Herpesvirus Entry Mediator and Nectin-1 Mediate Herpes Simplex Virus 1 Infection of the Murine Cornea[▽]

Andrew H. Karaba, Sarah J. Kopp, and Richard Longnecker*

Department of Microbiology-Immunology, The Feinberg School of Medicine, Northwestern University, Chicago, Illinois

Received 17 June 2011/Accepted 18 July 2011

Herpes simplex virus 1 (HSV-1) is a ubiquitous human pathogen that enters cells by the receptor-mediated fusion of the viral envelope with a host cell membrane. The envelope glycoprotein gD of HSV must bind to one of its receptors for entry to take place. Recent studies using knockout (KO) mice demonstrated that the gD receptors herpesvirus entry mediator (HVEM) and nectin-1 are the primary entry receptors for HSV-2 in the mouse vagina and brain. Nectin-1 was most crucial for the neuronal spread of HSV-2, particularly in the brain. HVEM was dispensable for infection in these models, but when both HVEM and nectin-1 were absent, infection was completely prevented. We sought to determine the receptor requirements of HSV-1 in an ocular model of infection using knockout mice. Wild-type, HVEM KO, nectin-1 KO, and HVEM/nectin-1 double-KO mice were infected via corneal scarification and monitored for clinical signs of infection and viral replication in various tissues. We report that either HVEM or nectin-1 must be present for HSV-1 infection of the cornea. Additionally, we observed that the infection was attenuated in both HVEM KO and nectin-1 KO mice. This is in contrast to what was reported for studies of HSV-2 in vagina and brain and suggests that receptor requirements for HSV vary depending on the route of inoculation and/or serotype.

Herpes simplex virus 1 (HSV-1), an alphaherpesvirus, is the leading cause of infectious blindness in developed countries (12). Infection begins in peripheral epithelial tissues, including the oral mucosal, skin, or corneal epithelium. After replicating in the epithelium, the virus infects adjacent neurons and travels via retrograde transport to sensory ganglia, including the trigeminal ganglia (TG) (1). In nerve cell bodies HSV is able to establish a life-long latent infection and reactivate at a later point or immediately undergo additional rounds of replication. During replication in neurons the virus can transit back down axons and reinfect the site of inoculation. HSV is also capable of spreading, in a zosteriform manner, to other peripheral tissues innervated by neurons from the infected ganglia. In rare cases in humans the virus can spread to the brain and cause encephalitis (7). HSV-1 is the predominant cause of ocular herpes infections and usually begins as conjunctivitis or epithelial keratitis. The reactivation of the virus can lead to subsequent damage to the cornea and stromal keratitis. This outcome depends on a variety of host and viral factors but often leads to blindness (2).

The infectious cycle of HSV-1 begins when the virion envelope fuses with a host cell membrane either at the plasma membrane or in endocytic vesicles. Fusion is a complex process that requires multiple glycoproteins, including gD, gB, and the heterodimeric complex gH/gL (4). While the precise function of each glycoprotein remains unclear, it is well accepted that gD must bind to a gD receptor on the host cell for efficient entry. Several gD receptors have been identified, including herpesvirus entry mediator (HVEM) (14), 3-*O*-*S*-heparan sul-

fate (21), nectin-1 (5), and nectin-2 (3). HVEM is a member of the tumor necrosis factor receptor superfamily and can contribute to the activation or suppression of lymphocytes, depending on its binding partner (19, 30). Nectin-1 and nectin-2 are members of the immunoglobulin superfamily and function in cell-cell adhesion (6, 16). Although nectin-2 can mediate HSV-2 entry, it does not mediate the entry of wild-type (WT) strains of HSV-1, and the murine nectin-2 homolog does not function in viral entry (10, 22, 31).

