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High susceptibility of a human oligodendroglial cell line to herpes simplex type 1 infection

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More than 20 infectious agents, ranging from retroviruses to mycobacteria, have been associated with multiple sclerosis onset or relapses in which oligodendrocytes, the myelin-forming cells of the central nervous system, are the initial target of the pathogenic status. In this work, the nature of the susceptibility of the human precursor oligodendroglial KG-1C cell line to herpes simplex virus type 1 (HSV-1) was investigated. Infection of KG-1C cells was characterized by a high level of virus production and a notable progression of the cytopathic effect. After infection, there was a significant shut-off of host mRNA translation, which was correlated with evident synthesis of viral proteins. An examination by electron microscopy of the infected cells revealed the presence of large clusters of mitochondria located in the proximity of intracellular HSV-1 particle groups. In addition, transmission electron microscopy and nuclear fluorescence analysis showed neither signs of chromatin condensation nor of apoptotic bodies. Furthermore, procaspase-3 remained uncleaved, suggesting that apoptosis does not take place, at least in this system. Finally, expression and localization of MAL2, a subpopulation of detergent-insoluble lipid raft protein, was studied. Detection of MAL2 significantly increased after infection and it was colocalized with HSV-1 proteins. From these findings the authors conclude that human oligodendrocyte-like cells are highly susceptible to HSV-1 infection. The implications of this for central nervous system viral infection are discussed. *Journal of NeuroVirology* (2005) **11,** 190–198.

Keywords: herpes virus; HSV-1; infection; KG-1C; MAL2; oligodendrocyte

Introduction

Herpes simplex virus type 1 (HSV-1) is an important neurotropic virus that can infect peripheral sensory neurons and, by retrograde axonal transport, reach the central nervous system (CNS) (Immergluck *et al*, 1998). A latent infection can be established once HSV-1 gains access to the neurons in sensory ganglia (Kramer *et al*, 2003). Periodically, HSV-1 emerges to cause acute infection and, in this circumstance, the virus could be responsible for a variety of disease states, such as encephalitis and some chronic and progressive neurodegenerative disorders (Itzhaki *et al*, 1998; Qiu and Abdel-Meguid, 1999). Nevertheless, little is known about the mechanisms underlying these processes of latency and reactivation.

In addition to the acute neurological disease resulting from the infection of the nervous system, HSV-1, as well as other members of the *Herpesviridae* family of viruses, has been included in models of virus-induced demyelination (Tsunoda and Fujinami, 2002; Kastrukoff and Kim, 2002; Fazakerley and

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Walker, 2003). There is molecular evidence of the involvement of HSV-1 in clinical acute attacks of multiple sclerosis (MS) (Ferrante et al, 2000). In this study, the prevalence of some herpesviruses in the peripheral blood of MS patients revealed that HSV-1 DNA was only found in acute MS, whereas both stable MS and healthy controls remained negative. Despite these recent data, HSV-1 infection in oligodendrocytes and oligodendrocyte-derived cell lines has yet to be completely characterized.

Differences in susceptibility to HSV-1 among oligodendroglia derived from different sources have been investigated in mice (Thomas *et al*, 1997) and humans (Kastrukoff and Kim, 2002). These differences, which were determined after viral adsorption but prior to the expression of immediate-early genes, were donor dependent and suggest differences in virus–host cell interactions that are probably determined by genetic factors (Thomas *et al*, 1997).

Traditionally, the inhibition of apoptosis has been recognized as one of the major hallmarks of HSV-1 infections (Derfuss and Meinl, 2002; for review see Goodkin *et al*, 2004). Nevertheless, programmed cellular death mechanisms have been implicated in recent investigations about the pathogenesis of herpes simplex encephalitis (HSE) (DeBiasi *et al*, 2002; Perkins *et al*, 2003). In fact, neuronal and glial apoptosis were clearly detected in acute HSE. This apoptosis induction was due to direct viral injury instead of inflammatory T-cell response (DeBiasi et al, 2002). Additionally, and in contrast to what occurs in human fibroblasts, antigen presentation by major histocompatibility complex class I molecules is not blocked after HSV-1 infection of human T-cell lines. Moreover, viral infection results in apoptosis of antiviral T cells (Raftery *et al*, 1999), which seems to be a mechanism of viral immune evasion.

In the present report, we address the characterization of the infection of a human oligodendroglial cell line by HSV-1. The human immature glioma cell line KG-1C, consisting of undifferentiated glial cells, is shown to be highly susceptible to HSV-1 infection. We examine whether this infection takes place through an apoptotic mechanism. Finally, we propose the involvement of a newly described raft protein of the MAL family in the infection of this human cell line.

