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# Nectin-1/HveC mediates herpes simplex virus type 1 entry into primary human sensory neurons and fibroblasts

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**Immunocytochemistry detects nectin-1/HveC, nectin-2/HveB, and HVEM/HveA on the surface of sensory neurons and fibroblasts grown as primary cultures from human dorsal root ganglia. Viral entry into these cultured cells was assayed by infection with a recombinant herpes simplex virus type 1 (HSV-1) expressing green fluorescent protein. Soluble, truncated nectin-1 polypeptide, as well as polyclonal and monoclonal antibodies against nectin-1, inhibited infection of neurons, whereas polypeptides and antibodies capable of inhibiting HSV-1 interaction with nectin-2 and herpesvirus entry mediator (HVEM) failed to prevent infection of neuronal cells. These results demonstrate that nectin-1 is the primary receptor for HSV-1 entry into human fetal neurons. Viral entry into fibroblasts was also reduced by soluble nectin-1 but not by soluble HVEM. However, in contrast to the results obtained with neurons, antibodies against receptors failed to inhibit entry into fibroblasts, indicating that unlike neurons, fibroblasts have multiple receptors or mechanisms for HSV-1 entry.** *Journal of NeuroVirology* (2005) **11,** 208–218.

**Keywords:** HSV-1; human neurons; receptors

## Introduction

Herpes simplex virus type 1 (HSV-1) is a human neuropathic virus that can establish latency in sensory neurons (Mitchell *et al*, 2003). The initial infection of humans involves predominantly epithelial cells of the oral and genital mucosa; subsequently, the virus infects sensory neurons that innervate the site of infection and lymphoid cells that traffic through the infected area. Infection of neurons leads to productive infection or the establishment of latency in sensory ganglia (Mitchell *et al*, 2003). HSV-1 infection proceeds either by attachment of free virions or by cell-to-cell spread. In either case, glycoproteins in the viral envelope interact with the cell membrane,

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leading to virion attachment, membrane fusion, and viral entry. The roles played by viral glycoproteins and cell receptors in attachment and fusion have recently been reviewed (Campadelli-Fiume *et al*, 2000; Spear *et al*, 2000). Mutant studies established an essential role for the viral glycoprotein D (gD) (Ligas and Johnson, 1988). Subsequent experiments with cells expressing gD led to the concept that the viral glycoprotein interacts with limiting amounts of receptor on the cell surface (Campadelli-Fiume *et al*, 1988; Johnson and Ligas, 1988). The ability of soluble gD to inhibit infection supported the concept of specific receptors and allowed an estimate of the number of receptors on a cell (Johnson *et al*, 1990). Genes for these receptors were identified and the gene products isolated (Geraghty *et al*, 1998; Montgomery *et al*, 1996; Warner *et al*, 1998). The first cellular receptor identified for HSV-1 entry was named herpesvirus entry mediator (HVEM) or HveA. HVEM is a member of the tumor necrosis factor (TNF) receptor superfamily, binds directly to gD (Whitbeck *et al*, 1997), and is abundantly present on lymphoid cells (Kwon *et al*, 1997; Montgomery *et al*, 1996). A second class of receptors, nectin-2/HveB and nectin-1/HveC, are members of the immunoglobulin superfamily and are found on neuronal and epithelial cells (Cocchi *et al*, 1998; Geraghty *et al*, 1998; Shukla *et al*, 2000). Nectin-1 is associated with the cell adhesion molecule afadin (Takai and Nakanishi, 2003) and undergoes changes in cellular location during infection (Krummenacher *et al*, 2003). Disruption of cell contacts exposes the gD binding site on nectin-1 and facilitates infection by HSV-1 virions (Yoon and Spear, 2002). A third type of receptor, 3-*O*-sulfated heparin sulfate, has been reported (Shukla *et al*, 1999), and a pH-dependent endocytic pathway has been identified (Nicola *et al*, 2003; Nicola and Straus, 2004). The identification of different types of receptors on different cell types explains in part the broad host range exhibited by HSV-1.

However, the presence of a receptor molecule on a cell does not guarantee that this receptor functions to allow viral entry. The amount and arrangement of a receptor on the cells' surface may influence its ability to function. This situation occurs with polarized cells (Schelhass *et al*, 2003) and with cells in organ culture (Visalli *et al*, 1997). A recent quantitative study of several cell lines by Krummenacher *et al* (2004) concluded that efficiency of infection depends on the amount of receptor expressed and the type of cell. This study also demonstrated that when there is more than one type of receptor on a cell, it can be difficult to determine their relative roles in viral infection.

The primary aim of the experiments described in this paper was to identify the receptors on human sensory neurons and their role in facilitating HSV-1 infection. A secondary objective was to obtain information on receptor usage by human fibroblasts subcultured from the neuronal cultures. These data have important implications in understanding HSV-1 pathogenesis and are especially relevant to the establishment of latency, because human sensory neurons are the natural site of HSV-1 latency. Neuronal cell cultures provide a well studied *in vitro* model of HSV-1 latent infection (Wilcox and Johnson, 1988; Wilcox *et al*, 1990). Previous studies with sensory neurons cultured from rat and mouse ganglia demonstrated that nectin-1 was the predominant cellular receptor (Richart *et al*, 2003). However, anti-nectin-1 antiserum raised against human nectin-1 failed to inhibit infection of mouse neurons, suggesting a species specificity to antibody-receptor interactions (Richart *et al*, 2003). Also, soluble nectin-1 efficiently blocked HSV-1 infection of rat fibroblasts even though these fibroblasts express abundant HVEM, indicating that nectin-1–gD interactions interfere with HVEM-gD interactions (Geraghty *et al*, 1998; Richart *et al*, 2003). The results with rat fibroblasts indicated that receptor specificity should be further studied with primary cultures of human cells.

