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Infection of human oligodendroglioma cells by a recombinant measles virus expressing enhanced green fluorescent protein

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One of the hallmarks of the human CNS disease subacute sclerosing panencephalitis (SSPE) is a high level of measles virus (MV) infection of oligodendrocytes. It is therefore surprising that there is only one previous report of MV infection of rat oligodendrocytes in culture and no reports of human oligodendrocyte infection in culture. In an attempt to develop a model system to study MV infection of oligodendrocytes, time-lapse confocal microscopy, immunocytochemistry, and electron microscopy (EM) were used to study infection of the human oligodendroglioma cell line, MO3.13. A rat oligodendrocyte cell line, OLN-93, was also studied as a control. MO3.13 cells were shown to be highly susceptible to MV infection and virus budding was observed from the surface of infected MO3.13 cells by EM. Analysis of the infection in real time and by immunocytochemistry revealed that virus spread occurred by cell-to-cell fusion and was also facilitated by virus transport in cell processes. MO3.13 cells were shown to express CD46, a MV receptor, but were negative for the recently discovered MV receptor, signaling leucocyte activation molecule (SLAM). Immunohistochemical studies on SSPE tissue sections demonstrated that CD46 was also expressed on populations of human oligodendrocytes. SLAM expression was not detected on oligodendrocytes. These studies, which are the first to show MV infection of human oligodendrocytes in culture, show that the cells are highly susceptible to MV infection and this model cell line has been used to further our understanding of MV spread in the CNS. *Journal of NeuroVirology* (2002) **8,** 24–34.

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Introduction

Measles virus (MV) has been identified as the etiological agent of the human CNS infections subacute sclerosing panencephalitis [SSPE] (ter Meulen *et al*, 1983) and measles inclusion body encephalitis [MIBE] (Agamanolis *et al*, 1979). Both diseases are characterized by infection of neurons and glial cells after incubation periods ranging from months, MIBE, to years, SSPE, following the primary infection. As yet, the site of viral persistence within the body remains unknown (Rima *et al*, 1995). Restrictions of viral gene expression have been demonstrated in brain tissues from patients with both diseases affecting the genes encoding the matrix, fusion, and haemagglutinin proteins (Baczko *et al*, 1986; Cattaneo *et al*, 1988; Billeter and Cattaneo, 1991;

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ter Meulen, 1997). Similar observations have been made in a rodent model of subacute measles encephalitis suggesting that transcriptional down-regulation of MV occurs in the early stages of infection (Schneider-Schaulies *et al*, 1989).

Neuropathological and immunopathological studies on autopsy tissue isolated from individuals with SSPE have established that gray matter neurons and their processes are infected at high levels and that many oligodendrocytes are infected in the white matter (Budka *et al*, 1982; Allen *et al*, 1996). Viral antigen is detected within both the nuclei and cytoplasm of oligodendrocytes in SSPE brain tissue (Allen *et al*, 1996). Although the infection is widespread in the hemispheric white matter, relatively low numbers of infected oligodendrocytes are observed in the brain stem and spinal cord. Astrocytes are also infected throughout the CNS but to a much lesser degree. The virus is distributed throughout the CNS, from the temporal and frontal cortices, to the medulla, pons, and cervical spinal cord regions. In some cases low numbers of antigen positive neurons have been observed in the cerebellum (Allen *et al*, 1996). Such studies have suggested transynaptic spread of the virus in a cephalo-caudal direction. Observations using the differentiated human neuron cell line, NT2, have given support to the hypothesis of transynaptic spread (Lawrence *et al*, 2000) but as yet the mechanism has not been elucidated. It is also unclear how the neurons or oligodendrocytes initially become infected or how the infection is propagated through the white matter.

Some recent studies on cultured astrocytes and neurons have shown the importance of extended cell processes in cell-to-cell spread of virus. Cultured human astrocytoma cells have intimately associated extended cell processes that are utilized by MV in the infection of surrounding cells (Duprex *et al*, 1999). Studies have also shown that cultured human neurons can become infected via the extended processes that are in contact with more readily infected neuroepithelial cells in mixed cell populations (McQuaid *et al*, 1998). Such a mechanism may be due to the presence of virus proteins in the neuroepithelial cell plasma membrane where it contacts the neurons. Furthermore, it has also been shown that viral spread in cultured neurons occurs in the absence of syncytium formation and with minimal extracellular virus production (Lawrence *et al*, 2000).

Nearly all of the published work on the susceptibility of CNS cells to MV infection have utilized astrocytic and, to a lesser extent, neuronal cell lines (Miller and Carrigan, 1982; Schneider-Schaulies *et al*, 1990, 1993; McQuaid *et al*, 1998; Duprex *et al*, 1999, 2000; Lawrence *et al*, 2000). Studies on glial cell lines have indicated that, in contrast to nonneural cells, MV transcription can be down-regulated by intrinsic host cell factors, whereas the differentiation state of the cells influences the translation of virus proteins (Schneider-Schaulies *et al*, 1993). Despite these investigations there are very few studies that have utilized either primary cells or oligodendrocyte cell lines to study MV infection in this important cell type. In one previous report, Atkins *et al* (1991) reported that a nonrodent-adapted strain of Edmonston strain of MV multiplied and produced a cytopathic effect in primary cultures of rat oligodendrocytes (Atkins *et al*, 1991). Viral infection in that study was only monitored by cytopathic effects and no viral immunocytochemistry or ultrastructural investigations were undertaken.

