A recombinant measles virus (MV) expressing red fluorescent protein (MVDsRed1) was used to produce a persistently infected cell line (piNT2-MVDsRed1) from human neural precursor (NT2) cells. A similar cell line (piNT2-MVeGFP) was generated using a virus that expresses enhanced green fluorescent protein. Intracytoplasmic inclusions containing the viral nucleocapsid protein were evident in all cells and viral glycoproteins were present at the cell surface. Nevertheless, the cells did not release infectious virus nor did they fuse to generate syncytia. Uninfected NT2 cells express the MV receptor CD46 uniformly over their surface, whereas CD46 was present in cell surface aggregates in the piNT2 cells. There was no decrease in the overall amount of CD46 in piNT2 compared to NT2 cells. Cell-to-cell fusion was observed when piNT2 cells were overlaid onto confluent monolayers of MV receptor-positive cells, indicating that the viral glycoproteins were correctly folded and processed. Infectious virus was released from the underlying cells, indicating that persistence was not due to gross mutations in the virus genome. Persistently infected cells were superinfected with MV or canine distemper virus and cytopathic effects were not observed. However, mumps virus could readily infect the cells, indicating that superinfection immunity is not caused by general soluble antiviral factors. As MVeGFP and MVDsRed1 are antigenically indistinguishable but phenotypically distinct it was possible to use them to measure the degree of superinfection immunity in the absence of any cytopathic effect. Only small numbers of non-fusing green fluorescent piNT2-MVDsRed1 cells (1 : 300 000) were identified in which superinfecting MVeGFP entered, replicated and expressed its genes.

INTRODUCTION

The presence of measles virus (MV) in patients with subacute sclerosing panencephalitis (SSPE) provides a prime example of the long-term persistence of a human RNA virus. Individuals suffer a lethal condition in which the brain is heavily infected with MV containing a variety of mutations (Baczko et al., 1993). These variants are derived from the MV involved in the acute infection (Rima et al., 1997). The virus persists in an unknown site in the body for a period of two to 30 years (Modlin et al., 1977). In SSPE, the virus primarily infects neurones and oligodendrocytes in the central nervous system and months to years can pass between the onset of symptoms and death. Persistent MV infection has also been implicated in a number of other human diseases including autoimmune chronic hepatitis, Paget’s disease, otosclerosis and Crohn’s disease. In almost all cases the evidence for such claimed association has not been confirmed by other investigators (reviewed by Rima, 1999). Whilst in SSPE MV infection occurs primarily, though not exclusively, in non-dividing cells, infection of bone marrow precursor cells, for example osteoblasts and osteoclasts, has been implicated, though not confirmed, in diseases such as Paget’s disease of bone and otosclerosis (Basle et al., 1986; Mills et al., 1994; Reddy et al., 1999).

Various types of persistently infected cell cultures have been generated and characterized. Some are so called ‘carrier cultures’ and these represent a dynamic equilibrium between replication of the virus and division of the host cell. Uninfected cells are present in these cultures and treatment with antiviral antiserum can ‘cure’ the cultures from the infection. These carrier cultures are often susceptible to crises in which most cells die due to extensive virus replication. Both interferons and defective interfering particles have been implicated in controlling...
the equilibrium (Collins & Flanagan, 1977). Other persistently infected cell cultures, which are probably more relevant to persistence in tissues, are characterized by infection of most, if not all of the cells. These divide normally and a constant level of virus replication and gene expression is observed. In some cases infectious virus is released. Several cell lines of this type have been generated with MV and related morbilliviruses using a number of strategies (Burnstein et al., 1974; Joseph et al., 1975; Rustigan, 1966). These 'true' persistently infected cells are typified by differing degrees of superinfection immunity. This extends to infection by related morbilliviruses but not to other paramyxoviruses or interferon-sensitive viruses from other families, indicating that cytokines are not primary controlling factors (Fernandez-Munoz & Celma, 1992; Rager-Zisman et al., 1984). It has always been a challenge to determine the extent of superinfection immunity, as assessment has relied on directly observing distinct cytopathic effects or indirectly detecting viral antigen.