## Results

### *Characterization of neurons and fibroblasts cultured from dorsal root ganglia*

Three to five days after establishment in culture, neurons express neurites (Figure 1A; Figure 2A, C, E). Neurite expression was facilitated by the association of neurons with support cells, and neurite bundles became thicker with time in culture (Figure 1B). Neuronal cell bodies appeared uniform in diameter (Figure 2A, C, and E) and unlike the situation found in animals (Lawson, 1995) or with purified human neurons obtained after autopsy (Cai *et al*, 2002), size classes were not observed. No attempt was made to study the stage of neuronal differentiation or whether neuropeptide and receptor expression change with time in culture. Cultured neurons expressed neurotrophin receptors (Mu *et al*, 1993), with approximately 90% showing moderately bright staining for TrkA and TrkB, whereas 10% gave bright staining for TrkC (data not shown). These values are in general agreement with those reported by Mu *et al* (1993) for developing dorsal root ganglia. Neurons were also positive for Nissl staining but negative for glial fibrillary acidic protein. Fibroblasts were negative for these markers and the differential staining verified the morphological criteria used to identify the neurons present in mixed cultures. At the time of infection, 7 to 8 days after establishment of the cultures, the cells present were predominantly neurons. Examination of 40 microscopic fields from two experiments showed 1895 ganglionic cells, of which 1237 were neurons (65%). The number of non-neuronal cells varied somewhat from experiment to experiment, and because these non-neuronal cells are

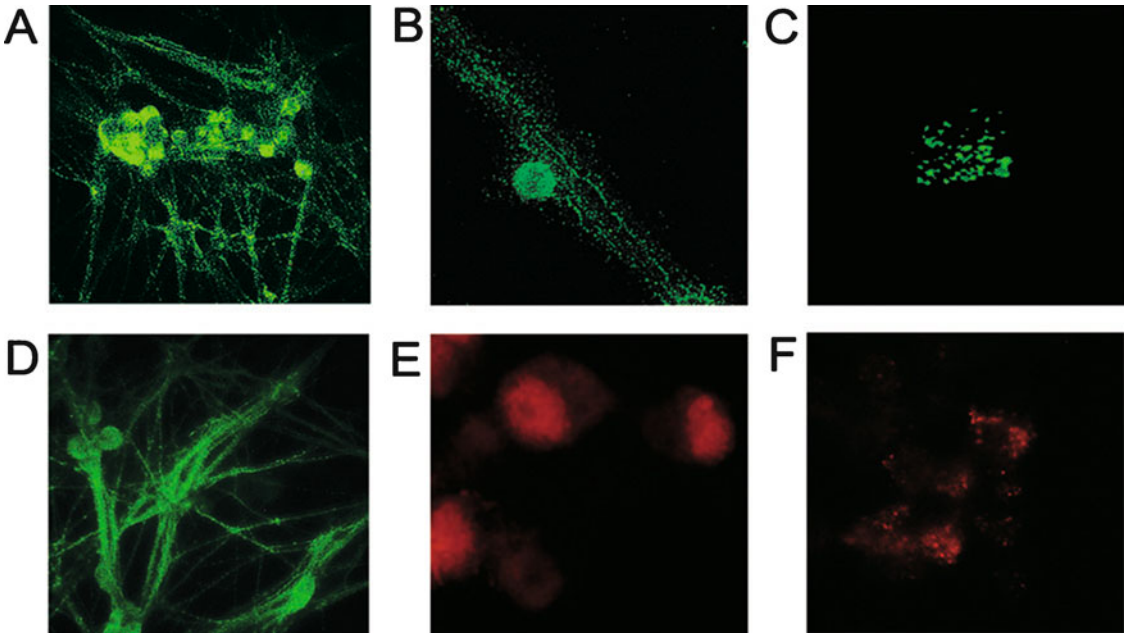


**Figure 1** Staining of HSV receptors on cultured human fetal neurons. **A**, **B**, and **C** show neurons incubated overnight with anti-nectin-1 antiserum R166 (271 $\mu$g/ml) followed by incubation with mouse anti-rabbit IgG Alexa-488 (*green*). The image in **A** was captured at 200× magnification; that in **B** was captured at 630× magnification. **C** shows a deconvolved image of the top of an acetone/MeOH-fixed cell body treated with anti-nectin-1 antibody captured at 1000× magnification. **D** and **E** show neurons incubated with anti-HVEM R140 antiserum (87 $\mu$g/ml) or anti-nectin-2 antiserum R146 (65 $\mu$g/ml, **F**), followed by incubation with chicken anti-rabbit IgG Alexa-594 (*red*) diluted 1:1000 (**E** and **F**) or mouse anti-rabbit IgG Alexa-488 (*green*; **D**). **E** and **F** were captured at 630× magnification, **D** was at 200× magnification.

readily infected, their presence could influence the effective HSV-1 multiplicity of infection (MOI) or the neurons.

Immunocytochemical methods indicate that nectin-1 (Figure 1A, B, and C) and HVEM (Figure 1D and E) are readily detected on the neuronal cell body and along neurites (Figure 1A and D). Nectin-1 was present on approximately 75% of the neurons, whereas HVEM was deteced on about 50%. Assuming the antisera against nectin-1 and HVEM have approximately the same affinity, both receptors appear to be present in similar amounts. Nectin-2 staining was weak (Figure 1F), could not be observed on neurites (data not shown), and varied in intensity from culture to culture. The specificity of antibody staining was demonstrated by blocking the antibody reaction with homologous soluble receptor; heterologous soluble receptor did not block (data not shown). Nectin-1 appeared as distinct foci on the surface of the cell body as well as along neurites and axons (Figure 1A, B, and C). Nectin-2 staining was also punctate (Figure 1F), whereas HVEM staining was diffuse on the cell body and along neurites (Figure 1D and E).

Cultured fibroblasts tended to aggregate with a characteristic colonial morphology (Figure 3A). Immunocytochemical staining detected abundant nectin-1 (Figure 3D) and HVEM (Figure 3B) with less pronounced staining for nectin-2 (Figure 3C). Nectin-1 and nectin-2 gave punctate patterns, whereas HVEM gave a more homogeneous, perinuclear stain that was distinct from that observed with neurons.