In this study, we aimed to establish a model of MV infection of oligodendrocytes and to utilize this model to determine if the virus infection was propagated via cell processes. MO3.13 is an immortal human-human hybrid derived by lectin-enhanced, polyethylene glycol-mediated somatic cell fusion between the thioguanine-resistant rhabdomyosarcoma mutant RD-TG.6 and primary human oligodendrocytes obtained from cultures of adult temporal lobectomies (Talbot *et al*, 1993; Ursell *et al*, 1995). MO3.13 cells are characterized as being positive by immunocytochemistry and Western blotting for the oligodendrocyte specific markers myelin basic protein (MBP) and proteolipid protein (PLP) (McLaurin *et al*, 1995). The OLN-93 cell line was established from primary cultures of glial cells prepared from the brains of 1-day-old Wistar rats (Richter-Landsberg and Heinrich, 1996). In some recently published studies (Duprex *et al*, 1999, 2000), we have come to appreciate the usefulness of a recombinant MV, which expresses enhanced green fluorescence protein (EGFP). This virus was therefore chosen to examine oligodendrocyte cell infection and virus spread from cell-to-cell. The MO3.13 cell line was also analyzed for expression of a receptor used by vaccine strains of MV, CD46 (Naniche *et al*, 1993) and for another recently described MV receptor SLAM (Tatsuo *et al*, 2000). In parallel investigations snap-frozen blocks of white matter from an autopsy case of SSPE were examined for the expression of CD46 and SLAM by immunocytochemistry.

Results

Characterization and expression of MV receptors on MO3.13 cells

At the split ratios used, MO3.13 and OLN-93 cells were highly proliferative and attained confluency within 3 days. Both cell lines expressed markers indicative of oligodendrocyte lineage (Figure 1A, B). Unfixed MO3.13 cells were examined for the presence of CD46 by indirect immunofluoresence. Expression of CD46 was present in localized patches on the cell surface of all cells (Figure 1C). MO3.13 cells were negative for SLAM expression (Figure 1D). As expected, the B-cell line B95a showed SLAM expression on all cells (Figure 1D1).

