CD46- and CD150-independent endothelial cell infection with wild-type measles viruses

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Measles virus (MV) infects endothelial cells of the skin, the brain and other organs during acute or persistent infections. Endothelial cells are supposed to play an important role in virus spread from the blood stream to surrounding tissues. CD46 and CD150 (signalling lymphocytic activation molecule, SLAM) have been described as cellular receptors for certain MV strains. We found that human umbilical vein and brain microvascular endothelial cells (HUVECs and HBMECs) were CD46-positive, but did not express SLAM. Wild-type MV strains, which do not use CD46 as a receptor at the surface of transfected Chinese hamster ovary cells, infected HUVECs and HBMECs to varying extents in a strain-dependent way. This infection was not inhibited by antibodies to CD46. These data suggest the presence of an additional unidentified receptor for MV uptake and spread in human endothelial cells.

INTRODUCTION

After acute infection of the upper respiratory tract, measles virus (MV) is rapidly transported to draining lymph nodes forming giant cells in the reticulo-endothelial system. MV then establishes a systemic infection and spreads to different organs, including the skin. In these organs, virus replicates primarily in endothelial cells (ECs), epithelial cells and monocytes/macrophages (Griffin & Bellini, 1996). ECs of dermal capillaries (Kimura et al., 1975) and small vessels throughout the body show clear evidence of MV infection. This appears to play a central role in pathogenesis, leading to changes in the skin, conjunctivae, mucous membranes and the brain (Cosby & Brankin, 1995), accompanied by vascular dilatation, increased vascular permeability, mononuclear cell infiltration and infection of surrounding tissues. In rare cases, the EC infection may extend to severe haemorrhagic infection with confluent haemorrhagic skin eruptions and intravascular coagulopathy, so-called haemorrhagic or black measles. Brain ECs and capillary endothelium of lymph nodes and the thymus have been found to be infected in fatal cases of acute measles (Esolen et al., 1993; Moench et al., 1988). In subacute sclerosing panencephalitis (SSPE) patients, brain ECs appear to be infected in addition to various neural cells (Allen et al., 1996; Isaacson et al., 1996; Kirk et al., 1991).

Following the identification of CD46 as a receptor for MV vaccine as well as wild-type strains (Erlenhoefer et al., 2001, 2002; Hsu et al., 2001; Ono et al., 2001a, b; Tatsu et al., 2000). SLAM is expressed on human B cell lines, primary activated B and T cells, memory cells and activated monocytes and monocyte-derived dendritic cells (Cocks et al., 1995; Minagawa et al., 2001; Ogimoto et al., 2001; Polacino et al., 1996; Punnonen et al., 1997), and its usage as a receptor can explain the tropism of wild-type MV for such cells, but not for epithelial, endothelial and neural cells, such as neurons, oligodendrocytes and astrocytes, which do not express SLAM (McQuaid & Cosby, 2002). On monocytes, the expression of SLAM is induced after infection with MV or treatment with UV-inactivated MV (Minagawa et al., 2001). Recently we demonstrated that this induction of SLAM is due to the interaction of wild-type MV with the Toll-like receptor 2, which is not a receptor for uptake of MV (Bieback et al., 2002).

Several observations with epithelial cell lines such as the African green monkey kidney cell line Vero suggest the presence of additional uptake mechanisms or unknown receptors on such cells (Hashimoto et al., 2002; Koumomo & Wild, 2002; Nielsen et al., 2001; Takeuchi et al., 2002). We recently demonstrated that most wild-type MV and recombinant viruses expressing the envelope haemagglutinin (H) and fusion (F) proteins of these strains do not use CD46 as a receptor (Erlenhoefer et al., 2002); however, they can infect SLAM-negative Vero and Hela cells (Johnston et al., 1999), which supports the suggestion of CD46- and SLAM-independent virus uptake. Here we have demonstrated that primary human umbilical vein endothelial cells (HUVECs) and transformed human brain microvascular endothelial...
cells (HBMECs) do not express SLAM, either with and without treatment with inflammatory cytokines or MV. Since certain wild-type MV strains can use CD46 as a low-affinity receptor on the surface of lymphoid cells (Manchester et al., 2000), we assessed whether CD46 might be involved in virus uptake by ECs. We found that, in the absence of SLAM and in the presence of CD46-blocking antibodies, wild-type MV could effectively infect ECs, suggesting the presence of an additional non-CD46/non-SLAM cellular receptor(s) for MV.