*Soluble receptors block HSV entry into neurons*

To determine whether HSV-1 uses HVEM, nectin-2, or nectin-1 for entry into human dorsal root ganglion neurons, virus was incubated with soluble recombinant receptors prior to adsorption to cells in the expectation that soluble receptors would compete with cellular receptors for viral gD and inhibit viral entry. To analyze infected neurons, cell cultures were first examined microscopically under bright-field to determine the location of neuronal cell bodies (Figure 2A, C, and E). The same field was then analyzed under fluorescence to determine the number of neurons infected with the green fluorescent protein (GFP)-expressing HSV-1 (Figure 2B, D, and F). When HSV-1 was treated with HVEM, a slight reduction in the number of GFP-positive neurons was observed (87% of control; [Figure 4A). As expected, treatment with nectin-2 had little effect (97% of control). However, treatment with soluble nectin-1 markedly reduced the number of GFP-positive neurons to approximately 13% of control. To examine the relationship between nectin-1 and HSV-1 infection in greater detail, a dose-response experiment with soluble nectin-1 was performed. Incubation of virus with a range of nectin-1 concentrations resulted in a

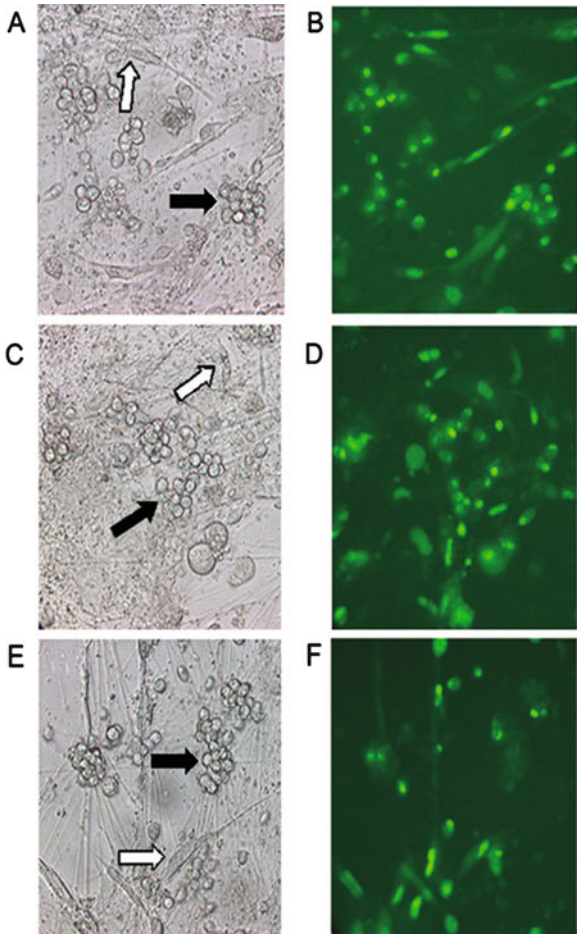


**Figure 2** Soluble receptor blocks HSV-1 infection of primary neuronal cultures. The images shown are representative neuronal cultures photographed 18 h after infection with HSV-1. **A, C,** and **E** are photographed under brightfield and **B, D,** and **F** are the same fields photographed under fluorescence. (**A, B**) HSV-1 alone, (**C, D**) HSV-1 plus soluble HVEM; (**E, F**) HSV-1 plus soluble nectin-1. Magnification 200×. Closed arrows indicate clumps of neurons. Open arrows indicate neuronal support cells.

dose-dependent reduction in viral entry into neurons (Figure 4B). These data demonstrate that treatment of virus with soluble truncated nectin-1, but not HVEM or nectin-2, effectively inhibits HSV-l entry into primary fetal dorsal root ganglion neurons. The positive controls in Figure 4A and B show that approximately 80% of the neurons were infected.

*Antibodies against cellular receptors block HSV-1 entry into neurons*

The effect of treating neuronal cultures with preimmune rabbit sera, monotypic serum against HVEM (R140), or nectin-1 (R166) was determined. Preimmune serum or anti-HVEM failed to reduce the number of infected neurons (95% and 93% of control, respectively; Figure 5A). However, treatment of neurons with anti-nectin-1 serum (R166) reduced the level of infection to about 40% of controls. When neurons were treated with increasing concentrations of anti-nectin-1 serum prior to adding HSV-1, a dose-dependent reduction of viral entry resulted (Figure 5B).

The results with rabbit serum were confirmed with monoclonal antibody CK41. Treating neurons with increasing concentrations of anti-nectin-1 (CK41) monoclonal antibody resulted in a dose-dependent inhibition of viral entry. At the highest concentrations tested, the CK41 antibody reduced the infection to 5% of control (Figure 5C). Monoclonal antibody CW10 binds HVEM but does not block HVEM-gD interactions, and does not inhibit HSV-1 entry. The results obtained with this antibody serve as a control on the specificity of CK41 blocking of nectin-1 rather than as a test of HVEM as a functional receptor. The antibody data demonstrate that anti-nectin-1 serum blocks the cellular receptor and effectively inhibits HSV-1 entry. Even though nectin-2 and HVEM can be detected on neurons by immunocytochemistry, it appears that these receptors do not function to facilitate viral entry. The antibody results support the conclusion from the soluble receptor experiments that nectin-1 is the main functional receptor on neurons for HSV-1.

