Herpes simplex virus type 2 (HSV-2) infections in the eye are becoming increasingly common in adults. The most likely point of entry for HSV-2 into the eye is through the cornea. By using primary cultures of human corneal fibroblasts (CFs), a natural target-cell type for infection, it was demonstrated that CFs are highly susceptible to HSV-2 entry and replication. RT-PCR and flow-cytometry analyses demonstrated expression of herpesvirus entry mediator (HVEM), a known mediator for HSV-2 entry into cells. Blocking of virus entry into CFs by anti-HVEM antibody implicated HVEM as a potential receptor for HSV-2 infection. These results indicate that HVEM may play a crucial role in HSV-2-induced corneal infections.

Despite the severity of HSV-2 corneal infections, it is not known which cellular receptor(s) is crucial for HSV-2 entry into the cells of the corneal stroma. It is known in general that HSV entry into host cells is a multi-step process that begins with the initial binding of viral envelope glycoproteins to host-cell surface receptors. It has been documented that glycoproteins B and C (gB and gC) mediate the initial attachment or binding of the virions to cell-surface glycosaminoglycans, the most prominent of which is heparan sulfate (HS) (Herold et al., 1991; Shukla & Spear, 2001). Attachment to HS is followed by interaction of gD with its receptor (Krummenacher et al., 1999, 2000). Thereafter, a concerted action involving gD, its receptor, three additional HSV glycoproteins (gB, gH and gL) and possibly an additional gH co-receptor triggers fusion of the viral envelope with a host-cell membrane (Gianni et al., 2006; Parry et al., 2005; Perez-Romero et al., 2005; Scanlan et al., 2003). Subsequently, viral capsids and tegument proteins are released into the cytoplasm of the host cell.

The entry receptors identified include cell-surface molecules derived from three structurally unrelated families. These receptors include herpesvirus entry mediator (HVEM), the nectin family of receptors and a modified
form of HS: 3-O-sulfated heparan sulfate (3-O S HS) (Campadelli-Fiume et al., 2000; O’Donnell et al., 2006; Shukla et al., 1999a; Tiwari et al., 2004, 2005a; Xia et al., 2002). Each type of receptor may have a different role in HSV infection and spread in human tissues. For example, nectin-1 is expressed widely in neuronal cells and tissues (Shukla et al., 2000; Simpson et al., 2005), whilst HVEM is expressed in lymphoid cells and the trabecular meshwork (Tiwari et al., 2005b). Recently, we demonstrated that 3-O S HS generated by 3-O-sulfotransferase 3 is a major entry receptor for HSV-1 in corneal fibroblasts (CFs) (Tiwari et al., 2006).

In an attempt to develop an in vitro model to study HSV-2 entry into the stromal region of the eye, we used primary cultures of human CFs. The CFs were cultured from human tissues derived from healthy eye donors (14 years; provided by the Illinois Eye Bank, Chicago, IL, USA) as described previously (Clement et al., 2006; Yue & Baum, 1981). Briefly, the CFs were grown in minimum essential medium (Invitrogen) supplemented with 10% fetal bovine serum and 5% calf serum (CS). To determine HSV-2 entry, cultured CFs, along with B78H1 melanoma cells, were plated in 96-well plates (2 x 10⁴ cells per well) at least 16 h prior to infection. The latter cells are resistant to HSV entry (Shukla et al., 1999b). A recombinant strain of reporter virus, HSV-2 (333) gJ⁻, engineered to contain a cytomegalovirus–lacZ cassette in place of part of the glycoprotein J gene, was used (Martinez & Spear, 2002). The virus expresses β-galactosidase upon entry into cells. Propagation and titration of HSV-2 (333) gJ⁻ were determined on Vero cells. After 6 h, the cells were washed, permeabilized and incubated with ImmunoPure ONPG (3 mg ml⁻¹; Pierce) substrate for quantification of β-galactosidase activity from the input viral genome. Enzymic activity was measured by a 96-well plate reader (Spectra Max 190; Molecular Devices).