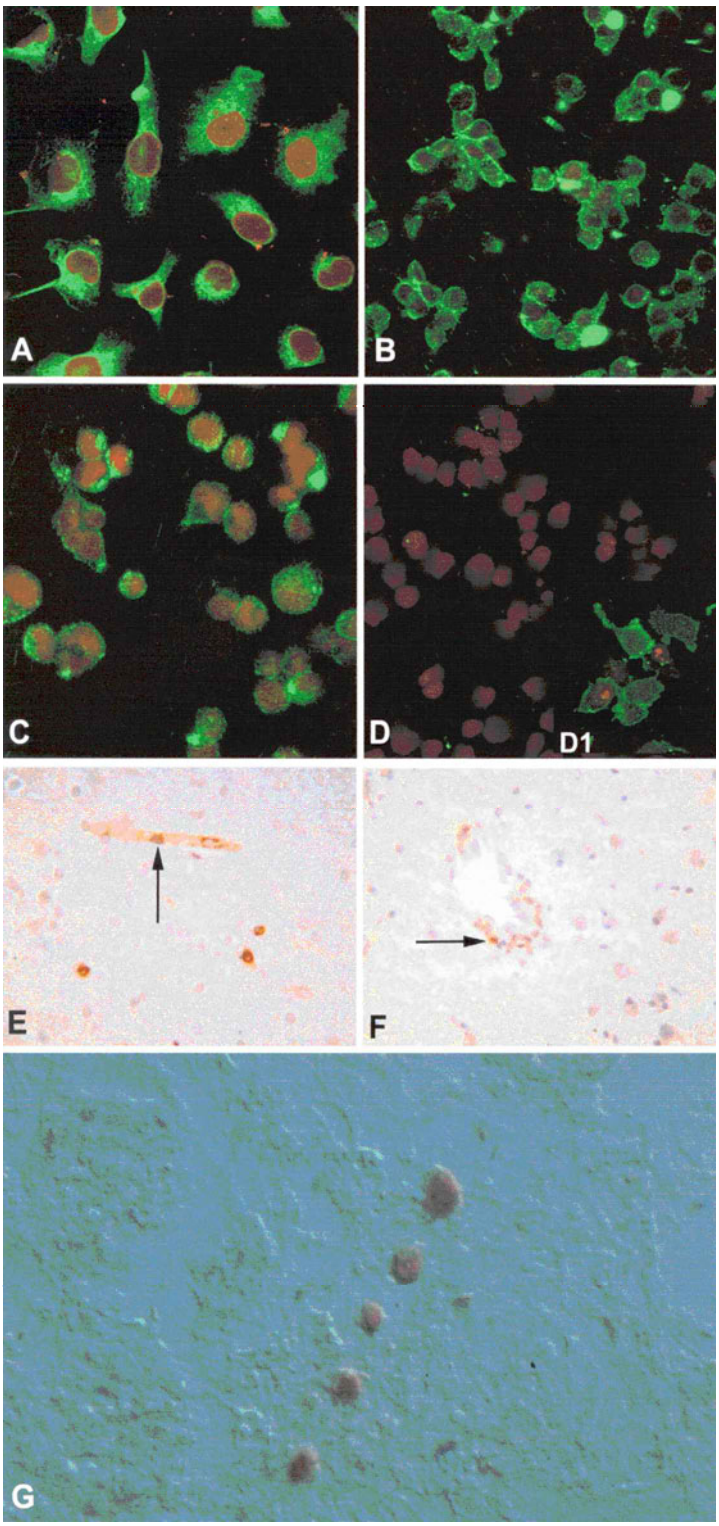

Figure 1 Immunoreactivity in MO3.13 cells, OLN-93 cells, and oligodendrocytes in SSPE. The fluorescent images are all composite confocal sections through 8–10 μm and cell nuclei are counterstained with propidium iodide. (**A**) Expression of oligodendrocyte-specific protein galactocerebroside on the surface of uninfected MO3.13 cells. (**B**) Oligodendrocyte-specific proteolipid protein expression on the surface of uninfected OLN-93 cells. (**C**) CD46 expression at the cell surface of MO3.13 cells. (**D**) Lack of expression of the MV receptor SLAM on MO3.13 cells. The insert shows typical SLAM expression on the surface of B95 cells. (**E**) CD46 is present on cerebral endothelium (arrow) and cells with the morphological characteristics of oligodendrocytes in the white matter in SSPE tissue sections. (**F**) SLAM expression on cells within the perivascular cuff in SSPE. Note the absence of expression in the surrounding parenchyma. (**G**) Detection of MV antigen in a chain of interfasicular oligodendrocytes in the white matter in SSPE. The photomicrograph was taken with differential interference contrast microscopy on a Leica Aristoplan microscope. (Magnification: A–D, X400; E–G, X250.)

Immunohistochemical staining of MV receptors in SSPE tissue

To extend these *in vitro* observations SSPE tissue sections were immunocytochemically stained for CD46 and SLAM. CD46 was expressed on all cerebral endothelium and on subpopulations of cells in the white matter with the morphological characteristics of oligodendrocytes (Figure 1E). Small numbers of neurons in the gray matter were also observed to express CD46. SLAM was absent from cells in the parenchyma of the brain but was expressed on cells within the perivascular infiltrates surrounding blood vessels (Figure 1F, arrow). SSPE tissue sections were immunohistochemically stained with MAb to measles virus nucleocapsid (N). Based on morphological criteria neurons in the gray matter and oligodendrocytes in the white matter were the cells types predominantly infected with MV from the nine cases of SSPE studied (Allen *et al*, 1996). In very rare instances chains of MV-infected interfasicular oligodendrocytes were observed in the white matter (Figure 1G). No viral antigen was detectable in processes between such groups of cells. The areas surrounding such chains of cells also contained scattered MV-positive oligodendrocytes (data not shown).

Infection of oligodendrocytes with MVeGFP

To establish an *in vivo* model of MV infection of oligodendrocytes, MO3.13 cells were infected with MVeGFP. The cells were readily infectable and up to 90% of cells became infected 48 h postinfection (h.p.i.). EGFP autofluoresence was observed in the cell bodies and fine processes of the infected cells. Infectious virus was recovered from both the supernatant and cell-associated components. MVeGFP was cultured by five passages on MO3.13 cells at an MOI of 0.01. Throughout this process, infected cells were evident by GFP fluoresence 24 h.p.i. and more than 90% of the cells were infected by 72 to 96 h.p.i. Titres of MVeGFP(verop1) and MVeGFP(MO3.13p5) were obtained in triplicate by TCID50, assay on Vero and MO3.13 cells. During this time, viral titres did not change (Table 1), indicating that the virus did not significantly adapt to MO3.13 cells. By comparison OLN-93 cells growing on 25-cm^3 flasks or glass coverslips could only be infected with MVeGFP at a very low level. EGFP autofluoresence was observed in small clusters of infected OLN-93 cells (approximately 35) at 112 h.p.i. Without the use of MVeGFP, virus-positive cells would have been very difficult to locate by phase microscopy as there was no obvious cytopathic effects. No further infection was observed from these infected cells when supernatant or cell-associated components were passaged onto OLN-93 or Vero cells. This indicated that these cells have no usefulness for studies of cell-to-cell spread of vaccine strains of MV.

Table 1 Titres of MVeGFP (verop1) and MVeGFP (MO3.13p5) viruses obtained following their growth on MO3.13 and Vero cells

Cell line	*MVeGFPverop1 titre (50% TCID$_{50}$/ml)*	*MVeGFP(MO3.13p5) titre (50% TCID$_{50}$/ml)*
MO3.13	2.45×10^4	1.2×10^4
Vero	3.16×10^4	2.14×10^4

Titres were measured as 50% tissue culture infectious doses/ml and are the averages obtained from a triplicate of each experiment.

Localization of viral antigen within infected MO3.13 cells

All experiments were carried out using MO3.13 cells infected with MVeGFP (MO3.13p5) virus at an MOI of 0.01. Immunocytochemistry with an anti-measles virus N MAb on cells at 48 h.p.i. showed the presence of cytoplasmic antigen in the presence of MVeGFP autofluoresence (Figure 2A). Infected cells expressed EGFP in the presence of MV nucleocapsid, detected with a MAb and visualized with Alexa 568 goat anti-mouse. A clear localization of EGFP to the nucleus of infected cells was observed as previously noted in other cell types. Such accumulation of EGFP in the nucleus only takes place in infected cells and at present is assumed to occur by a nonspecific mechanism (Duprex *et al,* 1999). The punctate nature of the nucleocapsid staining is typical of MV-induced intracytoplasmic inclusion bodies. It is also noteworthy that EGFP autofluoresence can be detected in cells that are negative for viral antigen (Figure 2A, arrow b) confirming previous observations and indicating that utilization of the recombinant virus is a very sensitive means to detect virus-infected cells.

