Short Communication

Rhesus brain microvascular endothelial cells are permissive for rhesus cytomegalovirus infection

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Endothelial cells (EC) are an important cell type for human cytomegalovirus (CMV) pathogenesis. To characterize better the role of EC in primate CMV natural history, rhesus macaque microvascular EC (MVEC) were purified from fetal brain and analysed for infectivity by rhesus cytomegalovirus (RhCMV). Rhesus brain MVEC (BrMVEC) in culture were positive for von Willebrand factor and CD105 expression, uptake of acetylated low-density lipoprotein, and formation of capillary-like tubules on Matrigel, all phenotypic hallmarks of EC. BrMVEC were fully permissive for infection by RhCMV strain 68-1, and detectable plaques formed within 5 days of infection. Infectivity of BrMVEC by RhCMV could be reduced, but not abolished, by treatment of cells either before or during infection with pro-inflammatory mediators tumour necrosis factor-α, interleukin-1β or phorbol 12-myristate 13-acetate. These results demonstrate that *in vitro* infection of rhesus BrMVEC is a dynamic process that is influenced by activation conditions.

Human cytomegalovirus (HCMV) has relatively low pathogenic potential during primary infection in healthy individuals, yet establishes a lifelong asymptomatic persistence after initial exposure (Alford & Britt, 1993). However, HCMV can be a significant pathogen in those with immune suppression. These distinctions in infection outcomes indicate that although host antiviral immune responses are essential for protecting the infected individual from a fulminate infection, they are insufficient to eliminate viral persistence.

HCMV infects multiple cell types during both clinical and subclinical infections (Plachter *et al.*, 1996). One potential advantage of a wide cell tropism is that it offers the virus multiple sites in which to establish and maintain a lifelong infection. Both monocytic precursor cells and endothelial cells (EC), in particular, have been implicated as key cell types in HCMV natural history. HCMV infection of EC has been documented in a variety of clinical settings (Plachter *et al.*, 1996), and EC are thought to play an important role in the haematogenous spread of the virus from the primary site of infection to distal tissues (Jarvis & Nelson, 2002; Myerson *et al.*, 1984). *In vitro* studies have demonstrated that EC function is profoundly altered following HCMV infection in ways that may recapitulate mechanisms of HCMV pathogenesis *in vivo* (Grundy *et al.*, 1998; Maidji *et al.*, 2002; Sedmak *et al.*, 1994; Waldman *et al.*, 1991; Yamamoto-Tabata *et al.*, 2004). However, *in vitro* studies have been complicated by strain-related differences in EC-tropism due to incompletely defined changes in the viral genome (Baldanti *et al.*, 2003; Bolovan-Fritts & Wiedeman, 2001, 2002; Gerna *et al.*, 2002, 2003; Hahn *et al.*, 2002; MacCormac & Grundy, 1999; Sinzger *et al.*, 1999).

The present study was initiated to determine whether EC propagated *in vitro* were permissive for infection by the pathogenic strain 68-1 of rhesus CMV (RhCMV) (Chang *et al.*, 2002b; Sequar *et al.*, 2002; Tarantal *et al.*, 1998). The results demonstrated that microvascular EC isolated from the brain of fetal rhesus macaques fully supported RhCMV replication.

Rhesus macaque microvascular endothelial cells (BrMVEC) were isolated from the brain tissue as described previously for human cells (Lamszus *et al.*, 1999; Unger *et al.*, 2002). By day 6 after primary cell isolation, a subconfluent monolayer formed. These primary cells were subsequently purified further by using biotinylated *Ulex europaeus* agglutinin 1 (UEA-1) (Vector Laboratories) and anti-biotin microbeads (Miltenyi Biotec), i.e. by positive selection using UEA-I binding to EC.