Recently, recombinant viruses expressing fluorescent proteins have been used to examine all stages of the virus life cycle (Duprex et al., 1999b; Elliott & O’Hare, 1999; Husain & Moss, 2001; Oomens & Wertz, 2004). Studies in plants, transgenic mice and zebrafish (Niwa et al., 1999; Moss et al., 1996; Villuendas et al., 2001) have reported no detrimental effects to these organisms upon green fluorescent protein (GFP) expression and a recombinant canine distemper virus (CDV) expressing enhanced green fluorescent protein (EGFP) retains its virulence in animals (von Messling et al., 2004). Thus, apart from very isolated examples (Huang et al., 2000; Liu et al., 1999), GFP is not considered to be cytotoxic. Thus, we reasoned that viruses expressing fluorescent proteins could be used effectively for the study of viral persistence. The emission and fluorophore matura- tion properties of the fluorescent protein DsRed1 (Matz et al., 1999) make it an obvious choice for the generation of an MV persistently infected cell line for superinfection experiments. Furthermore, red fluorescent proteins are the proteins of choice for use in two colour fluorescence microscopy with EGFP, which is expressed by the recombinant virus MVeGFP (Duprex et al., 1999b). A second recombinant MV (MVDsRed1) was therefore generated and, as expected, the level of fluorescence in syncytia was significantly less than that observed for MVeGFP, making it an ideal tool for the long-term labelling of persistently infected cells. Most importantly, these two viruses can be readily distinguished by fluorescence microscopy in living cells over time and are antigenically indistinguishable.

In this study, we used MVs expressing DsRed1 and EGFP to characterize 'true' persistent infections. We demonstrated that persistence can be maintained in the presence of fully functional viral fusion complexes and that cell surface reorganization of the MV receptor CD46 plays an important role in this process. This is also the first investigation that uses this approach to examine viral persistence and superinfection immunity and it could readily be extended to other viruses that establish such infections. Superinfection immunity is extremely effective in preventing gene expression of related viruses.

METHODS

Cells and viruses. Vero cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) containing 8 % (v/v) newborn calf serum (NCS; Gibco). These cells were used routinely for the growth of viruses that were propagated in DMEM containing 2 % (v/v) NCS. Chinese hamster ovary (CHO), CHO-CD46 and CHO-SLAM cell lines, obtained from Dr Jürgen Schneider-Schaulies, University of Würzburg, Germany, were grown in DMEM containing 10 % (v/v) fetal calf serum. Human teratocarcinoma cells (NT2) were grown as described previously (McQuaid et al., 1998). Etdag and MVeGFP were rescued from full-length infectious anti-genomic clones of MV using the MV-helper cell line (293-3-46) (Radecke et al., 1995). MVeGFP was propagated in Vero cells as described previously (Duprex et al., 1999b). Large plaque variant Onderstepoort is a vaccine-derived CDV isolate (Cosby et al., 1981). Virus stocks were generated in Vero cells, following plaque-purification, and titres up to a maximum of approximately $10^8$ TCID$_{50}$ ml$^{-1}$ were obtained. UV microscopy was used routinely to ascertain that expression of the fluorescent proteins was retained upon virus passage, and to ensure that mutants, which had lost the ability to express the proteins, were present below the limit of detection. Titres were obtained by 50 % end-point dilution assay and are expressed in TCID$_{50}$ ml$^{-1}$ determined by the method of Reed & Muench (1938). Virus-infected monolayers were fixed using 4 % (v/v) paraformaldehyde and cells were stained with methylene blue. Phase-contrast photomicrographs were obtained using a Leica DC 300F digital camera.

Generation of MVDsRed1. The open reading frame of DsRed1 was amplified from pDSRed N1 (BD Biosciences) using oligonucleotides priRFPNruI (5’-ATCCGGACCGTAGCCACATGTGCCTGCTCTCCAGAAC-3’) and priRFPNruII (5’-AACGATTCCGGACGTCAGTCTCAGGAACGTTGATTGG-3’). These primers contain MldI and NrdI restriction sites (underlined), respectively, which permit the insertion of the resulting 695 bp fragment into similarly restricted pMeGFPN5, a plasmid which contains the gene encoding EGFP flanked by MldI and NrdI (Hangartner, 1997). The resulting full-length anti-genomic clone of MV (pMDsRed1NV) contains an additional transcription unit of 810 nt and the genomic sequences conform to the rule of six (Calain & Roux, 1993). A recombinant virus (MVDsRed1) was recovered from this plasmid by using the 293-3-46 cell line, which stably expresses T7 polymerase and the N and P proteins of MV. Cells were monitored daily for cell-to-cell fusion, and UV microscopy was used to determine if the resulting syncytia were autofluorescent. MVDsRed1 stocks were produced following plaque purification and titres of up to $10^8$ TCID$_{50}$ ml$^{-1}$ were obtained. Virus stocks were stored at $-70 \degree$C.