*Functional receptors on ganglionic fibroblasts*

Published reports describe the presence of HVEM on fibroblasts and how this protein functions as a receptor (Krummenacher *et al*, 2004). Primary cultures of rat fibroblasts express both HVEM and nectin-1, but only soluble nectin-1 inhibited HSV-1 entry (Richart *et al*, 2003). This result indicates that interactions between nectin-1 and viral gD interfere with HVEM-gD interaction. In addition, anti-HVEM antibodies raised against human HVEM polypeptide failed to block viral entry into rat fibroblasts, indicating species specificity to antibody blocking. To test whether the results with rat fibroblasts could be generalized to the natural human host for HSV-1, experiments were repeated with primary cultures of fibroblasts from human dorsal root ganglia. The cell cultures had the morphological characteristics of fibroblasts (Figure 3A), and immunocytochemistry detected both HVEM and nectin-1 on the cell surface (Figure 3B and D). The GFP-expressing HSV-1 efficiently infected human fibroblasts and the infection was inhibited to approximately 50% of controls by soluble nectin-1 (Figure 6A). Increasing the concentration of nectin-1 in the preincubation with virus or supplementing soluble nectin-1 with soluble HVEM did not alter the extent of blocking (data not shown). These results indicate that cultured human fibroblasts, like cultured human neurons, can utilize nectin-1 as a functional receptor for HSV-1 entry, but in contrast to the results with neurons, the CK41 monoclonal antibody had no effect on infection of fibroblasts (Figure 6B). Soluble HVEM did not markedly inhibit viral infection. Pretreatment of virus with anti-gD serum neutralized viral infectivity of fibroblasts greater than 95% (Figure 6A), indicating that the

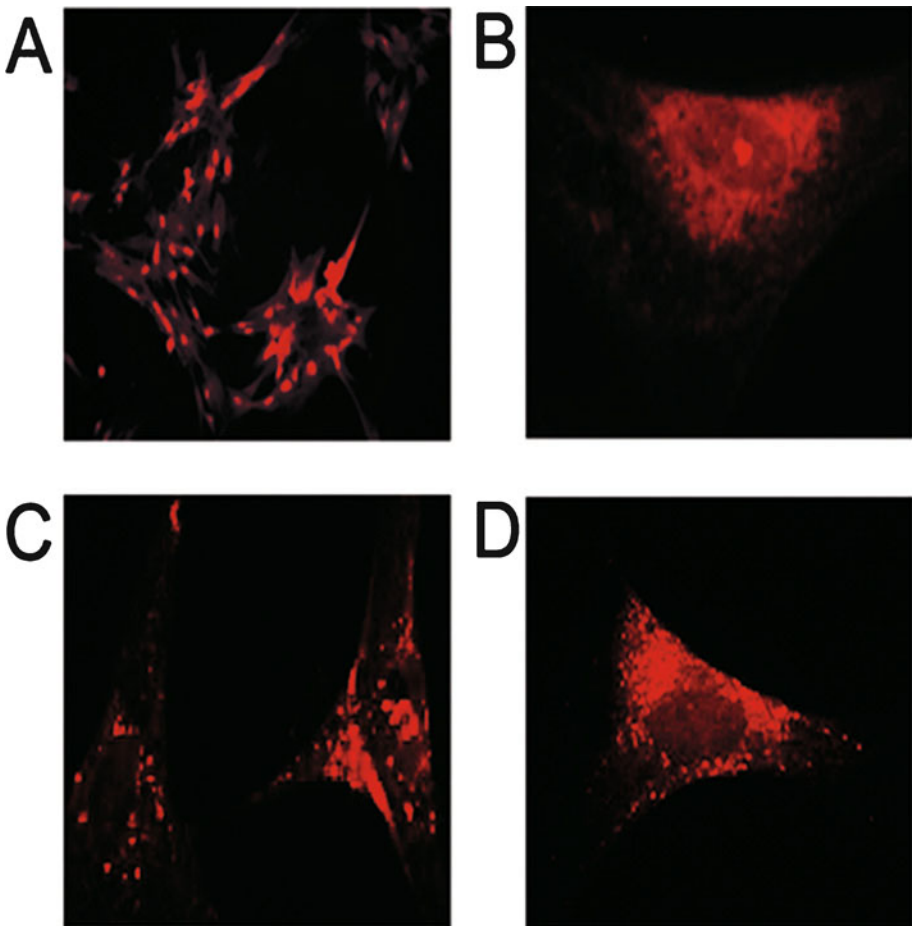


**Figure 3** Receptors on primary fibroblasts from fetal human neuron cultures. Primary fibroblast cultures were fixed and incubated overnight with rabbit polyclonal anti-receptor antisera followed by incubation with chicken anti-rabbit IgG Alexa 594 serum. (**A**) Anti-HVEM antiserum R140 (17 $\mu$g/ml), at 200× magnification; (**B**) anti-HVEM antiserum R140 (35 $\mu$g/ml), at 630× magnification; (**C**) anti-nectin-2 antiserum R146 (26 $\mu$g/ml), at 630× magnification; (**D**) anti-nectin-1 antiserum R166 (14 $\mu$g/ml), at 630× magnification.

appropriate conditions were used for testing antibodies. The results with anti-receptor antibodies support the proposal that multiple receptors can function on fibroblasts (Krummenacher *et al*, 2004).

## Discussion

The appreciation that HSV-1 infection involves specific cellular receptors has stimulated research into the nature of these membrane proteins and their role in directing viral infection to different cell types. The initial infection of the oral mucosa could involve nectin-1 or HVEM on epithelial cells (Hung *et al*, 2002). The spread of virus from the primary infection to adjacent cells, whether it involves cell-free virions or fusion between infected and uninfected cells, depends on the nature of the receptors on these cells and their availability to virus (Cocchi *et al*, 2000; Terry-Allison *et al*, 1998). At the primary site of infection are nerve endings from sensory neurons whose infection probably involves nectin-1 receptors (Mata *et al*, 2001). Nectin-1 is also involved in the development of synapses (Mizoguchi *et al*, 2002) and is widely distributed in the nervous system. Nectin-1 has a role in cell-to-cell interactions, but its functional role as a receptor for HSV-1 infection has not been demonstrated.