This method resulted in a highly enriched (99 %) population of cells that were phenotypically and functionally consistent with EC. One percent or fewer cells were positive for either α-smooth muscle actin or glial fibrillary acidic protein staining (data not shown), characteristic markers for smooth muscle cells or astrocytes, respectively. Confluent BrMVEC demonstrated characteristic 'cobblestone' morphology with no evident gaps between cells (data not shown).

The endothelial origin of the isolated cells was confirmed by positive immunostaining for von Willebrand factor (Factor VIII) (Dako) (Fig. 1a) and positive uptake for Dil-Ac-LDL (1,1′-dioctadecyl-1,3,3,3′-tetramethylindocarbocyanine perchlorate acetylated low-density lipoprotein) (Fig. 1b) in a reaction that is considered the most reliable parameter for the identification of EC (Craig *et al.*, 1998). Dil-AC-LDL uptake has been detected in BrMVEC derived from humans, as well as from other animal species (Carson *et al.*, 1989; de Vries *et al.*, 1995; Dorovini-Zis *et al.*, 1991; Gordon *et al.*, 1991; Greenwood, 1992; Stanness *et al.*, 1996; Steffan *et al.*, 1994; Unger *et al.*, 2002). Positive immunostaining was also shown by using FITC-labelled UEA-1 (Vector Laboratories) (data not shown), and immunostaining for anti-CD105 antibody (data not shown) (clone SN6h, mouse IgG1; DAKO) (Lamszus *et al.*, 1999; MacLean *et al.*, 2001). EC organize into newly formed capillary structures during angiogenesis (Lamszus *et al.*, 1999). When rhesus BrMVEC suspensions were seeded onto Matrigel (Kubota *et al.*, 1988) thin extensions between cells or small cell clumps could be observed within 4 h. At 12–16 h, a network of tubules was formed that organized in a way that left few solitary cells (Fig. 1c). This phenotype was recently demonstrated for human BrMVEC (Lamszus *et al.*, 1999).

Virus stocks of the pathogenic strain 68-1 of RhCMV (Tarantal *et al.*, 1998) were prepared in rhesus fibroblasts (Chang *et al.*, 2002a). Infectivity assays were performed by seeding BrMVEC at a density of 5×10^4 cells per well in 24-well plates 24 h prior to virus inoculation. Cells were infected at an m.o.i. equal to 1. After virus adsorption for 1 h, the viral inoculum was removed, and the cells were then washed with PBS before addition of complete MV-EGM-2 [microvascular endothelial cell medium 2 with growth supplements including 5 % fetal bovine serum (FBS), human epidermal growth factor, human fibroblast growth factor, vascular endothelial growth factor, ascorbic acid, insulin-like growth factor, hydrocortisone, gentamicin and amphotericin B; Clonetics/Bio-Whittaker/Cambrex]. Supernatants of four independently infected samples were collected at each time point and stored at −70 °C. Virus titres for the supernatants were determined by standard plaque assays on telomerized rhesus dermal fibroblasts (Telo-RF) in 24-well plates as described previously (Chang *et al.*, 2002a).

Virus replication kinetics demonstrated a large burst in virus production between 24 and 48 h post-infection that reached a plateau at 96 h (Fig. 2b). The kinetics of RhCMV replication in BrMVEC were comparable to the pattern of infection in telomerized rhesus fibroblasts (Chang *et al.*, 2002a). These results demonstrated that rhesus BrMVEC were fully permissive for RhCMV infection.

Previous studies have demonstrated that the responses of EC to activation stimuli are characterized by changes in both gene expression and the repertoire of surface adhesion molecules (Bierhaus *et al.*, 2000). An *in vitro* infection model was established to assess the effects of cellular activation on RhCMV infection of BrMVEC. Quantitative infectivity assays were performed by counting: (i) the number of infected cells per well in 96-well plates at 24 h post-infection by immunocytochemical staining for RhCMV immediate-early (IE) 1 protein (Lockridge *et al.*, 1999); or (ii) the number of viral plaques per well at 5 days post-infection by staining for IE1 and counting foci of clustered cells containing, at least, four stained nuclei. Replicates of eight wells were used for each data point. For each dataset, a one-way analysis of variance (ANOVA) was performed to determine differences between treatment groups and controls. If the ANOVA indicated significance, Tukey's method for pairwise comparison was used. Statistical significance was accepted at $P \leqslant 0{\cdot}05$.