Results

Susceptibility of KG-1C cells to HSV-1 infection

The human oligodendroglial KG-1C cell line was infected with HSV-1 (F strain) at a multiplicity of infection (m.o.i.) of 1 (plaque-forming unit [PFU] per cell). Infectious virus production was assayed over a time course. Figure 1 shows that virus production (measured as the log of total PFU) was similar to that observed in the highly susceptible Vero cell line. Twenty h post infection (p.i.), HSV-1 infected

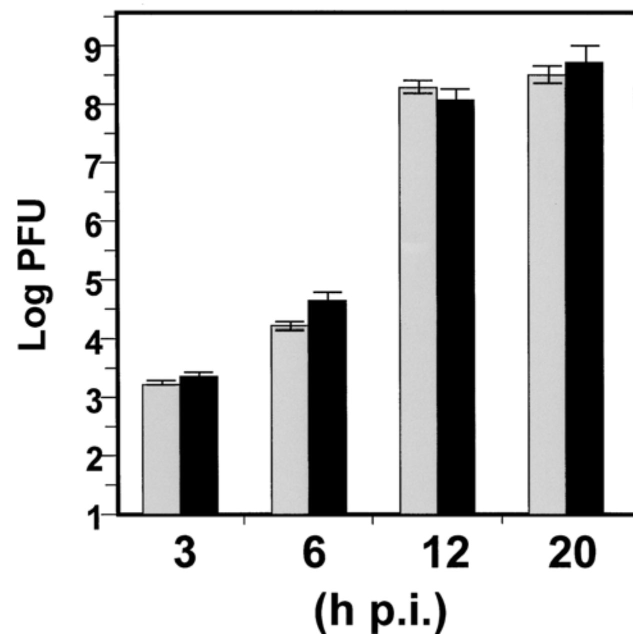

Figure 1 Susceptibility of KG-1C cells to HSV-1 infection. A total of 10^7 Vero (*gray bars*) or KG-1C (*black bars*) cells were infected with HSV-1 at 1 PFU per cell. After the indicated time, cells were harvested and extracts titrated for PFU production. Each value represents the mean of six samples; error bars indicate the standard deviations of the means.

more than 90% of the cells in both KG-1C and Vero cell cultures (data not shown). Comparable productivity between KG-1C and Vero cells was obtained after infection at lower (0.1) and higher (10) m.o.i. (data not shown). In addition, the cytopathic effect (CPE) could be noted from 20 h p.i. onwards (Figure 2A). The kinetics of protein synthesis in KG-1C cultures after HSV-1 infection showed that viral proteins were clearly detectable after 6 h p.i. by means of polyacrylamide gel electrophoresis (PAGE) analysis, in accordance with the production of infectious viral particles, as shown in Figure 1 (Figure 2B). HSV-1–induced shut-off was detectable from 12 h p.i., and most synthesized proteins were viral. Similar results were obtained when the accumulation of HSV-1 proteins was analyzed by Western blot using an anti-HSV-1–specific polyclonal antibody, providing further evidence of the viral nature of the proteins detected by PAGE analysis (Figure 2C).

Morphological and molecular changes in HSV-1–infected KG-1C cells

Modulation of apoptosis by HSV-1 infection has been extensively described (Derfuss and Meinl, 2002; DeBiasi *et al*, 2002; Perkins *et al*, 2003; Goodkin *et al*, 2004; Irie *et al*, 2004). Although HSV-1 possesses a battery of viral proteins involved in apoptosis inhibition (Derfuss and Meinl, 2002; Goodkin *et al*, 2004; Bloom, 2004), induction of this programmed cell death after CNS infection has also been