Entry of HSV nucleocapsids at nerve endings of primary sensory neurons initiates the spread of virus via retrograde transport in axons to neuronal cell bodies in the sensory ganglia. In the neuronal soma, a productive infection can take place producing infectious progeny, or an abortive infection can lead to latency. The neurobiology of infection and spread of alphaherpesvirus in the nervous system has been reviewed by Enquist *et al* (1999). The properties of the afferent sensory pseudounipolar neurons that transport the virus from the periphery to the ganglia are described in this review, together with the cellular components involved in nucleocapsid transport. The value of animal models in studying virus spread and the involvement of non-neuronal cells are also discussed together with the influence of cellular polarity on virus entry and exit (Enquist *et al*, 1999). Receptor usage in an animal could vary depending on

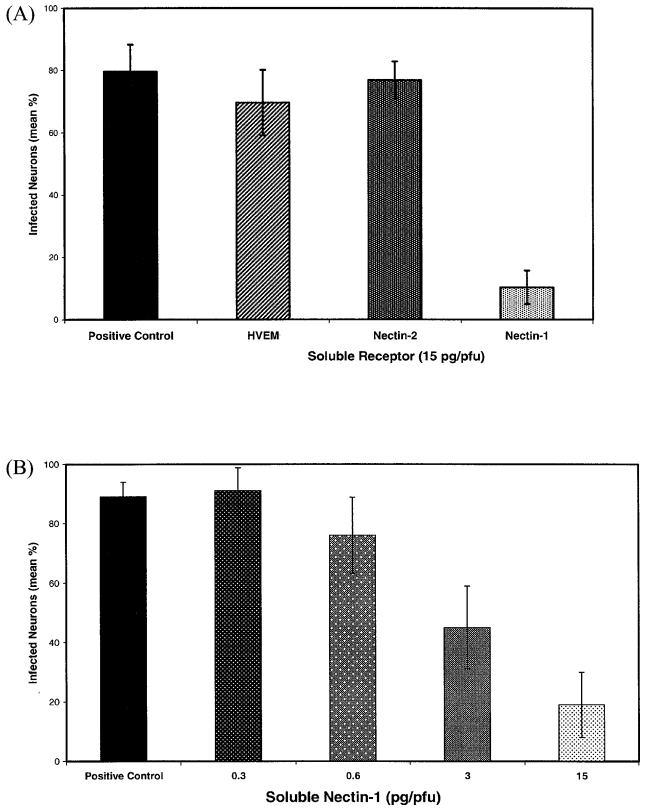


**Figure 4** Effect of soluble receptors on HSV-1 entry into neurons. (**A**) HSV-1 was pretreated with 15 pg/pfu of either HVEM, nectin-2, or nectin-1 and then added to neuronal cultures. The cultures were incubated, photographed, and the data analyzed as described in Materials and Methods. (**B**) HSV-1 was treated with increasing concentrations of soluble nectin-1, then added to neuronal cultures and the cultures processed as described in the methods. Error bars indicate one standard deviation.

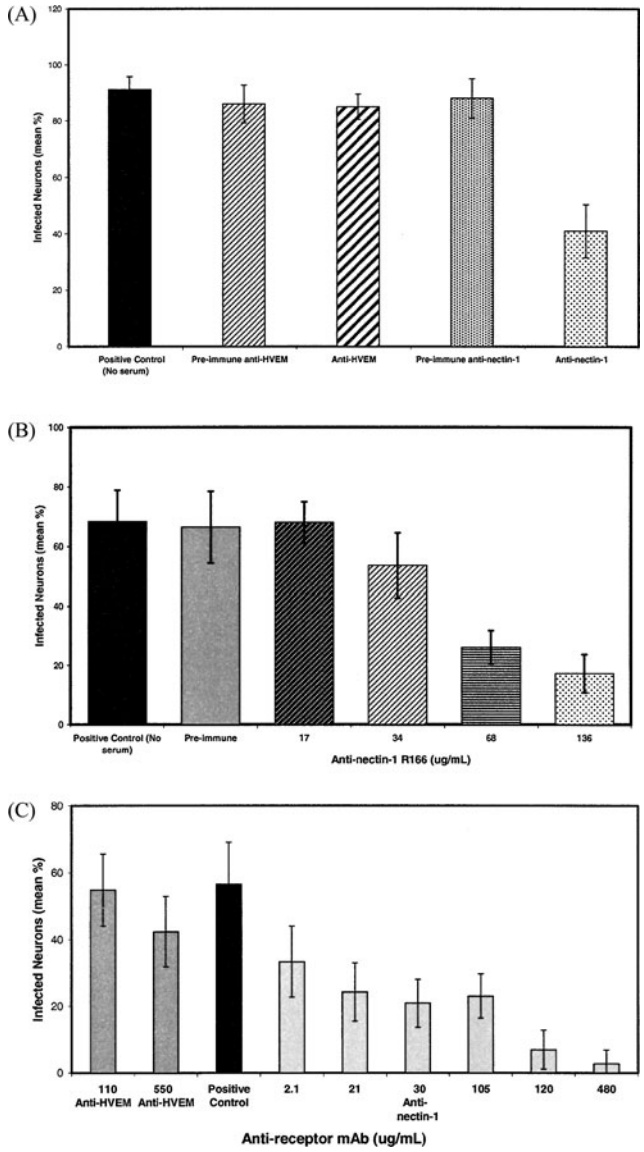


**Figure 5** Effect of anti-receptor polyclonal antibodies on HSV entry into neurons. (**A**) Neuronal cultures were treated with either preimmune, anti-HVEM antisera R140 (174 $\mu$g/ml), or anti-nectin-1 antisera R166 (68 $\mu$g/ml), infected with HSV-1, and processed as described in the Materials and Methods. (**B**) Neuronal cultures were incubated with increasing concentrations of anti-nectin-1 R166 serum and then infected with HSV-1. The cultures were processed and data collected as described in the Materials and Methods. (**C**) Neuronal cultures were incubated with increasing concentrations of anti-nectin-1 monoclonal antibody CK41 or anti-HVEM monoclonal antibody CW10 and then infected with HSV-1 as described in Materials and Methods. Error bars indicate one standard deviation.

topographic considerations and cell-to-cell associations. For example HSV receptor distribution differs in raft cultures compared to standard cell cultures. With these complexities in mind, one might question whether studies of receptor function on purified neurons and fibroblasts are pertinent to natural infection in animals. However, purified neurons allow a direct assay of receptor function. The results of the experiments described in this paper demonstrate that nectin-1 does function to allow HSV-1 to enter neurons, and HVEM, although present on these cells, does not. The presence of nectin-1 on neurites and neuronal cell bodies suggests that HSV-1 can enter neurons either by viral and cellular fusion with neurite extensions or with the cell body, and the electron microscope experiments of Lycke *et al* (1988) with cultured human sensory neurons confirm that HSV-1 enters neurons at either location. Based on the relative surface area of neurites and cell soma, one expects the cell body to be a more effective route for virus entry, but the close association between neuronal soma and satellite cells (Cai *et al*, 2002) might in the animal make the nectin-1 receptors on the cell body inaccessible to virus and restrict virus entry to the neurite terminal. However, because the pathogenesis of latency is believed to involve ascending infection of neurons from skin lesions, it is likely that receptors on neurites facilitate most viral entry into the nervous system. Viremia, which frequently occurs during severe primary infection, may also infect neurons via receptors on the cell body.