Murine homologs of HVEM and nectin-1 function in HSV-1 and HSV-2 entry (32). HVEM knockout (KO), nectin-1 KO, and HVEM/nectin-1 double-KO mice exist and have been used to investigate HSV-2 infection. The first study using these knockout animals demonstrated that either HVEM or nectin-1 must be present for HSV-2 to infect mice intravaginally (27). In that study, HSV-2 was able to infect the vaginal epithelium, spread to the nervous system, and cause death in both WT and HVEM KO mice at similar rates. Attenuated disease was observed for nectin-1 KO mice, where the vaginal epithelium was infected to a lesser degree and spread to the nervous system was reduced; however, nectin-1 KO mice still succumbed to the infection. Another set of experiments using KO mice showed that nectin-1 is the dominate receptor for HSV-2 in the brain (8). After intracranial inoculation the virus spread quickly in the brains of WT and HVEM KO mice, and they developed fatal encephalitis. In nectin-1 KO mice, HSV-2 was able to infect only ependymal cells, a cell type of epithelial origin, after cranial inoculation. Again, HSV-2 failed to infect double-KO mice in this model. These studies showed that HVEM and nectin-1 are the most important gD receptors *in vivo* and that nectin-1 plays a prominent role in HSV-2 spread in the nervous system.

We sought to determine if HVEM and nectin-1 also function as crucial receptors in HSV-1 infection of the cornea. Earlier studies found that HVEM and nectin-1 are expressed in the

* Corresponding author. Mailing address: The Feinberg School of Medicine, Northwestern University, Ward 6-245, 303 East Chicago Avenue, Chicago, IL 60611. Phone: (312) 503-0467. Fax: (312) 503-2803. E-mail: r-longnecker@northwestern.edu.

[▽] Published ahead of print on 27 July 2011.

epithelium of the murine cornea (9, 28). Additionally, a more recent report stated that HVEM and nectin-1 are expressed on human corneal epithelial cells and that antibodies directed at these receptors reduced HSV-1 infection (20). We used KO mice to explore the importance of these receptors in mediating HSV-1 infection of the cornea and spread to the TG. Interestingly, we found that the absence of either HVEM or nectin-1 resulted in attenuated disease, suggesting that both receptors play an important role in HSV-1 infection of the cornea, in contrast to HSV-2 infections.

MATERIALS AND METHODS

Cells and virus. HSV-1 strain 17 was obtained from David Leib (Dartmouth Medical School, Hanover, NH). Virus was propagated in Vero cells cultured in Dulbecco's modification of Eagle (DME) medium with 10% fetal bovine serum. When the entire monolayer showed cytopathic effect (CPE), the cell culture medium and cells were collected and frozen at -80°C . The harvested mixture was then thawed at room temperature and spun at $2,380 \times g$ for 5 min. The supernatant was collected and spun again at $7,711 \times g$ for 60 min. The supernatant was discarded, and the pellet was resuspended in 6 ml of DME medium, sonicated 2 times for 30 s, and spun at $214 \times g$ for 5 min. The supernatant was aliquoted, the titer was determined, and the supernatant was used for the infection of mice. Standard plaque titrations were performed on Vero cells.

Animal procedures. Animals were cared for and procedures were performed in accordance with institutional and NIH guidelines and approved by the Animal Care and Use Committee at Northwestern University. Mice were kept in a specific-pathogen-free environment until infection and were then transferred to a containment facility. Wild-type C57BL/6 mice from the Jackson Laboratory (WT) and *Tnfrsf14*^{-/-} (HVEM KO) (29), *Pvr11*^{-/-} (nectin-1 KO) (6), and *Tnfrsf14*^{-/-}/*Pvr11*^{-/-} (double-KO) (27) mice were used for the studies. Mice were anesthetized with an intraperitoneal injection of a mixture of ketamine and xylazine (20:3) and checked for the absence of a footpad reflex before inoculation. Each cornea was lightly abraded 10 times in a crosshatched pattern with a 25-gauge needle, and 2×10^6 PFU of HSV-1 strain 17 in 5 μl was dropped onto each cornea (11). Mice were weighed and monitored for clinical symptoms of HSV-1 infection each day beginning on the day of infection. Each mouse was given a lesion score from 1 to 5 (where 1 indicated a small area of broken skin of <0.5 cm; 2 indicated an area of broken skin of between 0.5 cm and 1 cm; 3 indicated broken skin, bleeding, scabbing, or pustules; 4 indicated an area of broken skin of >1 cm with multiple pustules or scabbing; and 5 indicated severe scabbing and/or bleeding with pustules) and a neurological score from 1 to 5 (where 1 indicated a ruffled fur and hunched posture but can easily be made to move around; 2 indicated a hunched posture and slow to move; 3 indicated a hunched posture, some movement, and labored breathing; 4 indicated a hunched posture, labored breathing, and little or no movement; and 5 indicated moribund or dead). Eye swabs were collected on days 1, 3, 5, and 7 postinfection (p.i.) by lightly anesthetizing the mice with isoflurane, gently proptosing each eye, and wiping a sterile cotton swab 3 times around the eye in a circular motion and twice across the center of the cornea in an "X" pattern. The swabs were placed into 1 ml of DME medium containing 5% fetal bovine serum, 1% gentamicin, 1% ciprofloxacin, and 1% amphotericin B (referred to as DMER) and stored at -80°C until the titer was determined. Before the titer was determined, the swabs were thawed and vigorously vortexed for 30 s. Some mice from each genotype were sacrificed on days 1, 3, 5, and 7 p.i. Eyes were enucleated, placed in OCT (TissueTek), snap-frozen, and stored at -80°C . Periocular skin (POS) biopsy specimens were taken by using a sterile biopsy punch (Sklar Instruments), and trigeminal ganglia and brains were removed and placed into 1 ml of DMER. These tissues were homogenized, sonicated, and stored at -80°C until the titer was determined. Before the titer was determined, brain samples were spun at low speed to remove debris.