On acetone-fixed cells at 48 h.p.i. MV antigens were detected using an SSPE serum, which predominantly detects N and phosphoprotein proteins, visualized by a FITC-conjugated rabbit anti-human secondary antibody. The intermediate filament, vimentin, was used as a counterstain to show both uninfected and infected cells. Vimentin was detected using a MAb visualized by an Alexa 568-conjugated goat anti-mouse secondary antibody. Once again large accumulations of nucleocapsid were seen in the cytoplasm of infected cells (Figure 2B inset). Antigen was also detected in the connecting processes between cells (Figure 2B, arrows) and in the fine, branching processes of singly infected cells (Figure 2B, asterisk).

Ultrastructural analysis of MV-infected MO3.13 cells

Monolayers of MO3.13 cells growing on 25-cm^3 flasks, infected at an MOI of 0.01, were fixed and processed for ultrastructural analysis. A high percentage of the cells viewed by EM were infected as evidenced by an abundance of spherical nucleocapsid inclusion bodies dispersed or aggregated throughout the cytoplasm (Figure 2C, dashed circle). This is consistent with the immunocytochemical data (Figure 2B, inset). Figure 2D is representative of nucleocapsids in cross-section observed to underlie

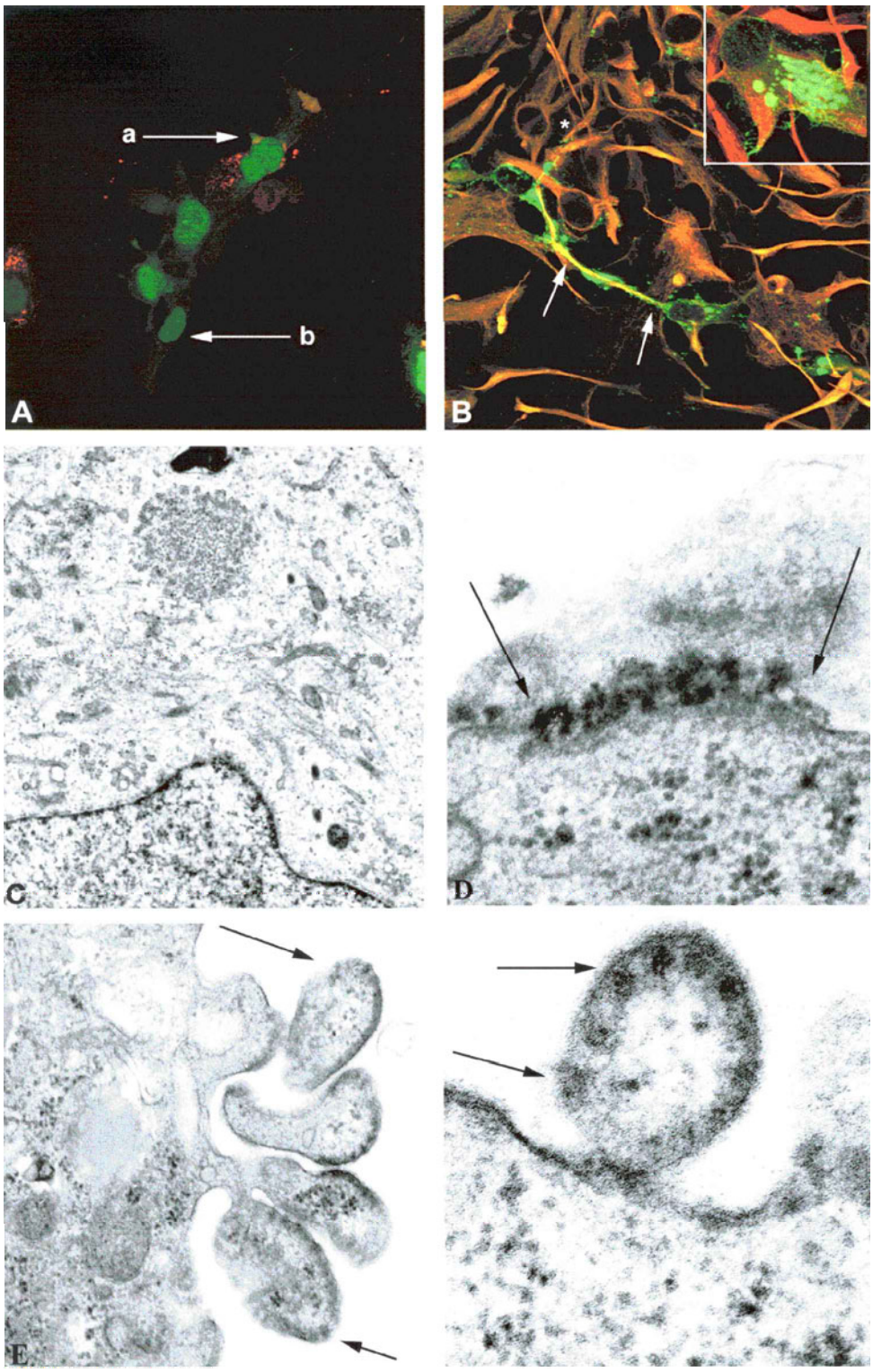

Figure 2 Immunocytochemical and ultrastructural analysis of MVeGFP-infected MO3.13 cells. (**A**) MV nucleocapsid (red) and GFP (green) expression in MO3.13 cells. Arrow **a** indicates expression of GFP- and MV-antigen in the same cell. Arrow **b** indicates a MVeGFP-infected cell that is negative for nucleocapsid. (**B**) Nucleocapsid and phosphoprotein antigens, detected with SSPE antiserum (green) and the intermediate filament vimentin (orange) in MO3.13 cells. Large accumulations of nucleocapsids are present in the cytoplasm of infected cells (insert **B1**) and in the connecting processes between cells (arrows). (**C**) Spherical mass of viral inclusions in the cytoplasm (magnification × 20250, encircled). (**D–F**) Various stages of the budding of MV virions from the surface of MO3.13 cells. (**D**) Protein thickening indicative of the budding process (magnification × 48500, arrows). (**E**) MV budding (arrows) at the cell surface (magnification × 24000). (**F**) Mature MV virion displaying nucleocapsid (arrows) dispersed within a thickened envelope membrane (magnification × 71000). The EM images were produced from EM negatives scanned into ADOBE Photoshop.