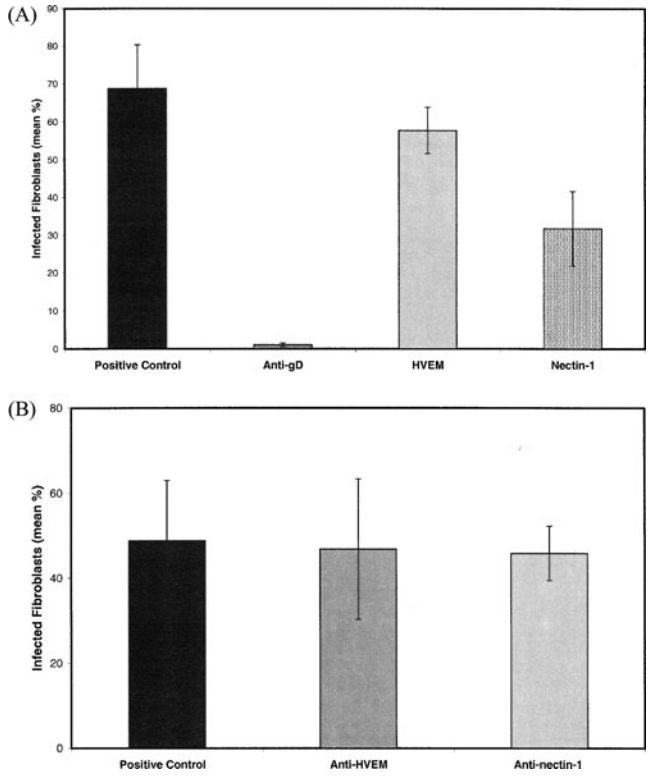


**Figure 6** Effect of soluble receptors and anti-receptor monoclonal antibodies on HSV-1 entry into fibroblasts. (**A**) Virus was treated with soluble receptor (15 pg/pfu) or anti-gD antibody (R45 1% *v*/*v*) and added to primary cultures of human fibroblasts. Infection proceeded as described in Materials and Methods. (**B**) Fibroblasts were treated prior to infection with monoclonal antibodies against nectin-1 (CK41) or HVEM (CW10) at 100 $\mu$g/ml. Antibody CW10 does not neutralize virus and thus serves as a control on CK41.

The primary aim of the experiments described in this paper was to identify the HSV-1 receptors on cultures of human neurons and subsequently determine whether these cellular receptors function to facilitate viral entry. Abundant nectin-1 was detected on neuronal cells and along the neurofilaments by immunocytochemistry, suggesting that virus could infect either the neuronal cell body directly or via neurite extensions. Staining for HVEM was diffuse throughout the neuronal cell and along neurite extensions, and could also be a functional receptor. Nectin-2 does not serve as a receptor for wild-type HSV-1 (Warner *et al*, 1998). The experiments with human neurons showed that soluble nectin-1 and anti-nectin-1 antibody blocked viral entry greater than 90%, indicating that human neurons, like rat neurons, use nectin-1 as the primary receptor. The failure of HSV-1 to use HVEM, an abundantly expressed receptor, is a novel and interesting observation. The phenomenon appears to be cell type specific, could depend on interactions with secondary cellular proteins, and warrants further investigation.

The other cells of interest were fibroblasts subcultured from the neuronal cultures. These cells express nectin-1, nectin-2, and HVEM. At saturation, soluble nectin-1 reduced infection to 50% of controls. Analogous to the results obtained with other fibroblast cultures, anti-nectin-1 antibodies failed to block viral entry into human fibroblasts. When more than one type of functional receptor is present on a cell, blocking one type will leave the cell susceptible to infection via other receptors or via alternate pathways.

Our results indicate there are multiple functional receptors on human fibroblasts (Shukla *et al*, 1999; Krummenacher *et al*, 2004). In addition, the endocytic pathway for infection could be active in fibroblasts (Nicola *et al*, 2003; Nicola and Straus, 2004). Fibroblasts differ from neurons in terms of receptor usage, and the characteristics exhibited by human fibroblasts derived from ganglia are shared by fibroblasts from other human tissues and other species (Krummenacher *et al*, 2004; Richart *et al*, 2003).

Another observation made with rodent fibroblasts and confirmed with human cells is that soluble nectin-1 inhibits viral entry even when HVEM is present. This result is similar to that obtained with Chinese hamster ovarian (CHO) cells transfected with the HVEM gene (Geraghty *et al*, 1998). An interpretation of this result is that nectin-1 binding to gD inhibits HVEM-gD interactions. Nectin-1 blocking could result from steric interference with HVEM binding to gD. However, antibody studies indicate that nectin-1 and HVEM interact with different regions of gD (Krummenacher *et al*, 2000; Nicola *et al*, 1998). It is possible that, as was found with neurons, HVEM on fibroblasts does not facilitate virus entry. A complete lack of HVEM receptor function appears unlikely because nectin-1–deficient fibroblasts can be infected by HSV-1 and this infection can be blocked by anti-HVEM serum (Krummenacher *et al*, 2004).

Crystallographic studies of gD and gD-HVEM cocrystals show that conformational changes take place when gD binds HVEM (Carfi *et al*, 2001). It is plausible that nectin-1 binding to gD interferes with the conformational changes that accompany gD-HVEM binding. As a consequence of interfering with the gD-HVEM conformational change, nectin-1 inhibits HVEM's ability to function as a receptor. Until crystallographic data are available for gD–nectin-1 binding, interpreting soluble receptor blocking of viral entry into cells that express multiple receptors requires caution and should be supported by antibody blocking data.