Trigeminal ganglion explants. On day 28 p.i. the remaining mice (5 WT, 6 HVEM KO, 6 nectin-1 KO, and 6 double-KO mice) were sacrificed, and TG were bisected and cocultured with a monolayer of Vero cells. Cultures were examined each day for CPE, and after 7 days of culture the TG and cells were homogenized, and titers were determined on fresh Vero cells.

Immunofluorescence. Eyes from mice sacrificed on day 1 p.i. were thawed, and corneas were dissected and placed into 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 5 h. They were then washed 3 times in PBS for 10 min and blocked in a solution containing 2% goat serum and 1% Triton X-100 in PBS for 1 h. Corneas were incubated overnight at 4°C with rabbit polyclonal

anti-HSV antibody (Dako) diluted 1:500 in PBS with 1% goat serum and 0.1% Triton X-100. The corneas were washed 3 times in PBS for 10 min and incubated with goat anti-rabbit polyclonal antibody conjugated to Alexa Fluor 568 (Invitrogen) diluted 1:2,000 in PBS with 1% goat serum and 0.1% Triton X-100 at room temperature for 2 h. Finally, the corneas were washed three more times in PBS for 10 min and flat mounted onto microscope slides with ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). Corneas were imaged with the TissueGnostics system at the Cell Imaging Facility at Northwestern University, using $2\times$, $20\times$, and $40\times$ objectives. Corneas were imaged in groups of 12 on different days. Images taken with the $40\times$ objective were analyzed for areas of antibody staining by a blinded observer using ImageJ software (National Institutes of Health).

RESULTS

HVEM KO, nectin-1 KO, and HVEM/nectin-1 KO mice do not develop clinical signs of HSV-1 disease. Wild-type (WT), HVEM KO, nectin-1 KO, and HVEM/nectin-1 double-KO mice were infected via corneal scarification with HSV-1 strain 17. Mice were weighed and observed daily for signs of HSV-1 disease as detailed in Materials and Methods. During these experiments WT mice typically began exhibiting clinical symptoms between 6 and 8 days postinfection (p.i.). These symptoms consistently included swelling of the forehead, periorbital hair loss, ruffled fur, and lesions near the eyes and on the forehead. Lesions and signs of neurological disease were scored daily. Interestingly, only WT mice developed lesions, and all WT mice developed lesions by 8 days p.i. (Fig. 1A and B). Similarly, none of the KO animals developed signs of neurological infection during the experiment, while all of the WT mice attained a neurological score of at least 1 at some point during the first 14 days p.i. (Fig. 1C). Additionally, WT mice lost a greater percentage of day 0 body weight than did the KO mice (data not shown). These results were unexpected, because when infected intravaginally or intracranially with HSV-2, HVEM KO mice developed signs and symptoms identical to those observed for WT mice (8, 27).

The WT average neurological disease score peaked on day 9 p.i., when one of the WT mice was immobile, was breathing shallowly, had exceeded the maximum percent body weight loss allowed by the protocol, and had to be sacrificed. All other mice survived past day 14 p.i. (Fig. 1D). The high survival rate of the mice was not unexpected, because C57BL/6 mice are considered to be more resistant to HSV than other "wild-type" strains of mice (13).