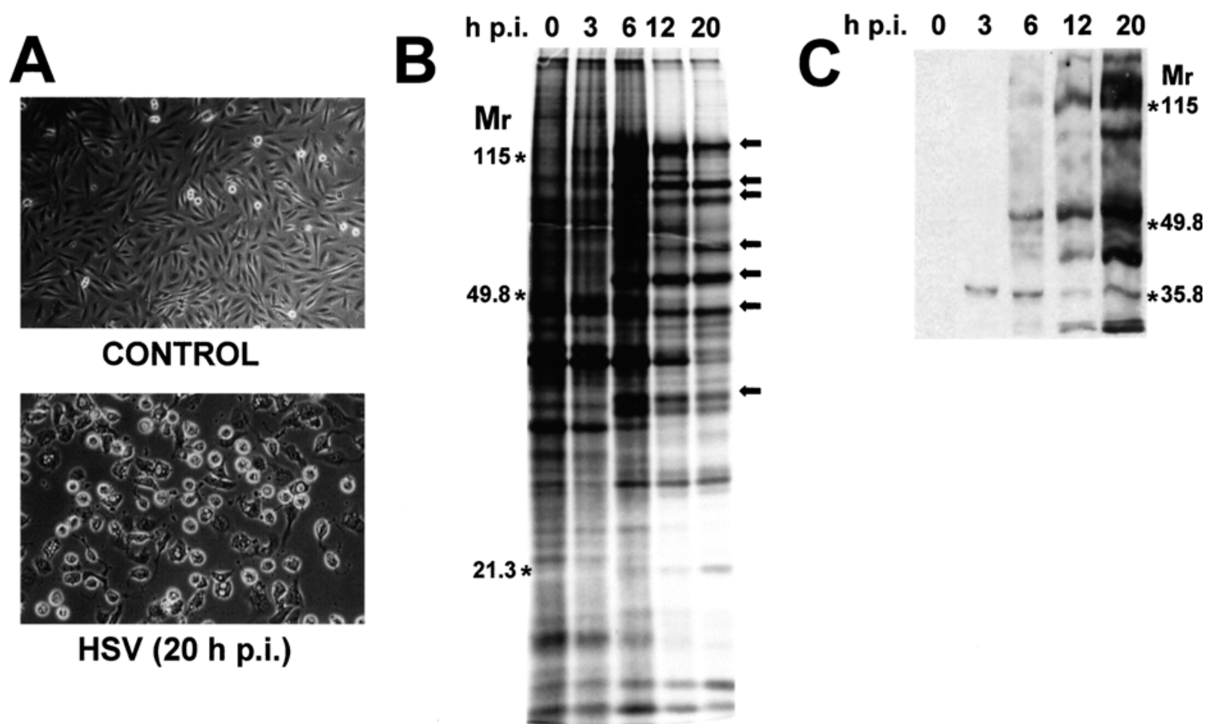

Figure 2 Productive infection of KG-1C cell line by HSV-1. Cultures were mock-infected or infected with HSV-1 at 10 PFU per cell. (**A**) Cytopathology of infected cells was visualized by phase-contrast microscopy. (**B**) SDS-PAGE analysis of ^{35}S proteins from HSV-1–infected and noninfected cells was carried out at the indicated times postinfection. (**C**) sixty microgram of total cell extracts were analyzed at the indicated times postinfection by Western blot using a polyclonal anti-HSV-1 antibody.

observed (DeBiasi *et al*, 2002; Perkins *et al*, 2003). In the current study, we investigated whether destruction of the oligodendroglial KG-1C cell line by HSV-1 could be due to an apoptosis pathway. For this purpose, nuclear fluorescence staining and the activation of procaspase-3 by means of Western blot was analyzed in HSV-1–infected KG-1C cultures. In our system, no signs of programmed cell death could be detected (Figure 3A and B), suggesting that infection of these glial cells by HSV-1 probably correspond to a necrotizing mechanism. In addition, no chromatin condensation or nuclear budding was produced (Figure 4). Internucleosomal DNA fragmentation assays were also sterile to detect apoptosis induction (data not shown). However, examination of infected cells by electron microscopy produced unexpected results (Figure 4). At 12 h p.i., most viral particles were located outside the nucleus. KG-1C cells infected with HSV-1 featured large clusters of mitochondria located in the proximity of cytoplasmic viral particles. This effect was clearly observed from 12 h p.i. and remained unchanged 8 h later (Figure 4, 2 and 3). Further investigation should determine the function of these large clusters of respiring organelles during the final stretch of the infection's course.

Finally, a recent report has shown that some viral proteins can interact with detergent-insoluble lipid rafts in HSV-1–infected cells (Lee *et al*, 2003). The vhs viral protein appears to be associated with rafts enriched in a cytoplasmic fraction with HSV particles (Lee *et al*, 2003). Of the raft-associated proteins so far studied, MAL2, a novel member of the MAL family (De Marco *et al*, 2002; Wilson *et al*, 2001), has recently been identified as an essential component of the cellular machinery for transcytosis. This molecule is present in specialized polarized cells, but at present it has been detected only in hepatocytes or epithelial cultures. As in the case of oligodendroglial cells, KG-1C was described as bipolar cells (Miyake, 1979). Therefore, we wondered whether MAL2 is expressed in this cell line and if HSV-1 infection could affect such expression. To this end, we used the species-specificity of the monoclonal 9D1 antibody to the human MAL2 protein. Fluorescence microscopy revealed basal expression of MAL2 in KG-1C cells (Figure 5). However, HSV-1 infection induced a significant increase of MAL2 accumulation. Double-label immunofluorescence of MAL2 and HSV-1 proteins (Figure 5) and preliminary confocal microscopy analysis (data not shown) suggested the possibility of colocalization of both components. The precise cellular distribution of this new member of the MAL family is currently being investigated. Finally, two controls of antibody specificity were used, allowing this result to be correctly interpreted (Figure 5, two bottom panels).