Table 1. Measles viruses used in this study

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Genotype</th>
<th>Isolated from:†</th>
<th>Year of isolation</th>
<th>Cells used for isolation</th>
<th>Cells used for passaging</th>
<th>Receptor usage*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edm</td>
<td>A</td>
<td>Blood (PBMCs)</td>
<td>1954</td>
<td>Vero</td>
<td>Vero</td>
<td>+ +</td>
</tr>
<tr>
<td>WTFb</td>
<td>C2</td>
<td>Blood (PBMCs)</td>
<td>1990</td>
<td>BJAB</td>
<td>BJAB</td>
<td>− +</td>
</tr>
<tr>
<td>Wu4797</td>
<td>D2</td>
<td>Urine (cells)</td>
<td>1996</td>
<td>BJAB</td>
<td>BJAB</td>
<td>− +</td>
</tr>
<tr>
<td>Wu5679</td>
<td>C2‡</td>
<td>Urine (cells)</td>
<td>1998</td>
<td>B95a</td>
<td>BJAB</td>
<td>− +</td>
</tr>
</tbody>
</table>

* Determined with CHO–CD46 and CHO–SLAM cells (Erlenhoefer et al., 2002).
† Viruses were isolated from hospitalized cases of acute measles.
‡ Most likely C2.

Fig. 1. CD46 and SLAM expression by endothelial cells. HUVECs (A–C) and HBMECs (D–F) were stained with mAbs to CD46 (13/42; A, D) and various antibodies to SLAM (5C6, IPO-3 and A12; B, C, E, F). Endothelial cells were grown in culture medium alone (A, B, D, E), or stimulated with 1000 U IL-1β ml⁻¹ (C, F). B95a cells were stained as antibody controls with SLAM-specific antibodies (G). Surface expression of the molecules was quantified by flow cytometry. The CD46- and SLAM-specific mRNA expression was investigated by RT-PCR (H–J). The lanes contained a size standard (lane 1), RT-PCR products with primers for CD46 (H), SLAM (I) and beta-actin (J) from cDNA of BJAB cells (lane 2), HUVECs (lane 3) and HBMECs (lane 4). The expression of TLR2 (K) was detected in HUVECs (lane 2) and HBMECs (lane 3).
METHODS

Endothelial cell isolation and culture. HUVECs were prepared from umbilical cords obtained from the maternity ward of the University Hospital, Würzburg, as previously described (Marin et al., 2001). HUVECs were cultivated in M199 medium (Gibco) containing 25 mM HEPES, 20% foetal calf serum (FCS; Biochrom), 5 U heparin ml⁻¹, 30 μg endothelial cell growth supplement (ECGS; Sigma) ml⁻¹ and 100 U penicillin/streptomycin ml⁻¹. SV40 large T antigen-transformed HBMECs (Stins et al., 1997) were grown in RPMI 1640 medium (Gibco) containing 25 mM HEPES, GlutaMAX I (Gibco), 10% FCS, 10% NuSerum IV (Becton Dickinson), 1% non-essential amino acids, 1% vitamins, 1 mM sodium pyruvate, 5 U heparin ml⁻¹, 30 μg ECGS ml⁻¹ and 100 U penicillin/streptomycin ml⁻¹. HUVECs were used up to passage 2 and HBMECs at passages 16–20. For both endothelial cell types, the surface of the plastic dish was coated with 0.5% gelatin (Sigma). The purity of the cell cultures was confirmed by staining with the lectin *Ulex europaeus* agglutinin 1 (Vector) and antibodies to von Willebrand factor or E-selectin (Pharmingen) after treatment of the cells with TNF-α for 6 h. Cells were regularly tested for mycoplasma using a PCR-based test kit (Sigma). Recombinant interleukin-1β (IL-1β) for the stimulation of ECs was used at a concentration of 1000 U ml⁻¹, TNF-α at 100 U ml⁻¹ (Strathmann), IFN-γ at 100 U ml⁻¹ and bacterial lipopolysaccharide (LPS) at 1 μg ml⁻¹ (Sigma).

Virus strains and antibodies. MVs used in this study were the vaccine-like strain Edmonston (Edm) and the wild-type strains WTFb, Wu4797 (Würzburg.DEU/96/4797) and Wu5679 (Würzburg.DEU/98/5679; Erlenhoefer et al., 2002). For further details see Table 1. Edm was amplified using Vero cells and the wild-type strains using the human Epstein–Barr virus (EBV)-transformed B cell line BJAB, which does not produce EBV. Titres of all viruses were determined using the monkey EBV-transformed B cell line B95a (Kobune et al., 1990).

