Virology 381 (2008) 203–214

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Role of class I human leukocyte antigen molecules in early steps of echovirus infection of rhabdomyosarcoma cells

Stéphane Chevaliez [a,*,1], Jean Balanant [a], Patrick Maillard [b], Yu-Chun Lone [c], Francois A. Lemonnier [c], Francis Delpeyroux [a]

[a] Unité de Biologie des Virus Entériques, Institut Pasteur, Paris, France
[b] Unité des Hépacivirus, Institut Pasteur, Paris, France
[c] Unité d'Immunité Cellulaire Antivirale, Institut Pasteur, Paris, France

ARTICLE INFO

Article history:
Received 7 May 2008
Returned to author for revision 23 June 2008
Accepted 4 August 2008
Available online 27 September 2008

Keywords:
Picornavirus
Enterovirus
Echovirus 11
Viral entry
Human leukocyte antigen class I
Receptors

ABSTRACT

Several echoviruses use decay accelerating factor (DAF) as a cell surface receptor. However, most of them require additional cell surface coreceptors. We investigated the respective roles of DAF and class I human leukocyte antigen (HLA) molecules in the early steps of the echovirus 11 (EV11) lifecycle in rhabdomyosarcoma (RD) cells. EV11 infection was inhibited at an early stage by anti-β2-microglobulin (β2m) and anti-HLA monoclonal antibodies and by a soluble monochain HLA class I molecule. Expression of class I HLA molecules restored the early steps of the EV11 lifecycle, but its expression was not sufficient for EV11 replication and particle production. Expression of HLA class I molecules was associated with leukocyte cell line permissiveness to EV11 infection. In conclusion, HLA class I molecules are involved in the early steps of EV11 infection of RD cells and appear to participate in a complex interplay of surface molecules acting as coreceptors, including DAF.

Introduction

Echoviruses are small, non-enveloped, single-positive-stranded RNA viruses belonging to the *Picornaviridae* family, genus *Enterovirus*. They are associated with a broad spectrum of clinical manifestations, ranging from non-specific illnesses to systemic disorders; they can also cause meningitis, encephalitis and severe systemic infections in neonates (Melnick, 1996; Pallansch and Roos, 2001). Most cases are sporadic, but certain serotypes have been associated with outbreaks (Kopecka et al., 1995; Mullins et al., 2004). Echovirus 11 (EV11) is one of the most commonly isolated enterovirus serotypes (Oberste et al., 2003). It is associated with various illnesses, including meningitis, uveitis, and severe multisystem haemorrhagic diseases in neonates and infants (El-Sageyer et al., 1998; Lukashev et al., 2003).

At least ten different picornavirus receptors have been identified (Bergelson et al., 1992, 1993; Berinstein et al., 1995; Greve et al., 1989; Goodfellow et al., 2001; Neff et al., 1998; Newcombe et al., 2003, 2004; Roivainen et al., 1994; Shafren et al., 1997a,b; Williams et al., 2004; Zautner et al., 2003, 2006). Many enteroviruses use decay accelerating factor (DAF or CD55) as a cell surface receptor (Clarkson et al., 1995; Karnauchow et al., 1996; Powell et al., 1998; Shafren et al., 1995; Shafren et al., 1997a). DAF is a 70-kDa glycophosphatidylinositol (GPI) anchor protein with four extracellular short consensus repeat (SCR) domains and a C-terminal serine/threonine-rich region and protects the serum exposed cells from complement-mediated lysis (Lublin and Atkinson, 1989). Previous genetic and biochemical studies of enterovirus binding to DAF have shown that enteroviruses can be divided into two major groups according to the involved SCR binding domain. With the exception of Coxsackie virus (CAV) 21 and enterovirus 70, which bind SCR1, enteroviruses bind SCR3, with additional binding to SCR4 and sometimes SCR2 (Bergelson et al., 1994; Karnauchow et al., 1996; Lea et al., 1998; Pettigrew et al., 2006; Powell et al., 1998; Shafren et al., 1995; Ward et al., 1994). Anti-DAF mAbs directed against SCR3 have been shown to inhibit the infection of human embryonic rhabdomyosarcoma (RD) cells by several echovirus strains, including EV7 and EV11 (Bergelson et al., 1994; Ward et al., 1994).