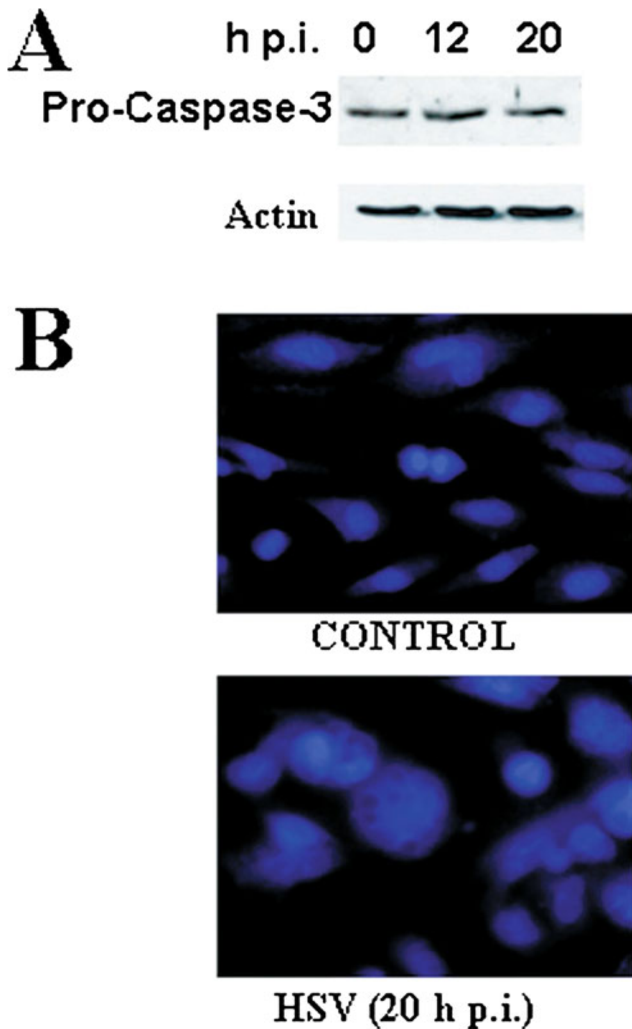

Figure 3 Effect of HSV-1 infection on apoptosis induction in KG-1C cells. Cell cultures were infected for the times indicated in Figure 2. (**A**) Aliquots of 60 μg of protein were analyzed by Western blot with a polyclonal anti-caspase-3 antibody. The band corresponds to full-length (32 kDa) proenzyme. A control with polyclonal anti-actin antibodies is shown. (**B**) Nuclear fluorescence analysis was carried out by staining with DAPI solution and fluorescence microscopy observation.

Discussion

Herein we have determined the level of susceptibility of the human precursor oligodendroglial KG-1C cell line (Uezono *et al*, 1998; Tatewaki *et al*, 1997; Miyake, 1979) to HSV-1 infection. HSV-1 is a neurotropic virus able to induce acute and/or latent infection in peripheral and central nervous systems (Baringer and Pisani, 1994; Schmutzhard, 2001). Nevertheless, although HSV-1 can infect neurons, oligodendrocytes, astrocytes, and microglial cells (Tsunoda and Fujinami, 2002; Kastrukoff and Kim, 2002; Fazakerley and Walker, 2003), and persists in the CNS indefinitely, its role in some chronic degenerative diseases, such as MS, remains a matter for speculation (Ferrante *et al*, 2000). In this sense, herpesvirus 6, another member of the *Herpesviridae* family, has been strongly associated with MS (Challoner *et al*, 1995; reviewed by Berti *et al*, 2000). Although it seems not to be absolutely necessary (Tsunoda and Fujinami, 2002), correlation between neurotropic virus infection and demyelinating diseases could be associated with the viral capacity to infect oligodendrocytes.