Rhesus CMV infection of BrMVEC was detected by strongly positive staining for IE1 gene product at 24 h post-infection. The infection was rapidly lytic with visible plaque formation within 5 days (Fig. 2a). These results indicated efficient cell-to-cell spread of RhCMV in BrMVEC.

For cellular activation experiments, BrMVEC were pre-incubated for 2 h with plain MV-EGM-2 with FBS-1 % as the only supplement. IL1β (2 ng ml^{-1}; Roche Molecular Biochemicals), TNF-α (10 ng ml^{-1}; R&D Systems) or phorbol 12-myristate 13-acetate (PMA) (100 ng ml^{-1}; Calbiochem) (diluted in MV-EGM-2/FBS-1 %) were added

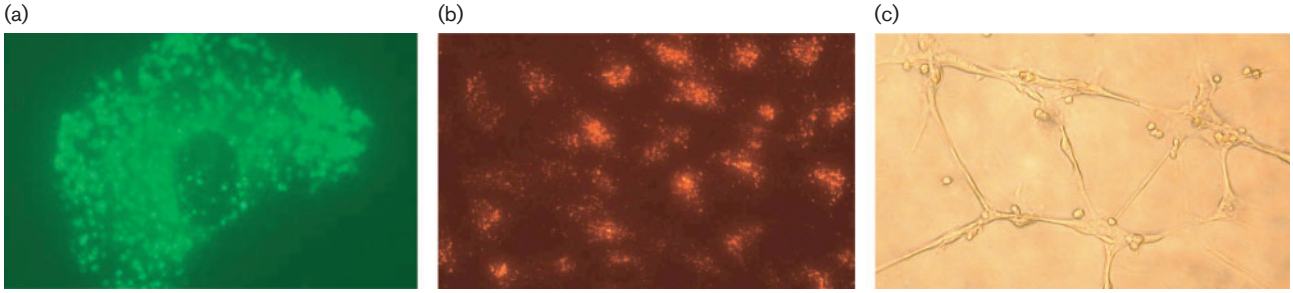

Fig. 1. Rhesus brain microvascular endothelial cells were characterized as EC by: (a) expression of anti-von Willebrand Factor VIII, (b) uptake of Dil-Ac-LDL (1,1′-dioctadecyl-1,3,3,3′-tetramethylindocarbocyanine perchlorate acetylated LDL), and (c) capillary-like tube formation after 12 h incubation on laminin-rich Matrigel.