HSV-1 replicates to higher titers in WT mice. To more closely monitor the infection, eye swabs were collected from the mice on days 1, 3, 5, and 7 p.i. The swabs were assayed for viral PFU in order to determine the amount of virus at the site of infection during the experiment. Eye swabs from WT mice consistently contained higher viral titers than swabs from HVEM KO, nectin-1 KO, and double-KO mice (Fig. 2A). This difference was significant (P value of <0.05) on days 1, 3, and 5 p.i. but not on day 7 p.i. No virus was detected from any of the eye swab samples from double-KO mice at any time point during this assay. There was no statistically significant difference between samples from HVEM KO mice and those from nectin-1 KO mice on days 1 and 3 p.i., but on day 5 p.i. the HVEM KO eye swabs had significantly more PFU/ml on average than did the nectin-1 KO samples. Both the HVEM KO and nectin-1 KO swabs had more PFU/ml than did the double-KO swabs on average on days 1, 3, and 5 p.i., with the

FIG. 1. Clinical symptoms of HSV-1 infection. Mice were inoculated with 2×10^6 PFU per eye and scored on a scale from 1 to 5 each day for both external lesions and neurologic symptoms. Data for groups of WT mice, HVEM KO mice, nectin-1 KO mice, and double-KO mice are shown ($n = 4$ for each group). (A) Average lesion score for each group on days 1 through 14 p.i. HVEM KO, nectin-1 KO, and double-KO averages are zero for each day. (B) Percentage of mice from each group with no lesions on the indicated day p.i. HVEM KO, nectin-1 KO, and double-KO averages are 100% for each day. (C) Average neurologic disease score for each group on days 1 through 14 p.i. One WT mouse received a score of 5 on day 9 and had to be sacrificed. That mouse was scored as 5 for the subsequent days. HVEM KO, nectin-1 KO, and double-KO averages are zero for each day. (D) Survival curve for each group. HVEM KO, nectin-1 KO, and double-KO averages are 100% for each day.

difference being significant on days 1 and 3. On day 5 p.i. the difference in PFU/ml between HVEM KO swabs and double-KO swabs remained significant, but the difference between nectin-1 KO and double-KO swabs was not. Although HVEM KO swabs contained more virus than the double-KO swabs on average, on day 7 p.i., this difference was not significant (P value of 0.175). These data imply that HSV-1 strain 17 can use either HVEM or nectin-1 to establish an infection in the murine cornea but that at least one of these receptors must be present.

To explore the spread of the virus in WT and KO animals, some mice from each group were sacrificed on various days p.i. Periocular skin (POS), trigeminal ganglia (TG), and brains were removed and homogenized, and titers were determined on Vero cells. Similarly to the eye swabs, POS from WT mice contained more PFU/ml than did POS from HVEM KO, nectin-1 KO, and double-KO mice on average (Fig. 2B). These differences between the WT and the KO groups were significant for each genotype at each time point measured. Again, POS from double-KO mice produced no plaques on any day p.i. in this assay. An intermediate phenotype was observed for the HVEM KO and nectin-1 KO samples. Both of these groups had fewer PFU/ml than the WT but more PFU/ml than the double-KO samples on days 1 and 3 p.i. There was no significant difference between HVEM KO and nectin-1 KO samples on either of these days.

A sharp distinction between POS titers in HVEM KO and nectin-1 KO samples appeared on day 5 p.i. (Fig. 2B). None of the POS samples from nectin-1 KO mice contained any detectable virus at this time point. On the other hand, the POS samples from HVEM KO mice contained more virus on day 5 p.i. than did HVEM KO samples from day 3 p.i. A similar increase was seen for the POS from WT animals between day 3 and day 5 p.i. This increase in viral titers in the POS on day 5 p.i. is consistent with zosteriform spread to the POS from the TG (25). Likewise, nectin-1 KO mice infected intravaginally with HSV-2 did not develop external lesions, indicating that viral spread from the nervous system back to the periphery was impeded (27). Therefore, these data suggest that while HSV-1 strain 17 in WT and HVEM KO mice is able to return to the periphery from the TG, the virus is unable to complete this circuit in nectin-1 KO mice.