Growth analysis. Vero cells were cultured to a confluency of 90 % in 25 cm$^2$ tissue culture flasks. Cells were infected at an m.o.i. of 0-1 with MV or MVDsRed1 for 1 h at 37 $\degree$C. The inoculum was removed and the cells were washed six times using PBS. Infected cells were incubated for 64 h and every 8 h the supernatant was removed and the cell sheet was scraped from the infected flask into serum-free DMEM (5 ml). Cell-associated virus was recovered by freeze-thawing the cell samples twice. Titres were obtained using the 50 % end-point dilution assay.

Generation of persistently infected cell lines. NT2 cells were infected at an m.o.i. of 0-01 with either MVeGFP or MVDsRed1. This resulted in the destruction of the majority of the cell monolayer by 5 days post-infection (p.i.). Growth medium was added every
3 days and the single autofluorescent cells that remained were observed to divide by 2 weeks p.i. At 6 weeks p.i., the resulting single colonies of autofluorescent cells were trypsinized and replated into 25 cm² flasks. Confluent cell monolayers were obtained at 8 weeks p.i. The persistently infected cell lines were routinely split (1:3) two times per week. Cell lines were monitored regularly for autofluorescence by UV microscopy to ensure that ‘true’ persistence had been attained and was continually maintained.

**Indirect immunofluorescence and confocal microscopy.** Cell lines were grown on glass coverslips to a confluency of 80% after which time they were rinsed twice in PBS. Cell surface expression of viral glycoproteins and the MV receptor CD46 was examined by incubating cells with the appropriate antiserum prior to fixation. The H glycoprotein was detected using monoclonal antibody (mAb) L77 (diluted 1:100 in PBS) (Moeller et al., 2001) and the F glycoprotein was detected using a polyclonal antiserum (diluted 1:500 in PBS), which were gifts from Jürgen Schneider-Schaulies, University of Würzburg, Germany. CD46 was detected using a polyclonal antiserum (diluted 1:100 in PBS), which was a gift from Fabian Wild, Institut Pasteur de Lyon, France. Cells were incubated for 1 h at 37 °C in the presence of the primary antibodies, after which time unbound antibodies were removed by three successive PBS washes. Cells were fixed for 10 min in 4% (w/v) paraformaldehyde and the coverslips were rinsed in PBS. Viral antigens were detected intracellularly in paraformaldehyde fixed cells using mAb L77, a polyclonal antiserum to the cytoplasmic tail of the F glycoprotein (diluted 1:500 in PBS) and a mAb directed against the MV nucleocapsid protein (diluted 1:1000 in PBS; Seralabs). Appropriate fluorescently labelled secondary antibodies were used, as previously outlined, to detect bound primary antibodies and a Leica TCS/NT confocal scanning laser microscope (CSLM), equipped with a krypton/argon laser as the source of the ion beam, was used to examine the samples (Duprex et al., 1999b). DsRed1 was visualized by virtue of its autofluorescence by excitation at 568 nm with a 564–596 nm band pass emission filter.

**Live-cell confocal microscopy.** MVeGFP and MVDsRed1 acutely infected Vero and NT2 cells, persistently infected (pi) NT2-MVeGFP and piNT2-MVDsRed1, and superinfected cells were examined for fluorescence by CSLM as described previously (Duprex et al., 1999b).

**Immunoblotting.** Vero, CHO, CHO-CD46, NT2 and piNT2-MVeGFP cells were lysed in Tris-glycine SDS sample buffer in the absence of a reducing agent. Western blots were performed by following standard procedures. CD46 was detected using an anti-CD46 rabbit polyclonal antibody (diluted 1:250 in PBS). An anti-rabbit immunoglobulin G (Fc) alkaline phosphatase conjugate (Promega) was used as a secondary antibody (diluted 1:5000 in PBS). Bound antibody was detected by immersing the gel in nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (NBT/BCIP; Promega).