**Fig. 1.** (a–e) HSV-2 enters and replicates in cultured CFs. (a) Cultured human CFs are susceptible to HSV-2 entry. Cultured CFs, along with B78H1 melanoma cells (negative control), were plated in 96-well plates and inoculated with twofold serial dilutions of β-galactosidase-encoding recombinant virus HSV-2 (333) gJ⁻ as indicated. After 6 h, the cells were washed, permeabilized and incubated with ONPG (3.0 mg ml⁻¹) substrate for quantification of β-galactosidase activity from the input viral genome. The enzymic activity was measured by spectrophotometry as A₄₁₀. Each value shown is the mean (± sd) of three or more determinations. (b) X-Gal staining of HSV-2 entry into CFs. CFs grown in Petri dishes were challenged with β-galactosidase-encoding recombinant HSV-2 (333) gJ⁻ at 5 p.f.u. per cell. After 6 h infection at 37 °C, cells were washed with PBS, fixed, permeabilized and incubated with X-Gal (1.0 mg ml⁻¹), which yields an insoluble blue product upon hydrolysis by β-galactosidase. Blue cells (representing virus entry) can be seen as dark cells. Microscopy was performed by using a ×20 objective of a Zeiss Axiovert 100. SLIDEBOOK software, version 3.0, was used for images. (c–e) HSV-2 infection of CFs results in plaque formation. Confluent monolayers of CFs (c) were infected with HSV-2 (333) gJ⁻ at 0.01 p.f.u. per cell for 120 min at 37 °C. Nectin-1-expressing CHO-K1 cells (d) and B78H1 melanoma cells (e) were also infected in parallel as positive and negative controls, respectively. After 48 h, the cells were fixed by using fixative buffer (2.0% formaldehyde and 0.2% gluteraldehyde) at room temperature for 20 min, followed by Giemsa staining for 45 min. The cells were again washed five times in PBS. Images were taken as described above by using a Zeiss Axiovert 100 microscope.
as $A_{410}$. Viral entry into cultured CFs was compared with that into B78H1 cells that have previously been reported to be naturally resistant to HSV-2 entry (Shukla et al., 1999b). As shown in Fig. 1(a), HSV-2 was able to enter CFs, but not B78H1 cells. This was further confirmed by using X-Gal (1.0 mg ml$^{-1}$; Invitrogen) staining. Cultured CFs exposed to $\beta$-galactosidase-encoding recombinants (5 p.f.u. per cell) of HSV-2 virions turned blue (Fig. 1b). These results demonstrate that cultured CFs are susceptible to HSV-2 entry.

We next determined whether HSV-2 was able to replicate in CFs by using plaque assays. Confluent monolayers of CFs were infected with HSV-2 at 0.01 p.f.u. per cell for 120 min at 37 °C. CHO-K1 cells expressing the nectin-1 receptor and B78H1 cells were also infected under similar conditions, as positive and negative controls, respectively. About 48 h later, plaques were fixed and stained. Images were taken by using a × 20 objective of an inverted microscope (Axiovert 100M; Zeiss). As indicated in Fig. 1(c), plaques were seen with the CFs and the nectin-1-expressing CHO-K1 cells, whereas no plaques were detected in B78H1 cells. Overall, the plaques were very similar in size and number in CFs and nectin-1-expressing CHO-K1 cells (data not shown).

In order to determine whether gD receptors played an important role in HSV-2 entry into cultured CFs, we performed a gD-mediated interference assay. This assay is based on the principle that cells which are normally susceptible to virus entry become resistant upon cellular expression of gD by sequestration of the receptors by cellular gD (Shukla et al., 1999a). Cultured human CFs were transfected with a plasmid encoding HSV-2 gD (Muggeridge, 2000). CFs transfected with vector alone (pcDNA3.1) were used as a control. The primary CF cultures were transfected with TransIT-TKO reagent (Mirus Corporation) according to the manufacturer’s instructions. Transfection efficiency in CFs was tested with a green fluorescent protein (GFP) expression plasmid. We estimated that over 80% of cultured CFs expressed GFP as a result of transfection. The transfection efficiency was estimated by using fluorescence microscopy (see Supplementary Fig. S1, available in JGV Online), which was very similar to a previously reported observation (Tiwari et al., 2006). Fourteen hours later, the cells were replated on 96-well plates and exposed for 6 h to $\beta$-galactosidase-encoding HSV-2 (333) gD$^+$, and were lysed. $\beta$-Galactosidase activities were determined as a measure of virus entry. (b) Cell-surface expression of HVEM in CFs. Monolayers of cultured CFs were incubated at 4 °C for 30 min with anti-HVEM antibody (1:200 dilutions). CHO-K1 cells stably expressing HVEM (CHO-K1-HVEM) and wild-type CHO-K1 cells were used as positive and negative controls, respectively. CFs and wild-type CHO-K1 cells stained only with FITC-conjugated secondary anti-rabbit immunoglobulin (IgG) were used as background controls. Cells were examined by fluorescence-activated cell-sorting (FACS) analysis after 45 min incubation with FITC-conjugated secondary anti-rabbit immunoglobulin (IgG) (1:500 dilutions).