Cell-surface coreceptors or accessory molecules in addition to DAF may be needed by some echoviruses to induce the formation of A-particles (sedimentation coefficient 135S), the product of receptor-mediated conformational changes in the native infectious virion structure (sedimentation coefficient 160S) (Pasch et al., 1999; Powell et al., 1998; Shafren et al., 1997a,b). For instance, soluble DAF (sDAF) blocks EV7 binding through steric hindrance. The interaction between

* Corresponding author. French National Reference Center for Viral Hepatitis B, C and delta, Department of Virology, Hôpital Henri Mondor, 51 avenue du Maréchal de Lattre de Tassigny, 94010 Créteil, France.
E-mail address: stephane.chevaliez@hmn.aphp.fr (S. Chevaliez).
[1] Current address: French National Reference Center for Viral Hepatitis B, C and delta, Department of Virology and INSERM U841, Hôpital Henri Mondor, Université Paris 12, Créteil, France.

0042-6822/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2008.08.006

sDAF and EV7 is reversible and does not lead to the formation of A-particles (Powell et al., 1997). In contrast, the interaction of human rhinovirus 14 and the soluble form of its receptor (intercellular adhesion molecule-1: ICAM-1) or of poliovirus and the soluble poliovirus receptor (PVR, CD155) induces irreversible conformational changes leading to the formation of A-particles, suggesting that these viruses do not need additional determinants at the cell surface (Casasnovas and Springer, 1994; Gomez Yafal et al., 1993).

Recent reports have suggested that β2-microglobulin (β2m) and CD59 — a GPI-anchored protein that regulates the complement cascade at a later stage than DAF — may also play a role as a coreceptor in the infectious cycle of EV7 (Goodfellow et al., 2000; Ward et al., 1998). However, there is no direct evidence that β2m or CD59 is involved in virus infection or A-particle formation. In addition, the class I human leukocyte antigen (HLA) molecules, and particularly the β2m light chain of the molecule, have also been shown to play a significant role in CAV9 internalization (Triantafilou et al., 1999, 2002).

EV11 is genetically related to EV7 and CAV9 (Huttunen et al., 1996). Some EV11 strains have been shown to recognize DAF as a receptor on RD cells. However, other surface molecules can be used in cell lines where DAF expression is reduced. This change in receptor use is generally associated with a few amino acid changes in the capsid protein sequence (Kim and Racaniello, 2007; Schmidtke et al., 2000; Stuart et al., 2002b). Infection of RD cells by EV11 is also inhibited by anti-β2m antibodies, with no blockade of virus binding or of translation and replication of transfected viral RNA, arguing against a physical association between EV11 and β2m (Ward et al., 1998). Although the panspecific anti-HLA-A, -B, and -C monoclonal antibody (mAb) W6/32 has no effect on EV infection (Ward et al., 1998), a role of class I HLA molecules in EV11 attachment and/or entry cannot be ruled out.

The goal of this study was to examine the respective roles of DAF and class I HLA molecules in the early steps of the EV11 lifecycle in RD cells. We show that the DAF molecule is not systematically involved in EV11 attachment to the surface of RD cells, and report a previously unknown interaction of EV11 with class I HLA molecules.

Results

EV11 infection of RD cells is inhibited by an anti-β2m monoclonal antibody and by the anti-HLA monoclonal antibody B9.12.1

We assessed the capacity of monoclonal antibodies directed against DAF (mAb BRIC216), β2m (mAb MCA1115) and the HLA class I heavy chain (mAbs W6/32, B9.12.1 and B1.23.2) to inhibit RD cell infection by EV11 strains, including the reference prototype strain Gregory and a clinical isolate of EV11 (HU-1108/89) responsible for a severe epidemic in Hungary (Chevaliez et al., 2004; El-Sageyer et al., 1998). The PV1/Mahoney strain, which recognizes a well-characterized receptor molecule (CD155) (Mendelsohn et al., 1989), was used as a control, along with EV7 (Wallace strain), for which RD cell infection is inhibited by anti-DAF and anti-β2 mAbs. The results are shown in Figs. 1a–d.