In our case, the great susceptibility of KG-1C to HSV-1 and their viral productivity were comparable to that observed in the classically studied Vero cell line (Marconi *et al*, 1996). To date, few publications have reported the study of human oligodendrocyte cell-line infection. Nevertheless, primary cultures of these cells were established from six different donors (Kastrukoff and Kim, 2002). Cultures of oligodendrocytes from donors differed in their susceptibility to HSV-1. Furthermore, it seems that resistance to HSV-1 of human oligodendrocytes could be genetically determined, which may be crucial to the development of CNS infection. Similar results, obtained by the same group, were previously reported with different strains of mice (Thomas *et al*, 1991, 1997). Differences in susceptibility, independent of the immune surveillance, may contribute to virus spread through the CNS and their possibility of demyelinating disease induction. Taking this into account, the molecular characterization of HSV-1 infection of oligodendrocytes or oligodendrocyte-like cells is clearly important.

Having observed the drastic effect on KG-1C cells provoked by HSV-1 infection, we wondered whether activation of an apoptotic mechanism could be involved. Numerous studies have confirmed the capacity of HSV infection to modulate apoptosis with both pro- and antiapoptotic effects (Derfuss and Meinl, 2002; Goodkin *et al*, 2004). Focusing on the CNS, HSV-1 infection has classically been described as a necrotizing process, but significant advances in the past decade have changed this perspective (DeBiasi *et al*, 2002; Perkins *et al*, 2003; Hunsperger and Wilcox, 2003). Apoptotic neurons and glia were detected in significant numbers in acute HSV-1–induced encephalitis (DeBiasi et al, 2002). In fact, a more recent report has associated the apoptotic component involved in viral encephalitis with activation of c-Jun and c-Jun N-terminal kinase (Perkins *et al*, 2003). Furthermore, activation of the proapoptotic proteolytic enzyme, caspase-3, seems to be crucial in both latent HSV-1 reactivation (Hunsperger and Wilcox, 2003) and encephalitic processes (Perkins *et al*, 2003). However, data presented here suggest that, at least in our system, HSV-1–induced CPE is not due to an apoptotic pathway. These results again suggest that the mechanism underlying HSV-1 infection of CNS-derived cells may be complex and differ from one cell type to another.

An important hallmark of apoptosis induction is the condensation of the nuclear chromatin. Consistent with the results presented above, no signal of chromatin condensation could be detected in

