Anterior cerebral cortex (A, B) and trigeminal ganglion (C, D) (M meningitis, P perivascular cuffing, L region of parenchymal infiltration of inflammatory cells, F focus of inflammation, N ganglionic neurons)

vealed less neurovirulence and less ability to cause a fatal disease (CNS disease and death: $P=0.26$, Fisher Exact test). These results suggest some effect on virulence as a result of having been grown in BHK-21 cells that was not completely overcome by the single passage back through Vero cells and that resulted in intermediate neurovirulence. However, an additional passage back through Vero cells resulted in the death of 6 of 10 rabbits (Table 4), results that are identical to the original virus grown in Vero cells. It appears, therefore, that two passages back through Vero cells were necessary to fully regain virulence.

Histological analyses

We next wanted to determine whether less damage occurred to the CNS after infection when BHK-21 cell-grown virus was used for infection. For this, we took two rabbit brains at 10 days post infection (dpi), two at 14 dpi, and three at 15 dpi. As demonstrated in Fig. 5, the location and severity of inflammation was the same at 14 or 15 dpi, indicating that the cell type used to grow the virus did not

Table 3 Disease due to BHK-21 cell-grown 17syn+ passaged one round back through Vero cells. Two groups of ten rabbits (591–600 and 601–610) were infected with 1×10^5 TCID per eye and monitored daily for evidence of disease or death

Rabbit	CNS disease		Death
	Seizures	Paralysis	
591	–	–	–
592	–	–	–
593	–	–	–
594	+	–	× ^b
595	–	–	–
596	–	–	–
597	+	–	× ^a
598	+	–	× ^a
599	+	–	× ^b
600	–	–	–
601	–	–	–
602	–	–	–
603	+	–	× ^b
604	+	–	× ^b
605	–	–	–
606	–	–	–
607	–	–	–
608	+	–	× ^a
609	–	–	–
610	–	–	–

^aRabbits that died from seizures

^bRabbits killed due to severe seizures

Table 4 Disease due to BHK-21 cell-grown 17syn+ passed two rounds back through Vero cells (*N.O.* not observed)

Rabbit	CNS disease		Death
	Seizures	Paralysis	
651	N.O.	—	× ^a
652	+	—	× ^b
653	+	—	× ^a
654	—	—	—
655	—	—	—
656	—	—	—
657	N.O.	—	× ^a
658	—	—	—
659	+	—	× ^a
660	+	—	× ^a

^aRabbits that died from seizures^bRabbits killed due to severe seizures

influence the ability of the virus to induce a significant inflammatory response. At 14 and 15 dpi significant inflammation was observed in the cerebral cortex that extended from the frontal lobes to the posterior portion of the occipital lobes, and from the ventral to the dorsal surfaces. Perivascular cuffs of inflammatory cells were quite common and were often accompanied by severe necrosis – especially in the entorhinal and pyriform cortices. The hippocampi were also significantly involved. Lesions were also found in the brain stem, thalamus, pons, and medulla. Evaluation of the severity of the pathology indicated the lesions ranged from 2.5+ to 4+ for the rabbits infected with Vero cell-grown 17syn+ (at 14 dpi) and from 3+ to 4+ for rabbits infected with BHK-21 cell-grown virus (15 dpi). The histopathological findings in representative regions of the nervous system for both sets of rabbits are summarized in Table 5. At 10 dpi and earlier, fewer inflammatory lesions were observed although, again, the location and severity of these lesions were similar in both sets of rabbits (data not shown).

In a separate experiment designed to examine infected rabbit brain sections at early time points, histopathologi-

cal analyses performed on rabbits infected with 17syn+ grown in Vero or BHK-21 cells at 5, 8, or 12 dpi demonstrated fewer inflammatory lesions that were of the same severity and at the same location in the two groups of rabbits.