As expected, poliovirus type 1 (PV1/Mahoney) infection of RD cells was not inhibited by any of the antibodies tested (Fig. 1d), whereas EV7 lytic infection of RD cells was efficiently inhibited by the anti-DAF BRIC216 and anti-β2m MCA1115 mAbs (Fig. 1c). In contrast, anti-DAF mAb BRIC216 had no effect on EV11 lytic infection, even at the highest concentration used (40 μg/ml) and whatever the EV11 strain (Figs. 1a and b). As shown in Figs. 1a and b, EV11 lytic infection of RD cells was inhibited by the anti-β2m mAb MCA1115 and by one of the three anti-HLA mAbs, B9.12.1. These two mAbs also inhibited EV7 infection (Fig. 1c). In order to rule out the role of a contaminant present in the hybridoma cell supernatant containing B9.12.1mAb, the same experiments were carried out with a purified IgG fraction of this mAb derived from a different batch of hybridoma supernatant. The purified B9.12.1 mAb inhibited lytic infection of RD cells by both HU-1108/89 and Gregory EV11 strains (Figs. 1a and b). RD cell infection by two other EV11 strains, RO-91/91 and FI-06666/89, isolated from clinical and environmental samples, respectively, was also inhibited by mAb B9.12.1 (data not shown).

We investigated whether the anti-β2m mAb MCA1115 and the anti-HLA mAb B9.12.1 functioned additively or synergistically by assessing the inhibitory effects of various doses of a combination of these two antibodies on EV11 (HU-1108/89) infection (Fig. 1e). These antibodies were found to inhibit EV11-mediated cell lysis synergistically.

*EV11 infection of RD cells is inhibited by a soluble β2m-HLA-A*2 monochain*

We then tested the ability of increasing concentrations of a recombinant soluble β2m-HLA-A*2 monochain (human β2m fused with the HLA-A*2 molecule (Lone et al., 1998; Motta et al., 1998) to inhibit EV11 infection of RD cells. A membranous version of this monochain was shown to efficiently present peptide antigens on rodent cells to human cytotoxic T cells (Firat et al., 2001; Pascolo et al., 1997; Snyder et al., 2004). As shown in Fig. 2, concentration-dependent neutralization of RD cell infection was observed 3 h post-infection. In contrast, soluble β2m-HLA-A*2 monochain did not affect viral infection by the control strain PV1/Mahoney (data not shown).

Inhibition of EV11 infection of RD cells by the anti-HLA mAb B9.12.1 occurs at an early stage

We studied the effect of the anti-HLA mAb B9.12.1 and the anti-β2m mAb MCA1115 added at various times before, concomitantly with, or after EV11 infection of RD cells on the level of viral multiplication (Fig. 3). Intracellular production of viral RNA was assessed 5 h after infection. Maximum inhibition of infection was observed when RD cells were incubated with these antibodies 30 to 60 min before infection (viral RNA reduction: 57% and 90% relative to untreated cells, respectively) (Figs. 3a and b). Adding the antibodies at the time of infection inhibited viral replication to a lesser extent (49% and 62%, respectively). Finally, no effect on replication was observed when the antibodies were added 30 to 120 min after infection (Figs. 3a and b). Viral RNA reduction was greater with anti-β2m mAb MCA1115 than with anti-HLA mAb B9.12.1, a finding in keeping with the strong inhibitory effect of anti-β2m mAb observed for EV11 infection in Fig. 1.

We then transfected RD cells with purified EV11 HU-1108/89 and PV1/Mahoney (negative control strain) viral RNAs after treatment with anti-DAF, anti-β2m and anti-HLA B9.12.1, B1.23.2 monoclonal antibodies and immunoglobulin isotypes were used as controls. With both viral RNAs transfected, the viral titers measured 5 h after transfection were not lower with mAb B9.12.1 and all other mAb tested than with the control antibodies (Figs. 4a and b). This suggested that mAb B9.12.1 does not act in the late stages of infection and that mAb B9.12.1 binding to the cell surface does not alter the cell machinery by interacting with virus-specific mechanisms. Overall, these findings, together with the observation that EV11 infection of RD cells was inhibited by soluble HLA monochain, suggested that the blocking effect of anti-HLA mAbs on EV11 HU-1108/89 infection occurred at an early step of the viral lifecycle.