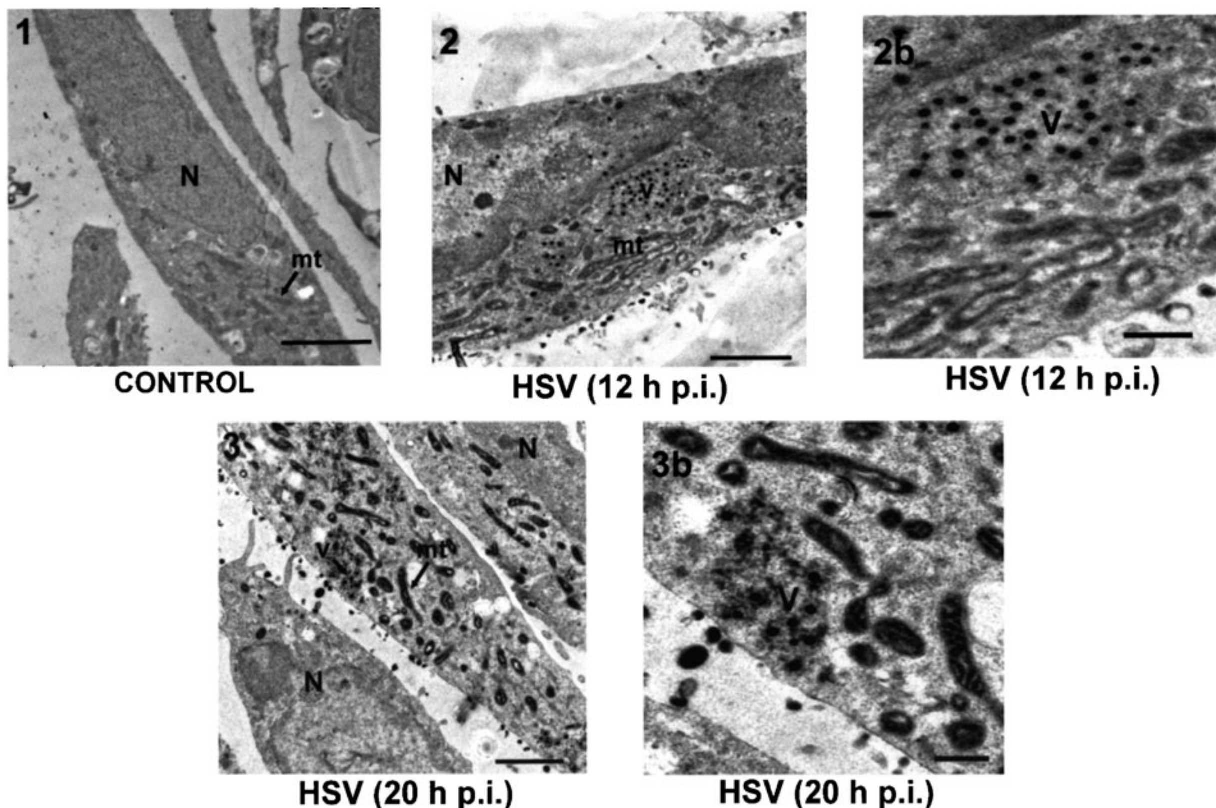

Figure 4 Effect of HSV-1 infection on mitochondrial cluster formation. KG-1C cells were mock-infected or infected for 12 or 20 h with HSV-1 at 10 PFU per cell. After infection, cells were collected by low-speed centrifugation and processed for electron microscopic analysis as described in Materials and Methods. N, nucleus; mt, mitochondria; v, viral particles. (**1**, **2**, and **3**) Bar, 2 μm; (**2b** and **3b**) bar, 0.5 μm.

HSV-1–infected KG-1C cells. Nevertheless, microscopic observation of these cells 12 and 20 h after viral infection (Figure 4) interestingly reveals the appearance of large clusters of mitochondria in the proximity of HSV-1 particles. Most of the mitochondria that surrounded the viral particles had a condensed ultrastructure, with a marked condensation of the cristae, which is a characteristic of the actively respiring mitochondrial state (Hackenbrock, 1966, 1968). Previous results performed in HSV-infected Vero cells showed mitochondrial migration to a perinuclear region in the cytoplasm (Murata *et al*, 2000), suggesting that these organelles respond to the stimulation of HSV infection at least until the middle stage of infection. Further experiments will be focus on the biogenetic or migrational aspect of this accumulation. Migration of actively respiring mitochondria to viral assembly sites has previously been reported in African swine fever virus (ASFV)-infected cells (Rojo *et al*, 1998). However, this complex animal-DNA virus multiplies almost entirely in the cytoplasm of the infected cells, and virus morphogenesis occurs in discrete cytoplasmic areas in the proximity of the nucleus known as viral factories (Breese and DeBoer, 1966). Therefore, it is reasonable to conclude that mitochondria supply the energy that ASFV morphogenetic processes require. In the case of HSV-1 infection, virion entry into, and intracellular transport within, mammalian cells has been widely studied (reviewed by Garner, 2003). HSV capsids are assembled and packaged with DNA in the cell nucleus. They then travel to, and accumulate within, organelles that have the biochemical properties of endosomes and the *trans*-Golgi network (Harley *et al*, 2001). Nevertheless, many aspects of virus morphogenesis and lipid envelope acquisition remain poorly understood and controversial. In this context, recent studies have indicated that some proteins of HSV-1 may be associated with detergent-insoluble lipid rafts. Moreover, this raft population was enriched in a cytoplasmic fraction containing assembling and mature HSV particles (Lee *et al*, 2003). Evidence that lipid rafts play a role in the assembly pathway of some viruses has been widely observed (Martin-Belmonte *et al*, 2000; Briggs *et al*, 2003), although the function of the raft machinery components is completely unknown at present. Thus, we have observed that MAL2, a novel raft protein of the MAL family (Rancaño *et al*, 1994; Wilson *et al*, 2001; De Marco *et al*, 2002), is detectable in the oligodendroglial KG-1C cell line. Although this result is interesting *per se*, because MAL2 has been characterized and is involved in transcytosis initially in hepatoma and epithelial polarized cells, confocal microscopy (data not shown), and