**RESULTS**

**Generation of recombinant MV expressing a red fluorescent protein**

A recombinant MV-expressing DsRed1 (MVDsRed1) was recovered 5 days post-transfection. Fig. 1(a) shows schematic diagrams of the plasmid pMDsRed1NV from which this virus was generated and the resulting recombinant virus genome. This plasmid is a derivative of pMeGFPNV, which contains the gene encoding EGFP in an additional transcription unit at the 3′ end of the genome (Duprex et al., 1999b). Due to the presence of the additional gene in this promoter proximal position large amounts of DsRed1 are generated upon infection. The f0.5 for maturation of the DsRed1 fluorophore is relatively slow when compared with that of EGFP (∼10 h vs ∼4 h) (Jakobs et al., 2000; Terskikh et al., 2002). Thus, in MVDsRed1-infected Vero cells the intensity of red fluorescence is less than that of MVeGFP-infected Vero cells at similar time points. Although single autofluorescent-infected cells were rarely observed, red fluorescent syncytia were readily identifiable (Fig. 1b). These were comprised of similar numbers of cells as syncytia produced by Edtag or MVeGFP at equivalent time points. The growth of MVDsRed1 was monitored to determine if the presence of the additional gene and/or the expression of the autofluorescent protein had a detrimental effect on virus growth (Fig. 1c). The virus grew at an equivalent rate to Edtag (Duprex et al., 1999a) and reached a similar titre of approximately 10⁶ TCID₅₀ ml⁻¹ by 60 h p.i., indicating that the presence of the additional transcription unit and the expression of DsRed1 did not have a major effect on virus replication. After this time the titre of the cell-associated virus decreased as the extensively fused cells began to detach from the monolayer, whilst the titres of the virus in the supernatant rose to over 10⁸ TCID₅₀ ml⁻¹.

**Generation and characterization of cell lines persistently infected with viruses expressing fluorescent proteins**

As uninfected and acutely infected sub-populations can confound the interpretation of biochemical population-averaged data obtained from persistently infected cell cultures a key requirement for the appropriate study of viral persistence in cell cultures is that all of the cells are infected. Thus, persistence is usually verified by detecting viral antigens by immunocytochemistry (ICC) in fixed cells. However, ICC detection can be less than satisfactory as viral antigen is detected indirectly and it is relatively insensitive. We reasoned that recombinant viruses expressing fluorescent proteins could provide a direct, sensitive and novel means to study persistence in living cells. NT2 cells were chosen as they are a well characterized neural precursor cell line (Pleasure & Lee, 1993) and neurones are the cell type most often infected in SSPE. Cells were acutely infected with either MVeGFP or MVDsRed1. This is followed by death of over 99-9% of the infected cells. By 2 weeks p.i., single fluorescent cells remained in the flask and these divided over the next 5 weeks to form colonies. After this time, the cells were trypsinized and allowed to settle in the same flask until confluency was attained. The resulting monolayers were considered to be passage 1 piNT2 cells. Two piNT2 cell lines were generated: piNT2-MVeGFP (using MVeGFP) and piNT2-MVDsRed1 (using MVDsRed1). These grew at the same rate and to a similar confluency as the NT2 cells. The piNT2-MVeGFP cells were phenotypically indistinguishable from the NT2 cells by phase-contrast microscopy (Fig. 2a). The cytoplasm of the...
piNT2-MVDsRed1 cells was less refractile when observed by phase-contrast microscopy possibly due to DsRed1 aggregates (Baird et al., 2000; Wall et al., 2000). Cells were examined regularly by phase-contrast microscopy during the establishment of persistence to confirm that they expressed the virus encoded autofluorescent proteins. No significant differences in morphology were observed between the persistently infected and uninfected NT2 cells (Fig. 2a). Autofluorescence was only observed by UV (data not shown) and confocal microscopy in the piNT2 cells (Fig. 2b). EGFP fluorescence was spread diffusely throughout the cytoplasm and the protein accumulated in the nucleus. This confirmed what has previously been observed in MVeGFP-infected Vero cells (Duprex et al., 1999b). DsRed1 fluorescence was less diffuse and the protein appeared to aggregate in the cytoplasm. Aggregation is reflected in the phase-contrast micrograph of the piNT2-MVDsRed1 cell line in which there is a degree of granularity in the cytoplasm of the cells (Fig. 2a). This is most likely due to the tetramerization of DsRed1, which can cause difficulties in studies that set out to examine intracellular localization of fusion proteins (Jakobs et al., 2000; Mizuno et al., 2001). It has been suggested that DsRed1 aggregates may have cytotoxic effects in eukaryotic cells (Sacchetti et al.,...
2002). The fact that piNT2-MVDsRed1 cells have been passaged more than 60 times indicates that in the context of a virus-infected cell this reported toxicity is not a significant issue.