Anti-HLA mAb B9.12.1, but not anti-β2m mAb MCA1115, inhibits EV11 binding to RD cells; interference with A-particle formation

We assessed the ability of anti-β2m and anti-HLA mAbs to inhibit the binding of EV11 (HU-1108/89) to RD cells. Anti-DAF mAb and the EV7/Wallace and PV1/Mahoney strains were used as controls. The effect of mAbs on binding was assessed by means of an antibody-binding assay using radiolabeled virus (Fig. 5). The binding of radiolabeled EV11 (HU-1108/89) to RD cells was inhibited by 36% by

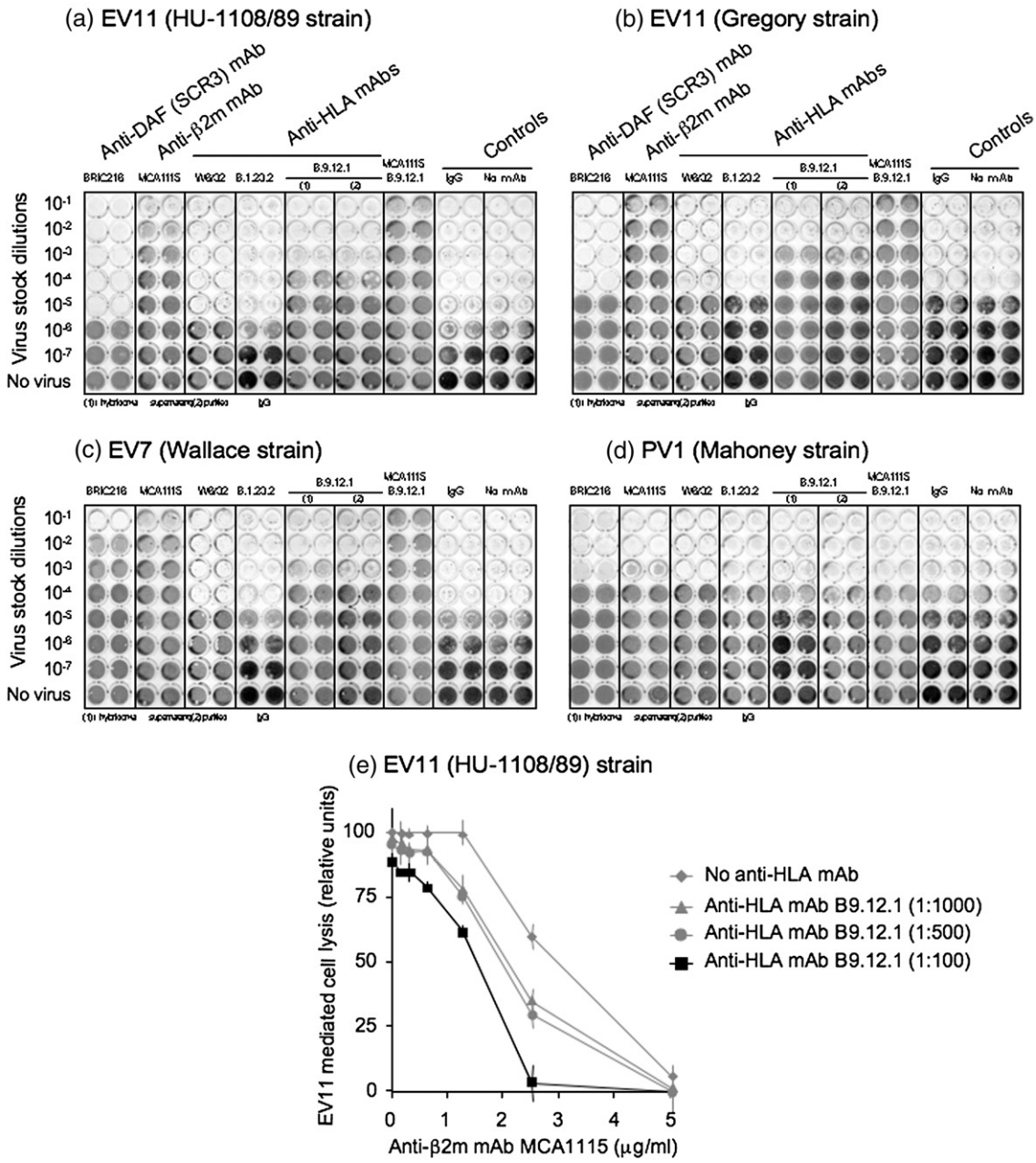


Fig. 1. Concentration-dependent inhibition of enterovirus infection of RD cells by monoclonal antibodies. (a to d) RD cell monolayers were incubated with fixed concentrations of anti-DAF (BRIC216), anti-HLA (W6/32, B9.12.1 from hybridoma supernatant (1) and purified IgG (2) and B1.23.2), and anti-β2m (MCA1115) mAbs and mouse IgG (control) for 1 h at 37 °C. RD cells were then challenged with serial dilutions of stocks of EV11 (HU-1108/89) (a), EV11 (Gregory) (b), EV7 (Wallace) (c) and PV1 (Mahoney) (d), and stained after 40 h of incubation. (e) RD cell monolayers were incubated with various concentrations of anti-β2m mAb MCA1115, alone or together with various concentrations of anti-HLA mAb B9.12.1. Cells were challenged by incubation for 40 h with 10^3 PFU of EV11 (HU-1108/89) per well. Cell survival was estimated by staining with crystal violet-ethanol solution and reading absorbance at 560 nm. An arbitrary value of 100 was attributed to the mean result of quadruple experiments performed in the absence of antibodies. The results of the experiments performed in the presence of antibodies are expressed as mean (±SD) values relative to 100.