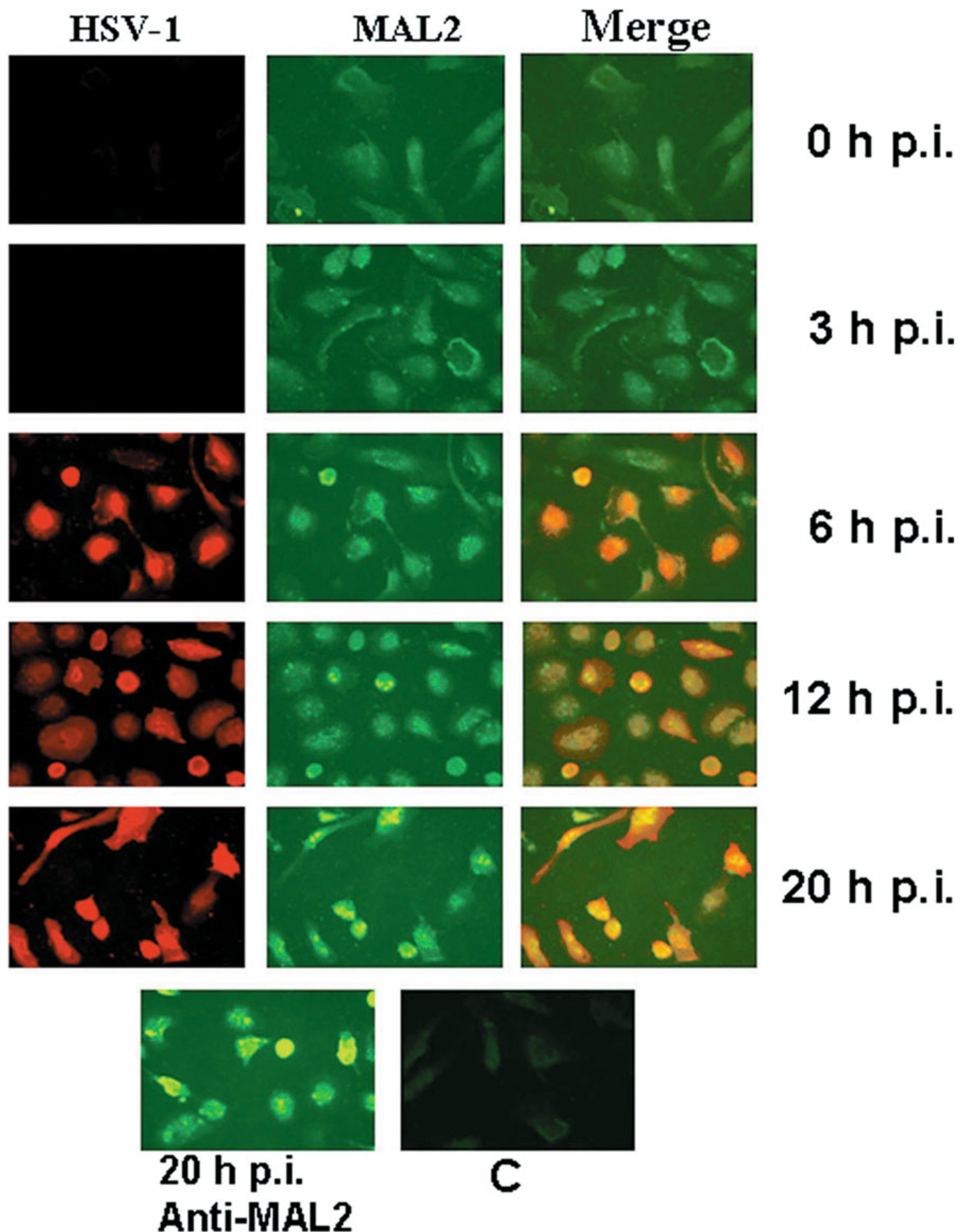

Figure 5 Distribution and increase of MAL2 in HSV-1-infected KG-1C cells. At the indicated times post infection, cultures were double-stained for HSV-1 and MAL2 and analyzed using a conventional fluorescence microscope. A control to assess labeling specificity included incubations with monoclonal anti-MAL2 antibodies alone (shown as anti-MAL2) or omission of the primary antibodies (C).

double-label fluorescence microscopy detected an increase of MAL2 in KG-1C cells after HSV-1 infection. MAL2 signal could not be ascribed to any particular area of the cell, although precise controls assured the specificity of the observations. Further analysis is needed to exclude the possibility that the increase of MAL2 signal is due to the unmasking of an antibody-recognized epitope during infection. Involvement of

mitochondria and MAL2 raft protein in the molecular mechanism of HSV assembling and trafficking through the infected cells requires further investigation, but our findings represent a first step in this direction.

In conclusion, we have characterized the HSV-1 infection of an oligodendroglial cell line. The high susceptibility of KG-1C to the infection and other results reported here open up new avenues for studying the involvement of HSV-1 on CNS degeneration and demyelinating disease induction.