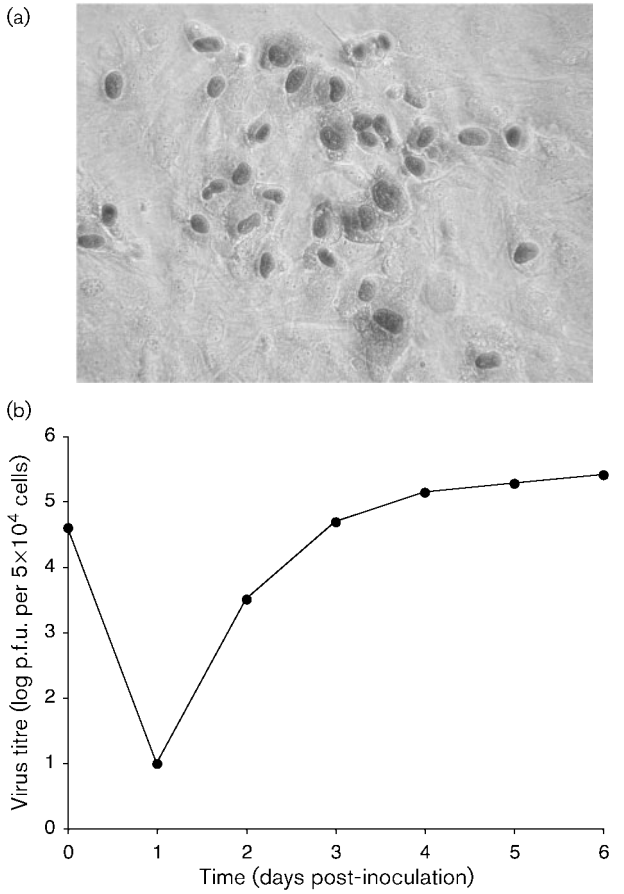

Fig. 2. Rhesus brain microvascular cells were infected with rhesus cytomegalovirus. (a) Positive staining of infected cell nuclei with anti-immediate-early 1 antibody within a plaque, 5 days after infection. (b) RhCMV replication kinetics in rhesus BrMVEC.

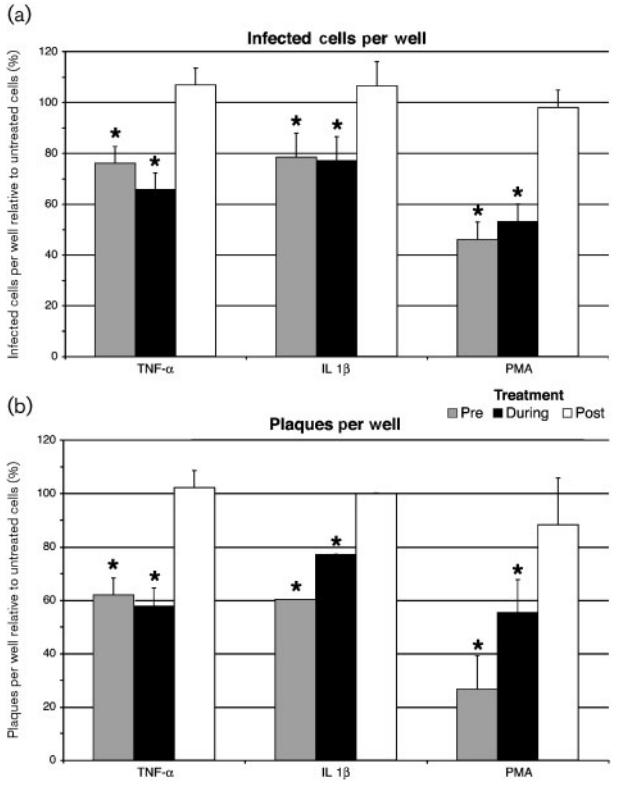

Fig. 3. RhCMV infection of BrMVEC was susceptible to treatment with TNF-α, IL1β or PMA when exposure was pre-infection compared with untreated controls. The effect of treatment was analysed by counting either (a) the number of cells per well that were positive for RhCMV IE1 protein expression 24 h post-infection or (b) the number of plaques per well. Triplicate experiments were performed with replicates of eight wells counted for each data point. A representative experiment is shown. Error bars represent the standard error of the mean.

at selected intervals before, during or after infection. Untreated cells served as controls for the IL1β and TNF-α treatments, whereas cells incubated with MV-EGM-2/FBS-1 %/0·1 % DMSO served as the controls for PMA treatment.

BrMVEC were treated with TNF-α, IL1β or PMA for 1 h intervals either (i) 1 h prior to infection (Fig. 3a and b), (ii) 1 h during infection (data not shown) or (iii) 2 h after infection (data not shown). Results of these experiments showed that treatment with all activators given prior to or during infection significantly reduced RhCMV infection of BrMVEC when assaying the number of infected cells 24 h post-infection (Fig. 3a) or the number of plaques 5 days post-infection (Fig. 3b). In contrast, when the cells were treated with each at an interval of 2 h immediately following infection, no significant differences were observed between treated and untreated cells. Since RhCMV infection of BrMVEC was not diminished by post-infection treatment with the activators, the results suggested that treatment of cells before or during infection altered the earliest steps in virus infection, such as attachment and/or entry.