cell membranes displaying modification or thickening (arrows) that are typical of the MV budding structures that form and release mature MV virions. The membranes of MO3.13 cells were examined at greater magnifications to identify areas of membrane thickening or modification and evidence of MV budding. Different stages of virus budding were observed along the membranes. In Figure 2E, the photomicrograph demonstrates a typical infected MO3.13 cell with five mature MV virions (arrows) at the cell surface. The formation of a virus bud begins with thickening of cell membranes, which project outwards from the cell until it pinches off to form a mature MV virion with characteristic surface projections (Figure 2F, arrows).

Cell-to-cell spread of MV in oligodendrocytes

Having established the MO3.13 cell line as a suitable model, we wished to examine the spread of MV in oligodendrocytes. MO3.13 cells were infected at a MOI of 0.01 with MVeGFP and infected areas observed regularly by UV microscopy from 24 h.p.i. Two representative time courses of MVeGFP-infected cells that illustrate both the spread of virus along cell processes and the fusion of virus-infected cell bodies are shown in Figures 3A and B. In Figure 3A, virus spread is mediated via interconnecting cell processes. At 24.75 h.p.i., EGFP is present in a cell process (arrow) of an infected cell. Within 15 min, GFP is present in the cell body of a neighbouring cell. By 26.5 h.p.i., the infection has progressed to adjacent cells. Figure 3B illustrates an example of fusion-mediated spread of MVeGFP. Three neighbouring small syncytia (27 h.p.i., arrows) fuse together over a 3.5-h time period, forming a larger syncytium. By 96 h.p.i., process- and fusion-mediated spread of virus led to infection of the complete cell monolayer (Figure 3A, insert). Cytopathic effect due to syncytia formation was observed throughout the monolayer.

Discussion

In the present study, we have demonstrated that MV is capable of infecting the human oligodendroglioma cell line MO3. 13. This is the first report of MV infection of a human oligodendrocyte cell line, an important cell type known to be infected in SSPE. The cells are readily infectible by a recombinant MV and infectious virus was produced. By comparison, the rodent oligodendrocyte cell line, OLN-93 displayed a low susceptibility to MVeGFP infection and infectious virus was not released. MO3.13 cells expressed high levels of cell surface CD46 but were negative for the recently described MV receptor SLAM. This is consistent with the observation in SSPE tissue sections where CD46 but not SLAM expression was detected on oligodendrocytes in the white matter. However, the numbers of CD46-positive oligodendrocytes detected by immunohistochemistry was only a small percentage of the total number of oligodendrocytes in the white matter of the brain areas examined. A more detailed description of CD46 and SLAM expression in the normal and MV-infected CNS and peripheral tissues is currently in preparation (McQuaid *et al*, unpublished results). It has been shown that not all cells that are susceptible to MV infection express detectable levels of CD46 (Yanagi *et al*, 1994; Dunster *et al*, 1995; Horvat *et al*, 1996), and SLAM is only constitutively expressed on immature thymocytes, $CD45RO^{high}$ memory T cells, and a proportion of B cells (Sidorenko and Clarke, 1993; Cocks *et al*, 1995). The mechanism of MV entry into these cells remains unclear and would seem to indicate that other MV receptors may exist on mammalian cells.

In a previous study, we made use of a human astrocytoma cell line to observe virus spread using a recombinant EGFP-expressing MV virus (Duprex *et al*, 1999). Individual infected cells, identified by EGFP autofluoresence, were monitored by CSLM and the virus spread was shown to be cell process-mediated with a rapid progression of GFP from cell-to-cell. Utilizing this technique, MO3.13-propagated MVeGFP virus was observed to spread in MO3.13 cell monolayers. Virus propagation through the monolayer was observed to be predominantly a cell process-mediated event. The actual mechanism of virus spread from an infected cell process to an adjacent uninfected cell process has not been established in these studies. It is possible that microfusion of cell processes occur with the accompanying passage of viral ribonucleoprotein into an uninfected cell process. However, infected cells were also observed to spread infection by cell body-to-cell body fusion. Adjacent cells become infected and then fuse to form syncytia. These observations have previously been made in MVeGFP-infected murine neuroblastoma cells where virus spreads from cell-to-cell both by fusion and via cell processes (Duprex *et al*, 2000).

MV infection of alpha/beta interferon receptor-defective mice expressing human CD46 has been used to suggest that replication is much more efficient in the rodent CNS than the peripheral nervous system, with the virus propagating mostly in the easily accessible ependymal cells (Mrkic *et al*, 1998). Viral RNA or antigen was often detected in contiguous cells, suggesting that in the brain of transgenic mice MV propagation may be based largely on lateral cell-to-cell contacts. This demonstrated both *in vivo* and *in vitro*, MV spread in the CNS most likely involves localized fusion events at cell-to-cell contact points without the requirement for specific viral receptor(s) (McQuaid *et al*, 1998; Duprex *et al*, 1999; Lawrence *et al*, 2000). Similarly, in the present study we have shown that human oligodendroglioma cells in culture spread MV infection both by fusion and along interconnecting processes.