The anti-SLAM monoclonal antibodies (mAbs) IPO-3 and A12 were obtained from Kamya Biomedicals and Pharmingen. The following mouse mAbs were grown and purified using protein G columns in our laboratory: anti-SLAM clone 5C6, anti-CD46 clones B97 and 13/42, anti-MV H clone K83 and anti-MV nucleocapsid (N) clone F227. Secondary FITC-conjugated goat anti-mouse and swine anti-human IgG antibodies and Alexa Fluor 488-conjugated goat anti-mouse IgG antibodies were obtained from DAKO and Molecular Probes.

Immunohistochemistry and infection inhibition assay. For immunohistochemistry, four- or eight-chamber glass slides (Nunc) were coated with 0.5% gelatin and 2% glutaraldehyde and washed three times with PBS prior to seeding of the cells. Cells were fixed for 7 min with 3.7% paraformaldehyde and permeabilized for 10 min with 0.25% Triton X-100. Non-specific antibody binding was blocked by incubation with 10% FCS for 45 min at 4˚C. MV infection was detected using mAb F227 to MV N (2 μg ml⁻¹) and goat anti-mouse IgG Alexa Fluor 488-conjugated secondary antibody (Molecular Probes). Cell nuclei were stained using DAPI nucleic acid stain (Molecular Probes).

For the infection inhibition assay, cells were incubated prior to infection with mAbs to CD46 at given concentrations at 37˚C for 1 h, infected at an m.o.i. of 0·1 with MV for 1 h, washed once with PBS and further incubated in culture medium supplemented with antibody for 2 days. MV was visualized using a human MV-specific hyperimmune serum of a SSPE patient and secondary FITC-conjugated swine anti-human antibodies.

Fig. 2. Virus binding assay with HUVECs and HBMECs. Increasing amounts of the MV strains Edm, WTFb, Wu4797 and Wu5679 (m.o.i. = 1, 2·5 and 5) were incubated with HUVECs (A, B) and HBMECs (C, D) at 37˚C for 1 h. Bound virus was stained with mAb K83 against the MV H protein and secondary antibodies and quantified by flow cytometry (B, D; m.o.i. = 5). Signals were standardized using binding signals of mock virus preparations from uninfected Vero cells (for Edm) or BJAB cells (for MV wild-type strains), which were set to 1% MV H-positive cells (A, C).
**RESULTS**

Analysis of CD46 and SLAM expression by endothelial cells

Staining of HUVECs and HBMECs with mAbs to CD46 and analysis by flow cytometry revealed a strong surface expression of CD46 by almost 100 % of the ECs (Fig. 1). In contrast, both HUVECs and HBMECs were negative after staining with the SLAM-specific mAbs IPO-3, A12 and 5C6. As a positive control for SLAM staining, we used B95a cells (Fig. 1G). Corresponding results were found by assessing SLAM expression by RT-PCR (Fig. 1H–J).

LPS and MV wild-type particles can activate cells via the Toll-like receptor 2 (TLR2) (Bieback et al., 2002). Therefore, we assessed the expression of SLAM by HUVECs and HBMECs by RT-PCR. Both cell lines expressed TLR2 mRNA (Fig. 1K). Since it is possible that SLAM expression might be induced in activated ECs, as found for dendritic cells (Kruse et al., 2001) and monocytes (Bieback et al., 2002; Minagawa et al., 2001), we treated HUVECs and HBMECs with LPS, IL-1β, TNF-α and UV-inactivated wild-type MV. None of the treatments induced the expression of SLAM mRNA or surface protein in HUVECs and HBMECs (shown for IL-1β in Fig. 1C, F), whereas typical markers, such as E-selectin on both EC lines after TNF treatment and MHC class II on HUVECs after IFN-γ treatment, were induced (not shown). Also, infection of HUVECs and HBMECs with MV did not induce the expression of SLAM (not shown). Using B95a cells were incubated with HUVECs and HBMECs for 1 h and viral envelope proteins present on the cell surface were quantified by flow cytometry. The vaccine strain Edm, as expected, bound very efficiently to a high percentage of HUVECs and HBMECs because of its high affinity to CD46 (Fig. 2). In contrast, the wild-type strains WTFb, Wü4797 and Wü5679 bound to only a fraction of the cells. Interestingly, the efficiency of virus binding varied substantially between the wild-type strains. Reproducibly more HUVECs and HBMECs bound strain Wü4797 (up to 40 and 60 %, respectively) than the other two wild-type strains, WTFb and Wü5679 (up to 15 and 20 %, respectively).