TG from WT mice contained more PFU/ml on average than did TG obtained from the three KO groups at all four time points assayed (Fig. 2C). This difference was significant for all three groups on days 1 and 5 p.i. It was significant for nectin-1 KO and double-KO samples on day 3 but was not significant for any group on day 7 p.i. WT titers in TG were low on day 1 p.i. and increased on day 3 p.i. before peaking on day 5 p.i. No TG sample from any double-KO mouse contained any detectable virus. HVEM KO samples had an intermediate amount of virus on days 3 and 5 p.i., and one nectin-1 KO

FIG. 2. Viral titers in eye swabs and tissue samples. Asterisks indicate a P value of <0.05 between the two designated data sets. P values were calculated by using a two-tailed Student t test assuming unequal variance. Genotypes are described in the legend of Fig. 1. The limit of detection was 5 PFU/ml. (A) Eye swabs were collected on the indicated days. Titers are reported as the logarithmic average of PFU/ml ($n \geq 6$ for each genotype at each time point). (B and C) POS biopsy specimens (B) and TG (C) were collected and homogenized in 1 ml of medium at the indicated time points. Titers are reported as the logarithmic average of PFU per ml of homogenate ($n \geq 6$ for each genotype at each time point). (D) Whole brains were collected and homogenized in 1 ml of medium at the indicated time points. Prior to the determination of titers, samples were spun at low speed to separate debris from medium. Titers are reported as the logarithmic average of PFU per ml of homogenate ($n \geq 3$ for each genotype at each time point).

sample contained a near-WT amount of virus on day 3 p.i. These data are consistent with previous observations made with the vaginal model of HSV-2 infection, where the virus was attenuated in its spread to the nervous system in nectin-1 KO animals but not completely ablated (27).

More PFU/ml were recovered from WT brains on average than from brains from the other genotypes on most days (Fig. 2D). This difference was significant on day 3 p.i. for all three genotypes and on day 5 p.i. for nectin-1 KO and double-KO mice. Virus was not detected in double-KO brains at any time point. Only one nectin-1 KO sample had a very low viral titer (5 PFU/ml on day 3 p.i.), which was not surprising given how crucial nectin-1 was for the direct cranial inoculation of HSV-2 (8). However, virus was detected in only two HVEM KO samples (5 PFU/ml on day 1 p.i. and 20 PFU/ml on day 5 p.i.), indicating that the spread of HSV-1 in the brains of HVEM KO mice was inhibited at some point along the path from the cornea to the brain.

TG reactivation is impaired in HVEM KO, nectin-1 KO, and double-KO mice. In order to confirm that the virus detected on days 3 and 5 p.i. in the TG was due to the infection of neurons and not just supporting cells, mice from each genotype were sacrificed after 28 days p.i. TG were extracted and cocultured with Vero cells for 7 days and monitored for latent virus reactivation from the TG. Virus was recovered from all

WT TG via this assay (Fig. 3). Virus reactivation was observed for only 25% of HVEM KO TG and 8% of nectin-1 KO TG. No double-KO TG contained virus. These results are consistent with the data from TG harvested on days 1, 3, 5, and 7 p.i.

FIG. 3. Trigeminal ganglion reactivation assay. At 28 days p.i., mice were sacrificed, and TG were cocultured with Vero cells. The culture was checked each day for CPE. At 7 days postexplant, the culture was homogenized, and titers were determined on fresh Vero cells. Percentages of TG that contained detectable virus are reported for each genotype ($n = 10$ for WT and $n = 12$ for HVEM KO, nectin-1 KO, and double-KO mice).

FIG. 4. Immunofluorescence of HSV-1-infected corneas. Corneas on day 1 p.i. were probed with primary rabbit polyclonal antibodies against HSV antigen and secondary antibodies against rabbit IgG with a red fluorescent tag. They were imaged by using the TissueGnostics system. Representative images of infectious foci from the indicated genotypes are shown with HSV antigen staining (red). No infectious foci were observed in corneas of double-KO mice.

and support the hypothesis that HSV-1 is attenuated in spread to the TG in both HVEM KO and nectin-1 KO animals.