Previous ultrastructural investigations of MV infected astrocyte and neuronal cell lines have demonstrated viral nucleocapsids dispersed or in small

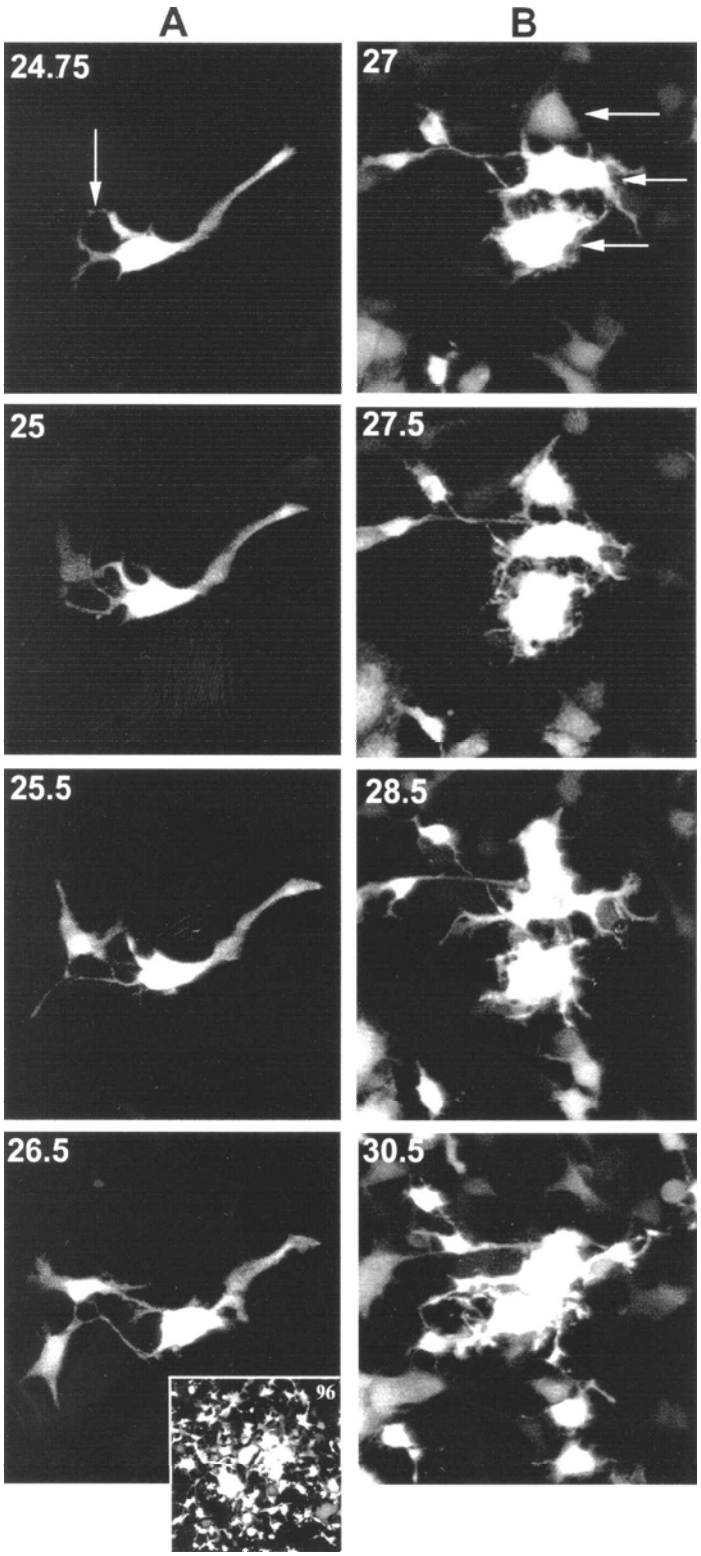

Figure 3 Cell-to-cell spread of MVeGFP in MO3.13 cells (see text for details). MO3.13 cells were infected with MVeGFP at an MOI of 0.01. Single or small numbers of infected cells were identified in the monolayer by UV microscopy and the time-lapse facility of the confocal system was used to acquire Z-series images every 20 min over a 48-h time period. Two representative time-course experiments are shown (**A** and **B**). Series A demonstrates process-mediated spread of virus from cell-to-cell. Series B demonstrates the fusion of three infected syncytia (arrows) over a 3.5-h time period. The insert at the bottom of series A demonstrates the extent of infection by 96 h postinfection. The images were collected in multiple optical sections through the entire thickness of the cells in single-excitation mode. The number of hours postinfection at which each autofluoresecent image set was collected is indicated. EGFP autofluoresence is shown in false-white color. (Magnification × 100.)

clumps throughout the cytoplasm (Macintyre and Armstrong 1976; McQuaid *et al*, 1998; Lawrence *et al*, 2000). EM observations of astroglial cultures displayed MV in various stages of assembly and extracellular virions were routinely observed. These mature virions have surface projections, thickened envelope membranes, and the nucleocapsid tubules are observed to be dispersed peripherally in a spiral form (Macintyre and Armstrong, 1976). Electron microscopy of undifferentiated and differentiated NT2 cells showed that viral budding occurred very rarely in undifferentiated cells and was not observed on the surface of differentiated NT2 cells (McQuaid *et al*, 1998; Lawrence *et al*, 2000). However, nucleocapsids were aligned at the cell membrane of differentiated NT2 cells, in neuronal processes and at presynaptic neuronal membranes. Ultrastructural analysis of MV-infected MO3.13 cells revealed an abundance of MV nucleocapsid within the cytoplasm, both in isolation and as aggregates. Typical plasma membrane modifications and various stages of budding (Fleury *et al*, 1980) were also observed consistent with the productive nature of MV infection from MO3.13 cells.