Binding data may reflect the presence of receptors on the EC surface but not necessarily the efficiency of infection of the cells. We therefore analysed the infectivity of the viral strains, which had all been titrated using B95a cells, for HUVECs and HBMECs. Cells were infected at an m.o.i. of 0·5 with Edm and the three wild-type viruses and incubated for up to 6 days. The expression of MV H on the cell surface was analysed by flow cytometry (Fig. 3). The expression of MV N in permeabilized cells was analysed by microscopy in a similar experiment after infection of cells at an m.o.i. of 0·1 (Fig. 4). All wild-type strains infected ECs with a

![Fig. 3. Virus growth analysis in endothelial cells. HUVECs (A) and HBMECs (B) were infected with MV strains Edm, WTFb, Wü4797 and Wü5679 at an m.o.i. of 0·5. After days 0–6, the percentage of MV H-positive cells was quantified by flow cytometry using mAb K83. For determination of 0 h values, virus was added to the cells and the mixture immediately processed for staining.](image-url)
reduced efficiency compared with the CD46-using strain Edm. To our surprise, we observed great differences in virus spread among the wild-type strains. The most pronounced infection was observed with wild-type strain Wu¨ 4797 in HUVECs. Wu¨ 4797 spread slowly but constantly in the culture (up to 60% infection at day 6 p.i.), while strains WTFb and Wu¨ 5679 infected only approximately 20% of the cells (Figs 3 and 4).

Infection inhibition assay with anti-CD46 antibodies

To investigate which MV strains can use CD46 on the surface of ECs as receptor, we performed an infection inhibition assay with antibodies that block the virus–CD46 interaction by binding to the short consensus repeat domain 1 of CD46. As a control, we used anti-CD9 antibodies, which bind to the cell surface but do not interfere with MV infection (not shown). Infection of HUVECs and HBMECs with MV strain Edm was specifically inhibited by CD46 domain 1 antibodies (Fig. 5). In contrast, infection of cells with wild-type MV strains was not affected by the antibodies used. These data indicate a CD46-independent virus uptake of the MV wild-type strains.

CD46 modulation

A further test for high-affinity MV receptor interaction is receptor modulation. We and others have demonstrated previously that vaccine and laboratory strains effectively modulate CD46 (Kracnik et al., 1995; Naniche et al., 1993b; Schneider-Schaulies et al., 1995a, b, 1996) and that MV wild-type strains modulate SLAM on the surface of infected

Fig. 4. Virus spread in cultures of HUVECs. HUVECs were infected with Edm, WTFb, Wu¨ 4797 and Wu¨ 5679 at an m.o.i. of 0.1 for 24, 48 and 72 h, fixed and permeabilized and the MV N protein stained with mAb F227 and Alexa Fluor 488-conjugated secondary antibodies (green). The nuclei were stained with DAPI (blue).
cells and after contact of the cells with viral glycoproteins (Erlenhöfer et al., 2001; Tanaka et al., 2002). We infected HUVECs and HBMECs with MV strains Edm and the wild-type strains (m.o.i. = 0-1) for 3 days and determined the CD46 and MV H expression by double staining and flow cytometry. The CD46 expression was evaluated by comparing the CD46-specific signals on cells of infected cultures with uninfected cultures (Fig. 6). Infection of cells with Edm led to a reduction in the CD46 signal of 25–40% depending on the target cell, whereas infection with the wild-type strains WTFb, WuÈ 4797 and WuÈ 5679 did not lead to a reduction in the CD46 signal.

**DISCUSSION**

Infection of ECs appears to contribute to the MV-induced pathology in vivo (Cosby & Brankin, 1995; Griffin & Bellini, 1996; Kimura et al., 1975). The infection of human ECs in tissue culture has been investigated with respect to certain aspects, such as comparison with other viruses (Friedman et al., 1981), adhesion of monocytes to infected ECs (Soilu-Hanninen et al., 1996) and strain variability (Mor, CAM-70, Edm) in the induction of ICAM-1 (Harcourt et al., 1999). However, infection of ECs with CD46-using and CD46-non-using strains has not been investigated systematically. We found that CD46-non-using wild-type MV infected HUVECs and HBMECs in the absence of the common MV receptor SLAM. The extent of infection varied from strain to strain, one spreading quite effectively in the HUVEC culture, whereas the others infected only approximately 20% of the cells.