Fig. 2. (a) Cellular expression of HSV-2 glycoprotein D (gD-2) interferes with entry into CFs. Cultured human CFs were transfected with a plasmid encoding HSV-2 gD (1.5 μg) or with empty plasmid pcDNA3.1 (1.5 μg) for 14 hours. The cells were replated on 96-well plates and exposed for 6 h to $\beta$-galactosidase-encoding HSV-2 (333) gD$^+$, and were lysed. $\beta$-Galactosidase activities were determined as a measure of virus entry. (b) Cell-surface expression of HVEM in CFs. Monolayers of cultured CFs were incubated at 4 °C for 30 min with anti-HVEM antibody (1:200 dilutions). CHO-K1 cells stably expressing HVEM (CHO-K1-HVEM) and wild-type CHO-K1 cells were used as positive and negative controls, respectively. CFs and wild-type CHO-K1 cells stained only with FITC-conjugated secondary anti-rabbit immunoglobulin (IgG) were used as background controls. Cells were examined by fluorescence-activated cell-sorting (FACS) analysis after 45 min incubation with FITC-conjugated secondary anti-rabbit immunoglobulin (IgG) (1:500 dilutions).

gnectin-1 (Tiwari et al., 2006). As 3-OS HS is not a receptor for HSV-2, we mainly focused on HVEM as the potential receptor for HSV-2 entry into CFs. To verify the presence of HVEM on the cell surface of cultured CFs, flow cytometry was performed by using a FACSCalibur system (BD Biosciences). Wild-type CHO-K1 cells, which do not express any gD receptors (Shukla et al., 1999a), were used as a negative control. CFs stained with fluorescein isothiocyanate (FITC)-conjugated anti-IgG secondary antibody alone were the background control. As shown in Fig. 2(b), both HVEM-expressing CHO-K1 cells and CFs were positive for HVEM expression. Next, to verify the role of
HVEM in entry, we used a previously characterized entry-blocking anti-HVEM antibody (Montgomery et al., 1996; Tiwari et al., 2005b). For the experiment, CFs plated on 96-well plates were incubated with twofold dilutions of the anti-HVEM antibody and a control antibody (α-TGF/βR II) for 90 min. Cells were then challenged at 37 °C with equal doses of HSV-2 (333) gJ at 5×10^5 p.f.u. per well. After 150 min, cells were washed and treated for 1 min with 0.1 M citrate buffer (pH 3.0). After further washing, cells were incubated at 37 °C for 4 h in PBS. The substrate, ImmunoPure ONPG, was prepared in PBS buffer with non-ionic detergent (120 μl 1% Igepal CA-630; Sigma) and β-galactosidase activity was read as A_410. The experiment was repeated three times with similar results.

![Fig. 3.](image)

**Fig. 3.** Anti-HVEM antibody inhibits HSV-2 entry into cultured human CFs. CFs plated in 96-well plates were pre-incubated at room temperature with twofold dilutions of anti-HVEM antibody (Ab) and a control Ab (α-TGF/βR II) for 90 min. Cells were then challenged at 37 °C with equal doses of HSV-2 (333) gJ at 5×10^5 p.f.u. per well. After 150 min, cells were washed and treated for 1 min with 0.1 M citrate buffer (pH 3.0). After further washing, cells were incubated at 37 °C for 4 h in PBS. The substrate, ImmunoPure ONPG, was prepared in PBS buffer with non-ionic detergent (120 μl 1% Igepal CA-630; Sigma) and β-galactosidase activity was read as A_410. The experiment was repeated three times with similar results.

Although unlikely, that the antibody can produce steric interference with some other, as-yet-unknown receptor.

In summary, the demonstration of the ability of HVEM to mediate HSV-2 entry into primary cultures of CFs provides novel information on its physiological relevance as a receptor for HSV-2. Although many reports have indicated its significance in HSV-1 entry (Montgomery et al., 1996; Tiwari et al., 2005b), ours is the first of its kind to demonstrate the importance of HVEM for HSV-2 infection. Our study is also unique because it demonstrates, for the first time, that the two serotypes of HSV prefer separate receptors for entering CFs. HSV-1 prefers 3-OS HS (Tiwari et al., 2006), whereas HSV-2 exploits HVEM. The use of HVEM, which is a member of the tumour necrosis factor receptor family, by HSV-2 might also have implications for the unusual immune response that is observed during HSK. HVEM is a regulator of the immune response mediated by T cells and antigen-presenting cells (Murphy et al., 2006). It is tempting to speculate that HSV-2 gD can modulate the normal activity of HVEM either by competing with its normal physiological ligands (Murphy et al., 2006) or by altering its normal expression, or both. Any such change can have profound immune consequences – a possibility that needs to be examined in the light of our discovery that HVEM is expressed by the cells of the corneal stroma and is used by HSV-2 for entry.

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