This pattern of MV nucleocapsid localization mirrored observations made with single- and dual-labeled immunofluoresence of MVeGFPp5-infected MO3.13 cells. MV nucleocapsid was observed throughout the cell cytoplasm with dense clumps of virus evident in the perinuclear regions. When dual-labeled for MV antigens and EGFP, it was evident that infected MO3.13 cells expressed GFP autofluoresence in the absence of detectable MV antigen. Such observations have led to the conclusion that EGFP expression provides an early indicator of MV infection *in vitro* (Duprex *et al*, 1999).

In the adult CNS, fully differentiated interfasicular oligodendrocytes occur in the white matter and are characterized by their many connections to segments (internodes) of myelin sheaths wrapped around axons. The processes, which link the oligodendrocyte cell body to the sheath, are narrow and tortuous (Knobler *et al*, 1974). The paranodal regions of oligodendrocyte can also contact other glial cells of the CNS such as astrocyte by gap junctions, indicating functional coupling (Berry *et al*, 1995). Gap junctions establish intracellular channels of communication through which ions and small solutes (<1300 D) can pass (Mugnaini, 1982). However, it is unlikely that viral nucleocapsid could pass from cell-to-cell via intact gap junctions. Occasionally interfasicular oligodendrocytes may be seen aligned in rows. Where this occurs, the cell membranes of adjacent cells are in intimate contact but lack specific junctional contacts. In very rare examples, in the white matter from cases of SSPE, chains of interfasicular oligodendrocytes were demonstrated to have detectable levels of MV. However, in such autopsy tissue, fixed for light microscopy, it is impossible to determine if virus is present in the very fine oligodendrocyte processes in the white matter. Oligodendrocytes also occur in the gray matter as perineuronal satellite cells. Individual satellite cells are thought to be in close contact with single neurons. In some pathological situations, such cells have been described to have processes extending to myelin sheaths (Ludwin, 1979). The extent of oligodendrocyte processes in the CNS and the findings, in the present study, of extensive infection of oligodendrocyte processes *in vitro* raises the possibility of oligodendrocyte-to-neuron infection in the CNS as a means of initiating spread of virus throughout the CNS. It is postulated that, once present in neurons, MV can spread transneuronally throughout the CNS (Allen *et al*, 1996; Lawrence *et al*, 2000), possibly by fast axonal transport (Oldstone *et al*, 1999).

Like a small number of other viruses, MV has been identified as potentially persisting in the CNS (Liebert, 1997). For example, Borna virus causes CNS disease in several species, and recent studies have suggested a potential role for these viruses in human mental health (Gonzalez-Dunia *et al*, 1997). The polyomavirus JC, which infects oligodendrocytes *in vivo*, is associated with most cases of progressive multifocal leukoencephalopathy, a demyelinating disease of the CNS leading to death within months of first presentation (Askamit, 1995; Eggers *et al*, 1999). However, the specific cell type or neuroanatomical location(s) of CNS viral persistence remains unknown. The finding that MO3.13 cells can sustain persistent coronavirus infections (Arbour *et al*, 1999a, 1999b) raises the possibility that other viruses, such as measles, may persist in oligodendrocytes *in vivo*.

A logical progression of these experiments will be to use primary oligodendrocyte cultures (McCarthy and De Vellis, 1980; Gates *et al*, 1985) to analyze virus spread. It would also be important to analyze CD46/SLAM expression on mature primary oligodendrocytes.

Materials and methods

Cell lines

Two cells lines of oligodendrocyte lineage, human MO3.13 (Talbot *et al*, 1993) and rat OLN-93 (Richter-Landsberg and Heinrich, 1996), were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 0.1% penicillin and streptomycin, and 4 mM glutamine in 25-cm^3 flasks at 37°C with 5% (MO3.13) or 10% (OLN-93) CO_2 added. OLN-93 cells have been shown to express the oligodendrocyte-specific markers, galactocerebroside, MBP, PLP, and myelin-associated glycoprotein (MAG) (Richter-Landsberg and Heinrich, 1996; Strelau and Unsicker, 1999). When confluent, cells were passaged at a 10:1 (MO3.13) or 20:1 (OLN-93) split ratio into 25-cm^3 flasks.

Virus infection of cells

MO3.13 and OLN-93 monolayers grown in 25-cm^3 flasks were infected with adapted MVeGFP virus that had previously been grown on Vero cells (Duprex *et al*, 1999). Cell monolayers (60% confluency) were rinsed with maintenance medium (DMEM supplemented with 2% FCS) and cells were infected with virus at a multiplicity of infection (MOI) of 0.01 and incubated for 1 h at 37°C. After this time unadsorbed virus was removed, maintenance medium was added, and cells were incubated at 37°C for varying time periods. Cell sheets were monitored by UV microscopy (Leica) daily for the appearance of MVeGFP positive cells. For adaptation of MVeGFP to oligodendrocyte cell lines, heavily infected monolayers (>90%) were freeze-thawed three times. Virus stocks were stored at −70°C. Virus titres were determined by $TCID_{50}$s (Reed and Muench, 1938), and the flasks were monitored by fluorescence microscopy to determine that MVeGFP expression was retained.