Monocytes/macrophages have previously been described as not expressing SLAM. However, it was recently found that activation of PBMCs with phytohaemagglutinin, LPS or MV particles leads to the induction of SLAM on CD14-positive cells (Minagawa et al., 2001). Interestingly, not only infection with MV but also UV-inactivated MV induced SLAM on such cells (Minagawa et al., 2001). The basis for this observation was recently elucidated when we found that wild-type MV interacts with TLR2, an interaction that induces intracellular signalling via the transcription factor NF-κB and the expression of SLAM (Bieback et al., 2002). While mediating the activation of monocytes

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**Fig. 5.** Infection inhibition assay with antibodies to CD46. HUVECs and HBMECs were incubated for 1 h at 37°C in the presence or absence of antibodies to CD46 (mAb B97; 10 μg ml⁻¹) prior to infection with the vaccine strain Edm (m.o.i. = 0-1) or wild-type MV WuÈ 4797 (m.o.i. = 0-1) for 48 h. Cultures were fixed and permeabilized and the MV N protein stained with mAb F227 and Alexa Fluor 488-conjugated secondary antibodies (green). The nuclei were stained with DAPI (blue).

**Fig. 6.** Receptor modulation from the surface of infected endothelial cells. BJAB cells, HUVECs and HBMECs were infected with vaccine and wild-type strains of MV (m.o.i. = 0-1) and the effect on the expression of CD46 was detected by flow cytometry after 3 days. As a control, cells were treated with mock virus preparations from Vero cells (for Edm) or BJAB cells (for MV wild-type strains) and the signals for the mean fluorescence intensity of CD46 were taken as 100%.
by MV, TLR2 is not an entry receptor for the virus (Bieback et al., 2002). Since ECs also express TLRs, which can be enhanced by LPS and IFN-γ (Faure et al., 2001), we asked whether treatment of the ECs with the TLR agonists LPS or MV could lead to the induction of SLAM. However, SLAM remained non-inducible on HUVECs and HBMECs.

In order to exclude a role for CD46 in the uptake of wild-type MV, we used strains that have been demonstrated to be unable to use CD46 as a receptor on the surface of transfected Chinese hamster ovary (CHO) cells (Erlenhoef er et al., 2002) and applied anti-CD46 antibodies to inhibit a potential interaction of wild-type MVs with this receptor on human ECs. The results indicated that MV wild-types can infect ECs in a CD46- and SLAM-independent manner. Receptor-independent mechanisms of virus spread, possibly as microfusion events at synapses, might circumvent the necessity for specific receptors for MV in the brain (Allen et al., 1996; Duprex et al., 1999; Lawrence et al., 2000; McQuaid et al., 1998; Meissner & Koschel, 1995; Urbanska et al., 1997), where CD46 is present on only a small proportion of cells and SLAM is not expressed (McQuaid & Cosby, 2002; Ogata et al., 1997). These findings are valid for virus spread in neurons in the brain, but not for infection of tissue culture cells with cell-free virus. The uptake of virus by ECs in tissue culture was relatively effective, which supports the assumption of a receptor-mediated process. Our data therefore suggest the presence of an additional unknown cellular receptor for MV on ECs. Our findings do not rule out the possibility that vaccine strains such as Edm may also use this additional receptor. We suggest this since, in the case of SLAM- and CD46-positive lymphocytes, virus attachment and infection with Edm can be blocked efficiently by antibodies to CD46, although Edm can also use the common MV receptor SLAM on cells in the absence of CD46 (Erlenhoef er et al., 2001, 2002). The molecular basis for this finding is not known.

It currently remains unclear why certain wild-type strains spread better on HUVECs than others. Various evidence has been accumulated indicating that subtle differences in the envelope proteins of MV can play a role in altering the tropism, virus uptake, cell-to-cell fusion and pathogenicity (Bartz et al., 1996; Bieback et al., 2002; Hsu et al., 1998; Johnston et al., 1999; Lecouturier et al., 1996; Moeller et al., 2001; Moll et al., 2001; Ohgimoto et al., 2001; Plemper et al., 2002; Shibahara et al., 1994; Takeuchi et al., 2002). To investigate the molecular basis for the differential spread of MV wild-types in cultures of HUVECs, we will further analyse the sequences of the envelope genes of the MV wild-types and intend functional studies with corresponding recombinant viruses.

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