BrMVEC have been isolated from various animal species including rhesus macaques and humans where they demonstrated *in vitro* maintenance of the phenotypic characteristics exhibited by the blood brain barrier (Abbott *et al.*, 1992; Audus *et al.*, 1990; Biegel *et al.*, 1995; Dorovini-Zis *et al.*, 1991; Rauh *et al.*, 1992; Takakura *et al.*, 1991; Unger *et al.*, 2002; Vinters *et al.*, 1987; Wolburg *et al.*, 1994). Rhesus BrMVEC were previously characterized for studies of simian immunodeficiency virus infection (MacLean *et al.*, 2001; Strelow *et al.*, 1998). Rhesus BrMVEC were shown to be activated in a manner similar to human cells (Stins *et al.*, 1997; Washington *et al.*, 1994; Wong & Dorovini-Zis, 1992). In addition, like cells from human origin, EC from the rhesus brain microvasculature were distinguished from those obtained from peripheral tissues with respect to growth, culture requirements, morphology and expression of surface molecules (MacLean *et al.*, 2001).

The BrMVEC that we isolated from fetal macaques demonstrated phenotypic characteristics similar to those described previously (MacLean *et al.*, 2001; Strelow *et al.*,

1998). In addition, we report an efficient method for purifying primary cultures by using biotin-labelled UEA-1 that bound to the BrMVEC before subsequent capture by anti-biotin antibody-coated magnetic beads. This method eliminated the need for the use of cloning rings (MacLean *et al.*, 2001; Strelow *et al.*, 1998) with no diminution in cell numbers or viability that has been reported previously (Lamszus *et al.*, 1999).

Although all EC share the property of forming a single cell layer separating flowing blood from the vessel walls and underlying tissues (Manconi *et al.*, 2000), EC isolated from different anatomical sites have demonstrated differences in physiological and biochemical characteristics (Page *et al.*, 1992; Turner *et al.*, 1987). It has been reported that HCMV infects human BrMVEC more efficiently than human umbilical vein EC (Fish *et al.*, 1998; Jarvis & Nelson, 2002), although others have found no EC-specific differences in infectivity (Kahl *et al.*, 2000b).

In the present study, we found that RhCMV strain 68-1 efficiently infects and replicates to high levels in BrMVEC. The formation of plaques demonstrates that infection is cytolytic, similar to HCMV infection of human BrMVEC (Jarvis & Nelson, 2002). Comparative studies using rhesus macaque EC derived from large vessels is now warranted to determine whether RhCMV replication is affected by the anatomical origin of the EC similar to HCMV (Jarvis & Nelson, 2002; Kahl *et al.*, 2000a). Treatment of rhesus BrMVEC with TNF-α, IL1β or PMA each caused a significant decrease in RhCMV infection. It should be noted, that however, this may be a reaction specific for BrMVEC, since our observation stands in contrast to the effects of PMA treatment on HCMV infectivity of human umbilical vein EC where CMV infection was enhanced by treatment (Slobbe-van Drunen *et al.*, 1997).

Our data show that BrMVEC from rhesus macaques can be easily isolated and cultured. These cells demonstrate phenotypic characteristics similar to those derived from human tissue and the replication kinetics of the lytic infection produced by RhCMV was similar to HCMV in human microvascular EC derived from brain tissue (Jarvis & Nelson, 2002; Kahl *et al.*, 2000a). These experiments indicate that *in vitro* assays using rhesus macaque-derived BrMVEC will enhance the RhCMV model for further neuropathogenesis research.

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