Discussion

We have demonstrated a dramatic attenuation of an HSV-1 infection in vivo. The strain of virus (17syn+) utilized, the amount of virus used to infect the rabbits (1×10^5 TCID), as well as the route of infection (ocular) were identical for all experiments. The only difference was the cell type used to grow the virus, indicating that the attenuation is specific to BHK-21 cells but not Vero cells. This suggests that strain 17syn+ grown in BHK-21 cells is “different” from strain 17syn+ grown in Vero cells and that this difference is responsible for the attenuation. Virus-shedding data and one-step growth curves in Vero, BHK-21, and rabbit corneal epithelial cells demonstrated that the ability of the virus to replicate was not impaired by growth in BHK-21 cells. We feel it is highly unlikely that the observed in vivo attenuation is due to mutation of strain 17syn+ when grown in BHK-21 cells as a low multiplicity of infection (0.01) was used and the virus was propagated for only one round in BHK-21 cells. However, this possibility cannot be excluded completely.

Prior to the histological analyses, it was tempting to predict that we would find differences in the areas of the brain affected and/or less severe inflammation in the CNS in the rabbits infected with 17syn+ grown in BHK-21 cells. One consequence of this hypothesis was that the Vero cell-grown virus would induce damage in areas of the CNS leading to seizures and death, while the same areas would be spared when virus was grown in BHK-21 cells. Such results could have provided a possible explanation for our in vivo observations. However, this was not the case, as histological analyses showed inflammatory cells throughout the brain for both sets of rabbits and the severity of the inflammatory lesions was similar. These

Table 5 Summary of nervous system histopathology in rabbits infected with HSV-1 strain 17syn+ grown in Vero or BHK-21 cells (*dpi* days post infection)

Cell type	dpi	Rabbit	Inflammation in:									
			Olfactory system			Trigeminal system						
			Bulb	Entorhinal/ pyriform cortex	Hippo- campus	Gan- glion	Descen- ding tract	Spinal nuclei	Brain stem	Thala- mus	Cerebral cortex	Menin- gitis
Vero	10	476	—	2.5+	—	1+	—	1+	1+	—	—	3+
	14	475	2+	4+	2.5+	—	4+	3+	1+	—	3+	2+
	14	478	—	1+	—	2+	2+	3.5+	1+	—	—	—
BHK-21	10	480	—	0.5+	—	1+	2+	1+	1+	1+	—	—
	15	481	—	4+	4+	4+	4+	2.5+	3+	—	4+	3+
	15	482	—	3+	—	—	4+	—	—	2+	1+	—
	15	484	—	3+	4+	3+	3+	3+	4+	3+	—	2+

results were quite surprising in light of the dramatic attenuation observed *in vivo*.

Our *in vivo* results raise some very intriguing questions, particularly about the differences between BHK-21 cells and Vero cells that are responsible for such a significant decrease in virulence. One obvious possibility could be glycosylation differences on one or more of the surface proteins that are involved in early interactions with cell surface components which, in turn, affect the infectious process. Two possible candidates are glycoproteins B and D (gB and gD), both of which are major viral membrane glycoproteins that play important roles in fusion, interactions with specific cell surface molecules, and rate of entry [29, 30]. Alterations of the glycosylation pattern on these or other viral membrane proteins could have profound effects on infectivity. Poss et al. [35, 36] demonstrated cell-type specific glycosylation of the surface glycoproteins of feline immunodeficiency virus to be responsible for a virus able to form syncytia and cause cell death in primary feline astrocytes versus a virus only able to cause an avirulent, persistent infection of Crandell feline kidney cells. Similarly, cell-specific differences in protein processing and/or glycosylation were shown to affect pathogenicity of Friend spleen focus-forming virus [16] and to alter host range of HIV [6]. It should be noted that in all cases, however, the above results were obtained using cultured cells and were not tested *in vivo*.

Another possibility that might provide an explanation for the attenuation could be reflected in differences in virus maturation in BHK-21 cells versus Vero cells. It is conceivable that the virus grown in BHK-21 cells may have incorporated a cellular component(s) that adversely affected the ability of the virus to cause disease. Alternatively, propagation in Vero cells may have resulted in incorporation of a cellular component that enhanced its virulence. A number of small RNAs, tRNAs and 5S ribosomal RNAs, have been shown to be packaged into retrovirus particles [33, 34, 40, 56]. In addition to cellular tRNA [54], HIV has also been found to incorporate a number of cellular components including cyclophilin A, intercellular adhesion molecule ICAM-1, HLA-DR, β 2-microglobulin, LFA-1, CD43, CD44, CD63, or CD71 [1, 5, 15, 21, 31]. Since the incorporation of some cellular proteins appears to be nonrandom [21], acquisition of such cellular components could conceivably alter the host range or influence the course of infection [27]. At the present time it is unknown whether HSV-1, whether grown in Vero or BHK-21 cells, packages cellular components that could affect the outcome of disease.