**CD46 is redistributed on the surface of persistently infected NT2 cells**

CD46 has been shown to be downregulated by contact with viral particles in acutely and persistently infected cells (Firsching et al., 1999; Naniche et al., 1993b). NT2 cells express the MV receptor CD46 uniformly over their surface (Fig. 2c and d). Therefore, a polyclonal antiserum was used in ICC to determine if the persistently infected cells had lower levels of CD46 on the surface. The receptor was detected at the surface in both the persistently infected cell lines, although rather than being uniformly distributed across the membrane the receptor had relocalized into discrete patches on the cell surface of both piNT2 cell lines (Fig. 2c and d). This is best illustrated by examining micrographs of the NT2 and piNT2-MVeGFP cells as CD46 was detected using the same secondary antibody and as such, differences in the emission intensity of fluorophore do not confound the comparison. Thus, CD46 is not downregulated from the surface of the piNT2 cells. Total amounts of CD46 were examined by non-denaturing PAGE and immunoblotting using a polyclonal antiserum, which recognizes CD46 for all cell types used in this study (Fig. 3). African green monkey CD46 was detected in Vero cells. Untransfected CHO cells do not express CD46, whereas a protein of the expected molecular mass (~55 kDa) was detected in lysates from stably transfected CHO-CD46 cells. CD46 was expressed at high levels in...
NT2 and piNT2-MVeGFP cell lines, and there were similar levels of expression of the BC (upper protein) and C (lower protein) isoforms. This suggests similar degrees of protein stability and that there is no intracellular degradation of CD46 following endocytosis. Thus, CD46 is not downregulated from the surface of the piNT2 cells.

A number of approaches were used to examine whether MV could attach to piNT2 cells via the redistributed CD46 receptors. First, binding potential was examined by determining the percentage of an input inoculum that failed to attach to cells. However, significant amounts of the virus adhered to all cell types irrespective of the presence or absence of an MV receptor. Second, a soluble form of the H glycoprotein, which contains a Flag-epitope tag in place of the transmembrane region (V. von Messling & R. Cattaneo, unpublished), was used for indirect immunofluorescence. In this instance, high, non-specific backgrounds were obtained in confocal microscopy studies. Hence, due to non-specific binding of MV and soluble H glycoprotein, we were unable to assess the functionality of the CD46 receptor molecules present on the surface of the piNT2 cells.

MV glycoproteins traffic to the cell surface

The piNT2-MVeGFP cells were examined using antibodies to MV proteins to determine if there were any changes in the intracellular localization of the N protein, the major structural component of the ribonucleoprotein complex or the cell surface distribution of the H and F envelope glycoproteins. Intracytoplasmic inclusions, typical of acutely MV-infected cells, were observed in permeabilized cells stained for the N protein (Fig. 4a). The H and F glycoproteins were readily detected in the cytoplasm and at the cell surface of permeabilized cells using a monoclonal anti-H antibody and polyclonal anti-F cytoplasmic tail antiserum (Fig. 4a). Unfixed, non-permeabilized piNT2 cells were examined by ICC to determine if the glycoproteins reached the cell surface. Both H and F were readily detected, indicating that there was no intracellular sequestration of the glycoproteins (Fig. 4b). As these antibodies recognize conformational epitopes these data suggest the proteins are correctly folded, cleaved and processed within the cell. As N is exclusively a cytoplasmic protein, no reactivity was observed when an anti-N mAb was used on non-permeabilized cells (Fig. 4b). In contrast to acutely infected NT2 cells, which release low levels of virus (~10° TCID₅₀ ml⁻¹), released virus was not detected in supernatants from persistently infected cells.

MV F and H glycoproteins are fusion active at the surface of persistently infected cells

Maintenance of persistence could be either due to mutations in the virus genome that lead to the expression of non-functional proteins or to changes in the cellular environment that influence the maturation of proteins and release of virions. Indirect immunofluorescence indicated that the F and H glycoproteins trafficked to the cell surface. However, this does not prove that the proteins are present in a fusion active complex. Thus, piNT2-MVeGFP cells were trypsinized and overlaid at a ratio of 1:1000 onto confluent monolayers of a number of cell types that express or lack utilizable MV receptors to address this question. CHO cells lack any known MV receptor. Cell-to-cell fusion was never observed when trypsinized piNT2 cells were overlaid onto CHO cells (Fig. 5a, arrows). However, when trypsinized piNT2 cells were overlaid onto stably transfected CHO cell lines, which express CD46 (Fig. 5b) or SLAM (Fig. 5c), cell-to-cell fusion was readily observed. Fusion ensued more rapidly in the CHO-SLAM cells and the syncytia reached a larger size at an equivalent time point. Equivalent data were obtained for the piNT2-MVDsRed1 cells (data not shown). Vero cells express the monkey homologue of CD46. Cell-to-cell fusion was observed after 16 h and significant levels of fusion were observed by 48 h (Fig. 5d). Moreover, high virus titres (10⁶ TCID₅₀ ml⁻¹) were released into the supernatant,
indicating that the virus genome in the piNT2 cells is not severely mutated. This virus was used to infect Vero cells, which it did at an indistinguishable level to MVeGFP or MVDsRed1. These experiments demonstrate that proteins expressed from the virus genome harboured within the piNT2 cells are fully functional. Furthermore, it indicates that the F and H glycoproteins, on the outside of the piNT2 cells, can induce fusion with cells that express MV receptors but not with persistently infected cells themselves. When piNT2-MVeGFP and piNT2-MVDsRed1 were trypsinized, mixed and allowed to settle no double fluorescent cells, cell-to-cell fusion events or syncytia were detected, confirming this observation (data not shown).