Indirect immunofluorescence

Indirect immunofluoresence was used to characterize the cells with commonly used oligodendrocyte markers and for the presence/absence of MV receptors. Cells were grown to confluence on glass coverslips, then fixed in ice-cold acetone. Primary antibodies were applied for 1 h at 37°C and the coverslips were washed twice for 5 min in PBS. Secondary antibodies, rabbit anti-mouse Alexa 488, or goat anti-rabbit Alexa 488 (Molecular Probes) were diluted in PBS (1:500) and incubated for 1 h at 37°C. Nuclei were then counterstained in propidium iodide (Sigma 2μg/ml) for 30 s, washed in PBS, and the coverslips mounted using Citiflour (Amersham). Primary antibodies used to characterize the cells were polyclonal antibodies to galactocerebroside (Chemicon, 1:20), PLP (Chemicon, 1:20), and MAb to vimentin (Dako 1:50).

A polyclonal anti-CD46 antibody (1:200, Gift from F. Wild, Institut Pasteur de Lyon, France) was used to examine the expression of the MV receptor CD46 on MO3.13 cells. The antibody was applied to unfixed cells overnight at 4°C. Cells were washed in PBS and fixed in ice-cold acetone. To examine expression levels of SLAM on MO3.13 cells, coverslips were fixed in 10% formalin and washed in PBS. MAb to SLAM (Kamiya Biomedical Company, 1:1000) was applied overnight at 4°C. For CD46 detection, cells were then incubated in Alexa 488 goat anti-rabbit (Molecular Probes 1:500) and for SLAM detection cells were incubated in Alexa 488 rabbit anti-mouse (Molecular Probes 1:500) for 1 h at 37°C. Following further washes, nuclei were counterstained with propidium iodide and the coverslips mounted in Citiflour. The B-cell line, B95a, was grown on glass coverslips and immunostained as described previously as a positive control for SLAM expression in cultured cells.

For virus detection, cells grown on glass coverslips in Petri dishes to 60% confluency were infected at an MOI of 0.01 as before. Infected cells were incubated for various time periods at 37°C. Cells were fixed in either ice-cold acetone or 4% paraformaldehyde for 10 min, depending on the antibody to be used. Anti-measles virus N MAb and hyperimmune SSPE serum were used as described previously (Duprex *et al*, 2000). Primary antibodies were incubated on the cells for 1 h at 37°C. Incubation was followed by two 5-min washes with PBS. The secondary antibodies used for the detection of single- or dual-labeling were Alexa 488 rabbit anti-mouse, Alexa 568 goat anti-mouse, and rabbit anti-human FITC (Dako 1:50). These were diluted in PBS and added to the coverslips and incubated for 1 h at 37°C. Coverslips dual-labeled with SSPE serum and vimentin were subsequently incubated in rabbit anti-human FITC and Alexa 568 goat anti-mouse. Coverslips were washed twice in PBS and mounted using Citiflour.

A Leica TCS/NT confocal scanning laser microscope (CSLM) equipped with a krypton/argon laser was used to examine the samples for fluoresence. Alexa 488-labelled samples or MVeGFP autofluoresence was visualized by excitation at 488 nm with a 506–538 band-pass emission filter. Alexa 568-labelled samples were imaged by excitation at 568 nm with a 564–596 band-pass emission filter.

Vital confocal fluorescence microscopy

Cells were grown to 60% confluence in 25-cm^3 tissue culture flasks. Cells were infected at an MOI of 0.01 with MVeGFP. As previously described, an inverted UV microscope was used to monitor the monolayers for the appearance of infected cells (Duprex *et al*, 1999). In initial experiments, flasks were oriented on the microscope stage and marked to permit the repeated observation of chosen groups of infected cells in the monolayers. Observations were made over a period of 24 h at hourly intervals. In additional experiments singly infected or infected groups of cells were selected and the time-lapse ability of the confocal system was used to acquire Z-series images every 20 min over 48 h (Duprex and Rima, 2001).

Ultrastructural analysis of cells

Infected MO3.13 cells were fixed and embedded for ultrastructural analysis. Monolayers were fixed in 2.5% glutaraldehyde for 90 min at 4°C, then rinsed in 0.2 M cacodylate buffer for 30 min at 4°C. Cells were then postfixed for 1 h at room temperature in 2% osmium tetroxide and rinsed in distilled water. Monolayers were dehydrated in graded alcohols and proplyene oxide was used to detach the cells from the flask. Cells were subsequently embedded in Agar 100 embedding resin as previously described (McCormack *et al*, 1983). Semithin sections were stained in toludine blue to identify areas with high numbers of cells and ultrathin sections (90 nm) were cut from these regions. Sections were lifted onto copper EM grids and stained with uranyl acetate and lead citrate. Sections were

examined on a Hitachi H-600 transmission electron microscope.

Immunohistochemical staining of SSPE tissue
Cryostat sections (12 μm) were cut from snap-frozen tissues from a SSPE case and fixed in 10% formalin. After blocking endogenous peroxidase in 0.5% H_2O_2 in methanol for 10 min, sections were incubated in polyclonal anti-CD46 or MAb to SLAM overnight at 4°C. Furthermore, selected blocks of predominantly white matter tissue from nine autopsy cases of SSPE were immunohistochemically stained for MV antigens as described previously (Allen *et al*, 1996). Bound receptor or viral antibodies were detected using diaminobenzidine or aminoethylcarbazole, as peroxidase substrate. Sections were counterstained with haematoxylin. Photomicroscopy was carried out on a Leitz Aristoplan fitted with differential interference contrast.

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