Recently, cell-specific effects have been observed in HSV-1-infected cells using the same two cell types as reported here. Initial observations with BHK-21 cells infected with HSV-1 yielded two types of virus particles on Ficoll gradients which were designated as H (heavy) and L (light) particles [51]. H particles are complete infectious virus particles, while L particles lack the viral capsid and DNA and are noninfectious [51]. In subsequent experiments, Yang and Courtney [58] demonstrated that the formation of L particles was cell-type specific as nearly

equal numbers of H and L particles were produced in BHK-21 cells, while in Vero cells the predominant particle formed were H particles; either no L particles were formed or they were formed in such small quantities as to prevent purification for analyses [58]. These results demonstrate a cell-specific effect on HSV-1, *in vitro*, that was observed with BHK-21 cells but not Vero cells. It is noteworthy that we also observed a cell-specific effect *in vivo* with the same cells. Support for a cell-specific effect came from our use of virus from BHK-21 cells that was passaged a single round back through Vero cells. While we had predicted that the virus would be as neurovirulent as when grown in Vero cells, the intermediate results that we obtained (Table 3) suggested that growth in BHK-21 cells prevented 17syn+ from re-gaining full virulence. We subsequently demonstrated that a second round through Vero cells was necessary before the virus yielded results similar in virulence to the original Vero cell-grown virus (compare Tables 1 and 4).

An interesting possibility is that a correlation exists between the attenuation observed when BHK-21 cell-grown virus was used to infect the rabbits and the production of L particles in BHK-21 cells, with L particles responsible for the attenuation observed *in vivo*. How could L particles be involved in attenuating the infection *in vivo*? One possibility is that L particle formation continues *in vivo* in the neural cells throughout the course of infection. In other words, H and L particles would continue to be formed in equivalent amounts throughout the course of infection in the rabbit brain. L particles would, therefore, be able to bind to the same cellular receptors utilized by the infectious H particles since L particles apparently contain similar glycoproteins in their envelope as complete virions [51]. Thus, L particles would be able to "compete" with H particles for receptor binding and prevent infectious virus from initiating successful rounds of replication. High numbers of L particles have recently been shown to interfere with infectious virus binding to cellular receptors *in vitro* [10]. Fewer infectious particles able to replicate in cells of the CNS could, conceivably, result in an attenuated infection. This idea also implies that L particles are as immunogenic as infectious H particles, which is consistent with our histological analyses (Fig. 5) where we found no differences in the severity or location of brain lesions between the two sets of rabbits. Likewise, our *in vivo* results using Vero cell-grown virus are also consistent with this idea in that lack of L particle formation in Vero cells yielded a virus that was not attenuated initially in the original inoculum nor subsequently as the virus replicated in the CNS. This virus was, therefore, able to cause overt symptoms of disease and a high mortality rate.

Additionally, Yang and Courtney [58] also found non-random association of the viral phosphoproteins ICP4 and ICP0 with L particles but not H particles from infected BHK-21 cells. ICP4 and ICP0 were also found associated with H particles from infected Vero cells, although they were unable to analyze L particles from these cells due to their insufficient numbers [58]. While the significance of

this apparent differential virion association remains unknown, it is possible that lack of or insufficient quantities of ICP4 and ICP0 in H particles from infected BHK-21 cells could affect virus replication and, subsequently, the outcome of disease as both of these proteins have been shown to play significant roles in viral expression early in infection [14]. It should be noted, however, that the biological significance of the virus particle-associated ICP0 and ICP4 and the role they play in the infectious cycle, if any, is currently unknown.

We feel that identification of the cell-specific differences observed in vivo (our results this report) and in vitro [58], represent another step forward in our efforts to unravel the complex nature of virus/host cell interactions. It is unknown at this time if the differences observed in vivo and in vitro are actually one and the same. However, such knowledge would almost certainly increase our understanding of the factors leading to serious CNS disease, such as herpes simplex encephalitis. Identification of such factors and determining how they function intracellularly would facilitate the development of more appropriate treatment regimens or novel methods for preventing HSV-1-induced CNS disease.

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