**Assessment of superinfection immunity in persistently infected cells**

Persistently infected cells are known to exhibit immunity to superinfection with homologous viruses and closely related heterologous viruses. Cells were infected with varying m.o.i. values of MV Edtag and the Onderstepoort large-plaque strain of the closely related morbillivirus CDV and syncytia were observed in fixed, methylene blue stained cell monolayers as unstained (white) areas (Fig. 6). Large numbers of syncytia were obtained in infected Vero cells, particularly in the case of Edtag which caused cells to detach from the tissue culture dish (Fig. 6a), whereas fusion was less pronounced in infected NT2 cells (Fig. 6b). Syncytia in the NT2 cells were never comprised of over 100 cells as by this stage they were seen to contract and detach from the support and this led to termination of infection. Fusion induced by the superinfecting virus was never observed in both piNT2-MVDsRed1 superinfected cells (Fig. 6c) and piNT2-MVeGFP (data not shown). Persistently infected cells were superinfected at an m.o.i. of 40 with the SBL strain of the more distantly related rubulavirus, mumps virus (MuV), to determine if a general soluble factor neutralized all infectivity. Mumps virus antigen was detected by indirect immunofluorescence using a mAb directed against the N protein and a CY3-conjugated secondary antibody. The virus could readily infect both persistently infected cell lines, indicating that the virus could both enter and replicate in the cells (Fig. 7a).

Recombinant viruses expressing fluorescent reporter proteins allow determination of the degree of superinfection immunity in the absence of any cytopathic effect. Moreover it is also possible to see if very small numbers of cells, which would be undetectable using standard biochemical assays, are superinfected. Superinfection immunity was quantified after infecting piNT2-MVDsRed1 cells with MVeGFP (m.o.i. of 0·1) as the EGFP fluorophore matures more rapidly than DsRed1 and can be detected at lower expression levels. Vero and NT2 cells were also infected as controls and no infection was observed using UV-inactivated virus, indicating that green fluorescence is dependent on virus replication. Fusion was observed in the Vero and NT2 cells by 54 h p.i. (Fig. 7b) and not in the piNT2-MVDsRed1 cells. Cells that exhibited both red and green fluorescence were observed in only 1 of ~ 300 000 (Fig. 7b, arrows and c). This suggests that superinfection immunity is virtually absolute. Superinfected cells were examined for 9 days. Although they moved as the surrounding cells divided, during this time no fusion was observed and no detectable MVeGFP virions were released. Superinfection appeared to have no detrimental effect on the cell and during the period of observations they divided and produced small foci of EGFP- and DsRed1-expressing cells.

**DISCUSSION**

Neurones and oligodendrocytes are the most commonly infected cell types in patients with SSPE (Allen et al., 1996; Esiri et al., 1982). In MV-infected brains, significant perturbations exist in expression levels of the H and F glycoproteins and the envelope associated matrix (M) protein, reviewed by Schneider-Schaulies and colleagues (2003) and Cattaneo & Billeter (1992). Along with a significant
decrease in the overall expression levels of promoter distal genes, due to greater levels of transcription attenuation at the intergenic junctions (Cattaneo et al., 1987), mutations also cause impaired envelope assembly and this might lead to defective viruses (Cattaneo et al., 1988). This predisposes the virus to an intracellular existence, thereby reducing or abolishing the likelihood of clearance by the immune system. Persistent MV infections have been established in epithelial cells (Rima et al., 1977; Rustigan, 1966) and astrocytes (Schneider-Schaulies et al., 1993), to attempt to replicate these observations in cell lines. However, no such studies have used human neural precursor or oligodendroglioma cell lines, despite the fact that neurones and oligodendrocytes are the cells most commonly infected in patients. This is the first study to establish and characterize a persistently infected human neural precursor cell line.