HSV-1 establishes more infectious foci in the corneas of WT mice than in those of receptor KO mice. Given that gD receptor-mediated entry takes place very early during the infectious cycle, we hypothesized that the attenuated phenotype observed for HVEM KO and nectin-1 KO mice is due to an entry defect soon after inoculation. To examine this possibility, corneas were removed from mice 1 day after infection, fixed, probed with a polyclonal anti-HSV antibody, and examined by using fluorescence microscopy. Areas of antigen staining representing viral infection were observed on WT, HVEM KO, and nectin-1 KO corneas (Fig. 4). No infectious foci were observed on corneas from double-KO mice. The number and area of the foci of infection were measured by a blinded observer. On average, WT corneas contained more infectious foci than did the HVEM KO and nectin-1 KO corneas (Table 1). Additionally, the total area occupied by these foci was greater in WT corneas on average. These data support the hypothesis that either nectin-1 or HVEM is sufficient for HSV-1 to establish an infection in the murine cornea. Furthermore, these results indicate that both receptors are needed for wild-type levels of infection in the cornea.

DISCUSSION

These studies confirm that either HVEM or nectin-1 is sufficient for HSV-1 infection of the cornea based on the fact that the virus was not detected in double-KO mice in eye swabs, POS, TG, and brains at any time point. Additionally, no viral

TABLE 1. Quantification of HSV antigen staining^a

Genotype (no. of corneas)	Avg no. of foci	<i>P</i> value	Avg area of all foci per cornea (μm ²)	<i>P</i> value	Avg area of each focus (μm ²)	<i>P</i> value
WT (8)	5.6	0.154	303,416	0.120	43,578	0.381
HVEM KO (7)	2.3		54,505		36,161	
Nectin-1 KO (7)	3.3		22,151		10,177	
DKO (4)	0.0		0		0	

^a Foci of antigen staining were counted and measured by a blinded observer using ImageJ software. The average number of foci per cornea, the average total area of antigen staining per cornea, and the average size of each focus are reported for each genotype. *P* values were calculated by using an analysis of variance (ANOVA) test for all groups. DKO, double knockout.

antigen was observed on the corneas of double-KO mice via immunofluorescence at 1 day postinfection. This result is consistent with data from two previous studies using HVEM and nectin-1 KO mice demonstrating that double-KO mice could not be infected with HSV-2 either intravaginally or via direct cranial inoculation (8, 27). Taken together, these three studies provide strong support for the hypothesis that HVEM and nectin-1 are the dominant functional gD receptors in murine-based models of HSV pathogenesis. Without a functional gD receptor, HSV is unable to infect a murine host.

Our studies also support the notion that nectin-1 plays a central role in the transit of HSV through the nervous system. The dorsal root ganglia (DRG) of nectin-1 KO mice infected intravaginally were infected at lower levels than those in WT and HVEM KO DRG (27). Similarly, the TG of nectin-1 KO mice infected with HSV-1 strain 17 via corneal scarification contained lower viral titers than did the TG of their WT and HVEM KO counterparts. These results indicate that nectin-1 mediates the majority of the spread from the periphery to sensory ganglia. Two additional sets of data suggest that nectin-1 also facilitates the anterograde spread of HSV from sensory ganglia back to the periphery. These are the observations that nectin-1 KO mice infected intravaginally with HSV-2 failed to develop external lesions and that POS from nectin-1 KO mice infected ocularly contained no detectable virus on day 5 p.i. It is unclear why HSV-1 was able to spread to only a small number of nectin-1 KO TG. Although nectin-1 is presumed to be the dominant gD receptor in sensory neurons, some infection can be observed even when nectin-1 is absent or blocked (17, 23, 27). HVEM has been detected in the TG of mice, and it is possible that it is able to mediate the small amount of HSV infection in sensory ganglia observed by this and previous studies (9). The possibility remains that an unidentified receptor permits HSV infection of sensory neurons in the absence of nectin-1.