anti-HLA mAb B9.12.1 (Fig. 5a). In contrast, neither anti-β2m mAb MCA1115 nor anti-DAF mAb BRIC216 affected EV11 binding to RD cells. Anti-β2m mAb did not increase the inhibitory effect of anti-HLA mAb B9.12.1 on EV11 binding (Fig. 5a). Other anti-HLA mAbs (B1.23.2 and W6/32) had no effect on EV11 binding (data not shown). Neither anti-HLA mAb B9.12.1 nor anti-β2m mAb MCA1115 affected the binding of radiolabeled EV7, whereas anti-DAF mAb BRIC216 totally inhibited EV7 binding, as already reported (Clarkson et al., 1995; Ward et al., 1994) (Fig. 5b). None of the antibodies tested affected the binding of radiolabeled PV1, the negative control (Fig. 5c).

We investigated the mechanism by which the anti-HLA mAb B9.12.1 and the anti-β2m mAb MCA1115 blocked EV11 and EV7 infections, by studying A-particle formation in the presence of mAbs during the binding and/or decapsidation phase. Radiolabeled EV11 (HU-1108/89) and EV7 (Wallace) was bound to RD cells at 4 °C before incubating the cells at 37 °C for 30 min or 2 h with these mAbs, respectively. Cell-associated and eluted viruses were measured by means of sucrose gradient sedimentation (Fig. 6). As shown in Figs. 6a and 6b, in the absence of antibody, cell-associated and eluted radioactivity of EV11 was detected in 160S, 135S and 80S particles. Small amount of 80S particles in the cell supernatant indicates that these particles have been detached from the surface or released from the cells. In the presence of mAb B9.12.1 during the binding phase at 4 °C and the internalization phase at 37 °C, cell-associated EV11 particles and subparticles were not detected

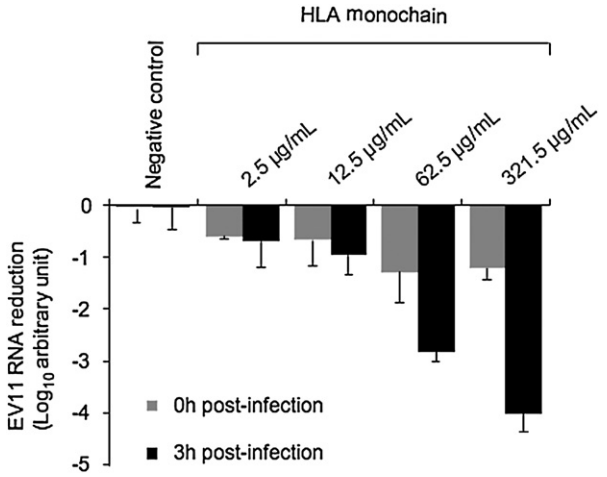


Fig. 2. Effect of increasing concentrations of the soluble monochain class I HLA molecule β2m-HLA-A*2 on EV11 infection of RD cells. The results are shown as the reduction in EV11 RNA, measured by means of quantitative real-time PCR, relative to the control experiment without soluble monochain. Total cellular EV11 RNA is shown in gray at the time of infection, and in black 3 h post-infection.