This investigation was undertaken to determine the degree of superinfection immunity in a disease relevant persistently infected human cell line using a fundamentally different approach to those used previously. Although superinfection immunity has been examined for many viruses, low levels of infection can never be ruled out as these studies have been restricted by the sensitivity of the detection methods employed (Bock et al., 1998; Breiner et al., 2001; de la Torre et al., 1985; Ho & Babiuk, 1979; Menna et al., 1975; Singh et al., 1997; Walters et al., 2004; Williams et al., 1981). Without having to rely on observing an overt cytopathic effect we have proven conclusively that superinfection immunity is extremely strong and can be overcome in only 1 : 300 000 cells. Interestingly, these few cells continued to divide to generate small foci of co-infected cells, illustrating the dominant effect that initial virus used to generate the persistently infected cells has on the superinfecting virus. Therefore, fluorescent persistently infected cell lines have resolved this question conclusively for the prototype morbillivirus. Finally, MuV superinfection of the piNT2 cells proves that superinfection immunity is not mediated by a general, soluble antiviral factor.

From a wider perspective these data allow us to propose particular mechanisms that make persistence possible and exclude those which are clearly not involved. For example, attachment interference due to desialylation, which has been described for Newcastle disease virus, Sendai virus and Human parainfluenza virus 3 (Horga et al., 2000; Morrison & McGinnes, 1989), can be ruled out as neuraminidase activity has not been demonstrated for MV, whereas attachment interference, due to receptor relocalization, could restrict MV entry. As infectious virus is released from the piNT2-overlaid Vero cell monolayers persistence also does not depend on the generation of mutations in the virus genome that inactivate assembly of virions. Furthermore, the fact that the F and H glycoproteins on the piNT2 cell surface are fusion competent demonstrates that loss of their function is not an essential step in the establishment of persistent MV infections. This correlates well with what is observed in SSPE viruses where alterations in the cytoplasmic tail of the F protein enhance, rather than abolish, fusion

**Fig. 6.** Assessment of superinfection immunity in piNT2-MVeGFP cells by observation of syncytium formation. Cell monolayers were infected at a range of m.o.i. values with MV (Edtag) and CDV. Infected monolayers were formaldehyde-fixed and methylene blue stained at 48 h p.i. and photomicrographs were obtained using a Leica DC 350F digital camera. Individual syncytia are visible as white areas of clearing in the methylene blue stained monolayer. (a) Large numbers of syncytia were obtained in Vero cells infected with Edtag and CDV. (b) Smaller numbers of syncytia were obtained in NT2 cells infected with Edtag and CDV. (c) No overt cytopathic effects were observed in piNT2-MVeGFP cells superinfected with either Edtag or CDV. Magnification × 1.
Fig. 7. Superinfection of piNT2-MVeGFP and piNT2-MVDsRed1 cells with MuV and MVeGFP. Infected cells were examined using CSLM in double excitation mode and EGFP and DsRed1 were visualized by virtue of their autofluorescence. The N protein of MuV was detected using a mAb and visualized using an anti-mouse CY3-conjugated secondary antibody (red). Photomicrographs represent 8–10 μm composite optical sections. (a) N protein of MuV was detected in large intracytoplasmic inclusions in permeabilized piNT2-MVeGFP cells infected at an m.o.i. of 40 with the SBL strain of MuV 48 h p.i., magnifications ×48 (left) and ×200 (right). (b) Vero, NT2 and piNT2-MVDsRed1 cells were infected at an m.o.i. of 0·1 with either UV-inactivated MVeGFP (UV) or MVeGFP. Small numbers of cells that were both red and green were observed very occasionally (arrows), magnifications ×25. (c) These double fluorescent cells are shown at a higher magnification for clarity, magnification ×212.
Receptor downmodulation and superinfection immunity have been examined extensively for a number of retroviruses. This phenomenon, originally identified in studies using *Rous sarcoma virus* (Rubin, 1960), is referred to as superinfection interference. Studies using human immunodeficiency virus (HIV) type 1 have shown that shortly after infection the primary receptor (CD4) is downmodulated from the cell surface. This is mediated by the Nef protein, which enhances CD4 endocytosis and lysosomal degradation (Garcia & Miller, 1991). CD4 is also bound by nascent gp160 synthesized within infected cells. This leads to an intracellular sequestration of the protein, ensuring that the virus receptor is not incorporated into budding virions and thus enhances the infectivity of the progeny virions (Bour et al., 1991). Thus, loss of CD4 from the cells makes them fully or partially resistant to infection by other retroviruses that utilize this receptor. Similar data have been obtained using amphotropic murine leukaemia virus (A-MuLV), which uses a sodium-dependent transporter (Pit-2) as a receptor. Upon A-MuLV infection, Pit-2 is downmodulated from the cell surface to intracellular aggregates and this leads to superinfection interference (Jobbagy et al., 2000). It is clear from our data that the mechanism of superinfection immunity in the persistent infections described in this report is fundamentally different for MV as we see neither internalization nor degradation of CD46. Rapid downregulation of CD46 from the cell surface of MV-infected cells was observed in the seminal studies that identified CD46 as an MV receptor (Naniche et al., 1993a). The mechanisms of CD46 removal from the cell surface by MV have not been examined in detail though some studies have suggested that CD46 is not endocytosed (Maison et al., 1997; Teuchert et al., 1999). Recently, two pathways that lead to CD46 internalization have been characterized (Crimeen-Irwin et al., 2003). In the ligand-independent pathway, CD46 is constitutively internalized in clathrin-coated pits, and localizes to multivesicular perinuclear inclusions from where it is transported back to the plasma membrane. In the second ligand-dependent pathway, binding of either an anti-CD46 multivalent antibody or MV leads to a process similar to macropinocytosis, which in turn causes the degradation of plasma membrane bound CD46. CD46 downregulation is minimal in pNT2 cells and there is no detectable decrease in overall expression levels when the protein is examined biochemically and microscopically. In contrast to a previous study, which examined CD46 downregulation in persistently infected monkey fibroblasts using only biochemical assays (Hirano et al., 1996), we consider that this study highlights the value of combining a cell biological approach, to examine CD46 localization, alongside a biochemical approach, to examine total levels of CD46. In pNT2 cells the F/H complexes present on the surface effect fusion only with cells that express MV receptors. A possible explanation is that CD46 is sequestered into specific domains at the plasma membrane, such as lipid rafts or caveolae, and that this renders the molecule incapable of acting as a receptor for the F/H complexes on neighbouring pNT2 cells. Whether CD46 relocalization plays a role in superinfection immunity remains to be determined. However, the fact that CDV could not also superinfect the pNT2 cells argues against this, as this virus does not use primate CD46, although whether canine CD46 can be utilized remains an open question. At present, we are attempting to generate a vaccine-based recombinant CDV, which expresses EGFP from an additional transcription unit to examine CDV superinfection using an equivalent approach. Mutations in domain 1 of the poliovirus receptor have been demonstrated to increase the resistance of persistently infected human neuroblastoma cells to virus mediated lysis (Pavio et al., 2000). Hence, we are determining the sequence of CD46 in the pNT2 cells to examine this potential determinant.