The most surprising finding from our current studies was the absence of clinical symptoms and reduced viral titers in HVEM KO mice. Previous studies of the vagina and brain demonstrated that HVEM was dispensable for HSV-2 infection (8, 27). We found that mice lacking HVEM failed to develop WT disease when infected with HSV-1 via corneal scarification. Furthermore, HVEM KO mice had significantly lower viral loads in eye swabs, POS, TG, and brain than did WT mice at several time points. Fewer HVEM KO TG reactivated after coculture with Vero cells. Finally, corneas from HVEM KO mice contained fewer foci of infection by immunofluorescence than WT corneas. These data suggest that HVEM is important early during the infection of the cornea and that in its absence, WT disease fails to develop. Given that HVEM KO mice had higher viral titers in the TG than did nectin-1 KO mice, the reduced viral spread to the nervous system is likely accounted for by the reduction of infectious foci in the cornea (compared to WT mice) and not due to an inability of HSV to infect neurons in HVEM KO animals.

There are several potential explanations for this novel finding in the ocular model of HSV-1 infection. One possibility is that the virus simply has fewer opportunities to bind to a gD receptor and enter cells when either HVEM or nectin-1 is absent. HVEM KO and nectin-1 KO mice had similar reductions in eye swab viral titers and POS titers at early time points

p.i., and the level of viral antigen was reduced in the corneas of HVEM KO and nectin-1 KO mice 1 day after inoculation. A recent study examining the role of nectin-1 and HVEM in HSV-1 infection of cultured human corneal epithelial cells found that infection was reduced when antibodies to either HVEM or nectin-1 were added to cells prior to the addition of virus (20). That study also found that HVEM and nectin-1 were expressed at very low levels in corneal epithelial cells. Taken together, it is conceivable that when one pathway of entry in the corneas is prohibited (either by blocking the receptor or by removing the receptor), HSV-1 is unable to enter as many cells and fails to establish a robust infection.

The role of HVEM as an immune modulator must also be taken into consideration. The gD-HVEM interaction was proposed previously to modulate cells in several ways, including preventing the apoptosis of infected cells, downregulating HVEM expression, and interfering with HVEM binding to its natural ligands LIGHT, LT α , and/or B- and T-lymphocyte attenuator (BTLA) (15, 18, 24). A study exploring the role of BTLA and HVEM in the pathogenesis of the Gram-positive bacterium *Listeria monocytogenes* found that HVEM KO mice had a more robust response to the bacteria and were protected from bacterium-induced lethality (26). Perhaps, in HVEM KO mice, HSV-1 is unable to bind to HVEM and modulate the immune system in its favor. However, this does not explain the results of the HSV-2 studies that found no difference between HSV-2 pathogenesis in WT mice and pathogenesis in HVEM KO mice.

The surprising contrasting results for HVEM KO mice between these HSV-1 ocular studies and previous experiments using HSV-2 in the vagina and brain could be due to receptor expression or the viral serotype. Although HVEM and nectin-1 mediate HSV-1 and HSV-2 equally well *in vitro*, the possibility remains that the two serotypes have different receptor requirements *in vivo* (10). Various degrees of HVEM and nectin-1 expression in the vagina versus the eye, different HSV-1 and HSV-2 gD-binding affinities for HVEM and/or nectin-1, or unknown virulence determinants of the serotypes could account for the difference between this study and previous studies. The increased virulence of HSV-2 and/or the greater efficiency of infection in the vagina and brain may have masked differences between WT and HVEM KO mice in previous studies.

We have shown that HVEM and nectin-1 are the primary mediators of HSV-1 strain 17 infection of the cornea and that in the absence of either receptor, disease is attenuated. This defect appears to take place early during infection, likely at the step of gD binding to one of its receptors. HSV-1 remains a significant cause of ocular infections and blindness. Therefore, an understanding of which host receptors the virus can use to initiate an infection in the eye and spread to the nervous system is a key step in the development of methods to prevent infection. Further research is warranted to clarify which serotype and/or which routes of infection depend on the presence of both HVEM and nectin-1 in order to target therapeutic strategies aimed at preventing infection.

ACKNOWLEDGMENTS

We thank David Leib and members of his laboratory for providing essential reagents and help making these experiments possible. We

thank the members of the Longnecker laboratory for help and support, particularly Nan Susmarski for providing cell culture assistance and Sarah Connolly and Cynthia Rowe for help with manuscript preparation.

This research was supported by Public Health Service grants R21 EY021306 (R.L.) from the National Eye Institute and T32 AI060523 (A.H.K.) from the National Institute of Allergy and Infectious Diseases.

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