Materials and methods

Cells and virus
The human oligodendroglial KG-1C cell line was cultured in Dalbecco's modified Eagle medium (DMEM) (Life Technologies, Paisley, United Kingdom) supplemented with 10% heat-inactivated fetal calf serum (FCS) in a 5% CO_2 atmosphere at 37°C. HSV-1 (F strain) was propagated in Vero cells. This cell line was used as a control of HSV-1 infection. Virus titration by plaque assay was performed on Vero cell monolayers with a final concentration of 0.8% low-melting-temperature agarose (Gibco BRL, Life Technologies). The m.o.i. was expressed as the number of PFU per cell. Exponentially growing cultures were used for all experiments. After infection, the establishment of cytopathic effects was observed microscopically. Survival of HSV-1-infected cells was determined by the trypan blue exclusion technique.

Protein labeling and PAGE analysis
In vivo labeling of newly synthesized proteins was carried out by giving 1-h pulses with 40 μCi of L-[^{35}S] Pro-mix (approximately 70% L-[^{35}S]methionine [>1000 Ci/mmol] and 30% L-[^{35}S]cysteine; Amersham Life Science, Little Chalfont, Buckinghamshire, United Kingdom) per milliliter in methionine- and cysteine-free medium at 37°C. After this, samples were washed extensively and subjected to sodium dodecyl sulfate (SDS)-PAGE analysis under reducing conditions, as described previously (López-Guerrero *et al*, 1989).

Western blotting
For immunoblot analysis, cultures (10^6 cells) were infected with HSV-1 (10 PFU per cell) and treated basically as described by López-Guerrero *et al* (2000). Briefly, cells were collected on several occasions post infection and suspended in 370 mM Tris-HCl pH 6.8; glycerol 17%, dithiothreito (DTT) 100 mM, SDS 1%, and bromophenol blue 0.024%. Samples were subjected to SDS-PAGE in 12% acrylamide gels under reducing conditions and transferred to nitrocellulose membranes (Hybond ECL, Amersham Europe). After blocking with 5% nonfat dry milk, 0.05% Tween-20 in phosphate-buffered saline (PBS), blots were incubated with rabbit anti-HSV-1 (Dako, Glostrup, Denmark) or anti-caspase-3 polyclonal antibodies (PharMingen, San Diego, CA, USA). As control, anti-actin polyclonal antibodies were used. After several washes, blots were incubated for 1 h with goat anti-rabbit immunoglobulin G (IgG) antibodies coupled to horseradish peroxidase, washed extensively, and developed using an enhanced chemiluminescence Western blot kit (ECL, Amersham).

Immunofluorescence analysis
Cells were cultured on slides and mock-infected or infected with HSV-1 (10 PFU per cell). At the indicated times after infection, cells were fixed by adding 4% paraformaldehyde (made fresh) for 20 min at room temperature and washed with PBS. Subsequently, cells were incubated for 5 min with 10 mM glycine, rinsed twice with PBS, permeabilized with 0.2% Triton X-100 in PBS, and incubated with 3% bovine serum albumin (BSA) in PBS for 15 min. Cultures were double-stained with a 1:100 dilution of anti-HSV-1 polyclonal (Dako) or anti-MAL2 monoclonal antibodies (the generous gift of Dr. M. A. Alonso, CBM, Madrid, Spain) for 1 h at 37°C. After incubation with the indicated primary antibodies, cells were rinsed several times with 1% BSA in PBS, and incubated for 1 h with the appropriate fluorescent secondary antibodies (Pierce, Illinois, USA). Controls to assess labeling specificity included incubations with control primary antibodies or omission of the primary antibodies. Images were obtained using a conventional fluorescence microscope (Zeiss). Nuclei were stained by incubating cells with 100 ng/ml DAPI (4′,6-diamidino-2-phenylindole; Sigma) in PBS for 30 min following standards protocols, in parallel with immunofluorescence analysis.

Electron microscopy
Cells were processed by freeze substitution. Cultures were mock-infected or infected with HSV-1 (10 PFU per cell). At 20 h p.i., cells were fixed for 60 min with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). Cells were then washed and embedded in 10% gelatine in PBS. Subsequently, 0.5- to 1-mm^3 cubes of gelatine-embedded cells were cryoprotected with 2 M sucrose at 4°C overnight, plunge-frozen in liquid propane, and immediately transferred to an Automatic Freeze-Substitution System (AFS, Leica). Freeze substitution was carried out at −85°C in methanol containing 0.5% uranyl acetate for 50 h. After raising the temperature to −35°C at a rate of 5°C/h and washing several times with pure methanol, samples were infiltrated with Lowicryl k4M and polymerized by ultraviolet (UV) light irradiation at −35°C for 2 days. Ultrathin Lowicryl sections were cut in a Reichert-Jung Ultracut E ultramicrotome. Samples were examined at 80 kV under a JEM 1010 electron microscope.

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