The results presented in this paper not only have intrinsic value to the field of neurovirology in that they address questions of HSV-1 infection of human neurons, but also identify areas of concern when studying cellular receptors. The experiments reported in this and our previous paper (Richart *et al*, 2003) utilized cell-free virions. From the perspective of viral pathogenesis, it would be important to determine whether soluble nectin-1 inhibits viral transmission from infected fibroblasts or keratinocytes to neurons via direct cell-to-cell transmission.

## Materials and methods

*Primary neuronal cultures*

Procedures for harvest of human dorsal root neurons were adapted from previous publications (Wilcox *et al*, 1990; Smith and Wilcox, 1996). Neuronal cultures were prepared from elective abortions. Written consent was obtained for the use of this tissue for research purposes (IRB approval 00-719). All fetuses were between 52 and 54 days of gestation. Dorsal root ganglia were excised and placed in "WM-Ø" medium (L-15 media containing 10% heat-inactivated newborn calf serum [NCS; Gibco-BRL Life Technologies, Carlsbad, CA], 200 units/ml of penicillin, 200 $\mu$g/ml of streptomycin, and 0.50 $\mu$g/ml amphotericin B). Ganglia were digested with 3 mg/ml of type I collagenase (Boehringer-Mannheim, Indianapolis, IN) in WM-Ø medium for 1 h at 37°C. Cells were then centrifuged at 1000 × *g* for 1 min and washed with 10 ml of WM-Ø medium. After three consecutive 10-ml washes, the tissue fragments were suspended in 5 ml of Dulbecco's modified Engle's medium (DMEM) plus 10% newborn calf serum, supplemented with 100 ng/ml 2.5 S mouse nerve growth factor (NGF; Harlan Bioproducts, Indianapolis, IN), 100 units/ml of penicillin, 100 $\mu$g/ml of streptomycin, and 0.25 $\mu$g/ml amphotericin B. The tissue was converted to a single cell suspension by gentle trituration with a sterile small-bore Pasteur pipet. Approximately $8 \times 10^3$ cells in 100 $\mu$l of medium were placed in 8-chamber type I rat-tail collagen-coated slides (BD Discovery Labware, Bedford, MA). After 6 to 24 h at 37°C with 5% $CO_2$, fresh DMEM/10% NCS medium was added to a final volume of 800 $\mu$l/well. To reduce non-neuronal cells in the cultures, culture medium was cycled every 2 to 3 days between DMEM/10% NCS and this same medium supplemented with 20 $\mu$M fluorodeoxyuridine and 20 $\mu$M uridine (Sigma-Aldrich, St. Louis, MO).

*Support cell cultures*

Support cell (astrocytes and fibroblasts) cultures were established after the human fetal tissue was processed to obtain neuronal cultures. The residual 100 to 200 $\mu$l of cell suspension containing $1–2 \times 10^4$ neurons and non-neuronal cells was placed in a 60-mm-diameter sterile Petri dish and allowed to attach to the plastic surface. The cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1× penicillin-streptomycin-amphotericin B solution (Gibco-BRL), and 1% nonessential amino acid solution (Gibco-BRL). Over the course of the subsequent 2 weeks, the culture was subcultured twice using trypsin-EDTA. After the second subculture few, if any, neuronal cells remained in the culture, and the cells predominantly had the appearance of fibroblasts. After 14 to 17 days, the cells were trypsinized and replated at $10^4$ cells per well in eight-chamber glass slides. In some experiments, to improve the dispersal of cells and increase ease of scoring, 12-well plastic plates were used. The cells were allowed to attach and grow from 12 to 48 h to reach a suitable cell density. Approximately $6\times 10^3$ cells routinely attached to the slide. To ensure the desired MOI, the cell number was determined by direct counting immediately preceding the experiment.

*Virus*

HSVEGFP4 is a recombinant HSV-1 (17+ strain) expressing (GFP) fused to the C-terminus of the immediate-early gene product ICP4. Gene expression is controlled by the native ICP4 promoter. The virus was generated by recombining the ICP4-GFP construct into the ICP4 deletion mutant D30EBAΔ (Paterson and Everett, 1990). The recombinant virus behaves essentially as wild-type virus (C. L. Wilcox, unpublished data), producing a fluorescent signal in neurons 12 to 24 h after infection and in non-neuronal cells by 6 to 8 h after infection. The virus was propagated in rabbit skin cells (Stenberg and Pizer, 1982) and the viral titer in rabbit skin cells used to calculate the MOI for infecting neurons and fibroblasts.

*Polypeptides and antibodies*

Recombinant nectin-1/HveC (346t), nectin-2/HveB (361t), and HVEM (200t) are baculovirus-expressed, truncated, soluble forms of the receptors produced as previously described (Geraghty *et al*, 1998; Warner *et al*, 1998; Whitbeck *et al*, 1997). Polyclonal antibodies anti-HVEM (R140) and anti-nectin-1/HveC (R166) were obtained by immunizing rabbits with the soluble polypeptides (Nicola *et al*, 1998; Shukla *et al*, 2000; Whitbeck *et al*, 2001). Preimmune sera were collected from rabbits before immunization. Immuno-globulin (IgG) concentrations in rabbit antisera were measured by enzyme-linked immunosorbent assay (ELISA). Monoclonal antibody CK41 binds a conformational epitope on nectin-1 (Krummenacher *et al*, 2000), and monoclonal antibody CW10 binds HVEM but does not block infection (Krummenacher *et al*, 2004). Monoclonal antibodies were purified on protein A sepharose.