The utility of recombinant viruses that express fluorescent proteins to study persistence has been clearly demonstrated in this study. Persistence does not depend on the generation of mutations in the virus genome, inactivation of the fusion complex on the cell surface, perturbation of M protein/ribozyme interactions or a general diminution in viral gene expression. This approach could be applied to any RNA or DNA virus for which there are rescue systems available, for example, it could be used to study HIV infections, where questions of superinfection interference and receptor downregulation are highly relevant (Altfeld et al., 2002; Locher et al., 1997) and where persistently infected cells and EGFP-expressing recombinant viruses have been generated (Mahlknecht et al., 2000; Tanaka et al., 1999). Replication-defective Sindbis viruses (SINV), which express either EGFP or DsRed1 with a palmitoylation signal at the amino terminus of the fluorescent protein, have been produced. The palEGFP SINV was useful in vivo as an anterograde and retrograde neuronal tracer and it produced Golgi apparatus-like labelling of neurones, whereas the DsRed1 equivalent was unsuitable for this purpose due to a significant amount of fluorescent protein aggregation (Furuta et al., 2001). Furthermore, SINV has been shown to persist in the brains of mice for up to 17 months (Levine & Griffin, 1993). It would be interesting to ascertain if the DsRed1-expressing SINV could be used in an analogous manner to MVDsRed1 not only to generate persistently infected cell lines but to facilitate studies into the long-term persistence of the virus in vivo using this animal model. Finally, fluorescent laser capture microdissection is a new method that can be used to select and procure cell clusters from tissue sections and cell monolayers (Suarez-Quian et al., 1999). This methodology could allow the study of host genes that permit superinfection of single cells by targeted amplification of
candidate host genes by RT-PCR. Such information could be useful in the development of general strategies for controlling virus infections.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council (Grant 63438) and the Hellen C. Levitt Visiting Professorship to W. P. D. M. L. was supported by a studentship from the Research and Development Office of the Northern Ireland Department of Health and Personal Social Services (Grant EAT/850/98). We thank Paula Haddock and Sompong Vongpunsawad for excellent technical assistance.

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