*Viral infection*

Neuronal cultures were maintained for at least 8 days prior to infection with HSVEGFP4 and the culture medium was changed to anti-mitotic–free DMEM/10% NCS medium 24 h before adding virus. After the growth medium was removed, 100 $\mu$l of medium containing virus or virus plus receptor was added to achieve an MOI of 100 plaque-forming units (PFU)/neuronal cell. Cultures and virus were incubated for 1 h at 37°C with gentle rocking; then the inoculum was removed and replaced with fresh medium supplemented with 100 $\mu$M acyclovir (Sigma-Aldrich). Eighteen to 24 h after initiating infection, infected neurons were identified as GFP-positive cells. Two methods of scoring infection were

used. In initial experiments, neurons in a microscope field were identified by their morphology under brightfield, and fluorescence microscopy of the same field used to determine the number of neurons infected. In later experiments, microscope fields randomly chosen were photographed under both brightfield and fluorescence. These digital photography images were opened at equivalent screen size in AdobePhotoshop 7.0 for Windows. Using a transparency sheet attached to the computer screen, the total number of neurons per field were counted and marked within the brightfield image. The corresponding fluorescent image was subsequently superimposed on the brightfield image and the GFP signal used to identify HSV-positive neurons. For each experiment, four microscopic fields were randomly examined per chamber and four chambers were studied for each experimental group. The slides were coded and examined without indication of the experimental conditions. Data were calculated as percent of GFP-positive neurons ([number of GFP-positive neurons per field/number of total neurons per field] $\times$ 100). Both methods of scoring infected neurons gave equivalent data but the transparency overlay procedure was more efficient. All experiments were performed at least twice and critical experiments (e.g., receptor blocking) were carried out five times.

Fibroblasts were infected at an MOI of 10 pfu/cell. The culture medium was aspirated, virus alone or virus after treatment with soluble receptor was added to the cells, and the cultures incubated at 37°C for 1 h. The inoculum was then removed and the cells washed twice with DMEM supplemented with 2% fetal bovine serum (FBS). DMEM supplemented with 100 $\mu$M acyclovir was added and the cells were incubated at 37°C for 24 to 48 h. Four fields per well were randomly chosen and photographed under brightfield, to locate the cells, and fluorescent microscopy, used to identify infected cells. A total of 16 photos were obtained per experimental condition. The data for each experimental condition were compared with that obtained with the untreated control.

*Effects of soluble receptors on HSV-1 entry*

The procedure to measure soluble receptor inhibition of viral entry into neurons or fibroblasts from human ganglia was similar to that published previously for cell lines (Geraghty *et al*, 1998) and rodent neurons (Richart *et al*, 2003). Soluble, truncated forms of HVEM/HveA [HveA(200t)], nectin-2/HveB [HveB(361t)], or nectin-1/HveC [HveC(346t)] were preincubated with the virus for 2 h at 4°C with periodic mixing. The soluble receptor concentration in the preincubation was 15 pg/pfu. After preincubation, the virus and soluble receptor mixture was added to neurons or fibroblasts and virus allowed to adsorb for one hour. The inoculum was then removed, replaced with medium, and the infection allowed to proceed.

*Effects of antibodies against cellular receptors on HSV-1 entry*

Blocking of infection by antibodies against cellular receptors was assayed as previously described (Richart *et al*, 2003). Briefly, neuron or fibroblast cultures were preincubated for 60 min at 37°C with preimmune serum, polyclonal immune rabbit antisera, or purified monoclonal antibody. After the preincubation, the medium with serum was removed and the cells infected with virus.

*Immunostaining of fetal human dorsal root ganglia cell cultures*

Dorsal root ganglia cells cultured on eight-chamber glass slides were rinsed in phosphate-buffered saline (PBS) (pH 7.4). Cells to be stained for membrane surface proteins were fixed for 30 min at room temperature in 4% paraformaldehyde. Those to be stained for intracellular proteins were fixed in $-20$°C acetone/methanol (1:1) for 2 min. Cells fixed by either method were rinsed briefly in PBS prior to staining and were not permitted to dry during staining. To minimize nonspecific staining, slides were submerged in blocking buffer (3% bovine serum albumin [BSA] diluted in SuperBlock; Pierce) for approximately 1 h at room temperature or overnight at 4°C. This buffer, supplemented with 5% pooled normal goat serum, was used for all antibody dilutions.

Rabbit polyclonal antisera (R140) (Whitbeck *et al*, 1997) reactive with human HVEM, nectin-2 (R146) (Warner *et al*, 1998), or nectin-1 (R166) (Krummenacher *et al*, 1998, 2000) were titrated for specific reactivity with primary fetal neurons and primary fibroblasts. Staining controls included primary staining with either preimmune sera, pooled normal rabbit sera, pooled rabbit IgG, or secondary antibodies applied directly to cells without prior addition of primary antibody. Secondary antibodies used for indirect staining included Alexa Fluor 488 goat F(ab')$_2$ anti-rabbit IgG, Alexa Fluor 594 chicken anti-rabbit IgG, Alexa Fluor 594 chicken anti-mouse IgG, and Alexa Fluor 488 goat F(ab')$_2$ (Molecular Probes). Antibodies used to characterize cultured neuronal cell phenotypes included anti-TrkA, anti-TrkB, anti–glial fibrillary acidic protein (Santa Cruz Biotechnologies), and anti-TrkC (Oncogene Research Products). NeuroTrace fluorescent Nissl stain (Molecular Probes) was also used to distinguish neuronal from non-neuronal cells.

Cells were stained with anti-HVEM, anti-nectin-2, or anti-nectin-1 overnight at 4°C or with antibodies against neuronal cell markers for 1 h at room temperature. Cells were then washed with five changes of PBS/0.1% Triton $\times$ 100/0.05% Tween-20 buffer and incubated with dilutions of a secondary antibody at room temperature for 20 min. Following the incubation with the secondary antibody, cells were again washed extensively in PBS. Slides were air-dried, mounted in OPDA medium

(2 mg/ml *o*-phenylenediamine-di-HCl [Sigma]/0.1 M Tris-HCl, pH 8.5/90% glycerol), and stored at −20°C.

Images were captured by a Zeiss Axiophot microscope equipped with a CCD camera and controlled by SlideBook 3.00.2 software (Intelligent Imaging Innovations, Denver, CO). In some experiments, fluorescent image sets were collected and further resolved using deconvolution analysis. Alternatively, images were captured by a Nikon Eclipse TS100 microscope equipped with a Nikon COOLPIX995 digital camera.

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