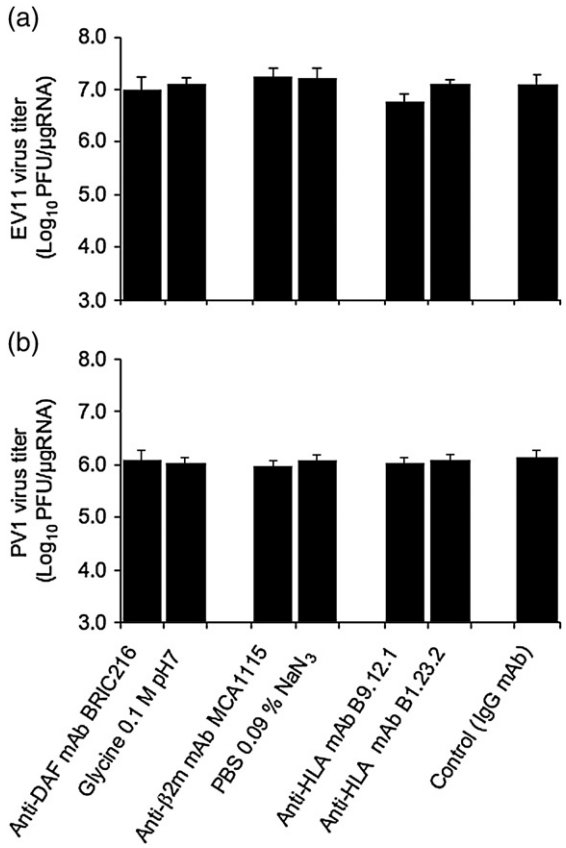


Fig. 4. Infectivity of the viral RNA in the presence of anti-DAF SCR3 (BRIC216), anti-β2m (MCA1115), anti-HLA-A, -B, and -C (B9. 12. 1 and B1. 23. 2), isotype control (IgG mouse) or negative controls (PBS 0.09% NaN_3, glycine 0.1 M pH7). RD cell monolayers were incubated in the presence of anti-DAF (40 µg/mL), anti-β2m (5 µg/mL), anti-HLA-A, -B, and -C (1:100), IgG mouse (40 µg/mL) or the absence of antibodies (PBS 0.09% NaN_3, glycine 0.1 M pH7) for 1 h at 37 °C. The cells were then transfected with 1 µg of purified viral EV11 (HU-1108/89) RNA (a) or purified viral PV1/Mahoney (b) using Effectene® reagent and the infection was allowed to proceed for 5 h at 37 °C in 5% CO_2. The infectivity of each sample was assessed by plaque assay on RD cells and the results were expressed in number of PFU per µg of viral RNA±SD. Pretreatment with RNAse (100 µg/mL) abolished infectivity.

in the cell pellet, confirming that this antibody inhibits EV11 binding (Fig. 6a, gray line). In contrast, anti-β2m MCA1115 mAb did not appear to inhibit EV11 binding, whereas they appeared to slightly affect subparticle formation. Analysis of eluted viral particles detached from the cell surface in the presence of both mAbs showed that far fewer 135S particles were detected (Fig. 6b). In contrast with EV11, anti-HLA B9.12.1 and anti-β2m MCA1115 mAbs appeared to affect EV7 subparticle formation but were not able to inhibit its binding (Figs. 6c and d).

In order to study the influence of anti-HLA mAb B9.12.1 and anti-β2m mAb MCA1115 on A-particle formation, independently of their inhibition of cell binding, RD cells bound at 4 °C with radiolabeled EV11 (HU-1108/89) or EV7 (Wallace) were incubated with the two mAbs alone at 37 °C for 30 min or 2 h, respectively. The production of 135S particles was slightly reduced when the cells were treated with anti-HLA mAb B9.12.1 and, to a lesser extent, with anti-β2m MCA1115 mAb, suggesting that these antibodies directly or indirectly interfere with A-particle formation (data not shown). Anti-DAF mAb BRIC216 had no effect on A-particle formation (data not shown).

Class I HLA molecules and DAF contribute to EV11 binding in transfected CHO cells

We investigated the role of human DAF and HLA molecules in the binding of EV11, by stable transfection of CHO cells expressing human DAF (CHO-DAF/A9) (Clarkson et al., 1995; Coyne et al., 1992) with a plasmid encoding a membranous version of the β2m-HLA-A*2 monochain (see Materials and methods). Flow cytometry showed correct expression of both DAF and β2m-HLA-A*2 on the transfected CHO cells (CHO-DAF-β2m-HLA-A*2) (data not shown).

Surprisingly, CHO-DAF/A9 cells expressing only DAF bound radiolabeled EV11 (HU-1108/89) and this binding was totally inhibited by

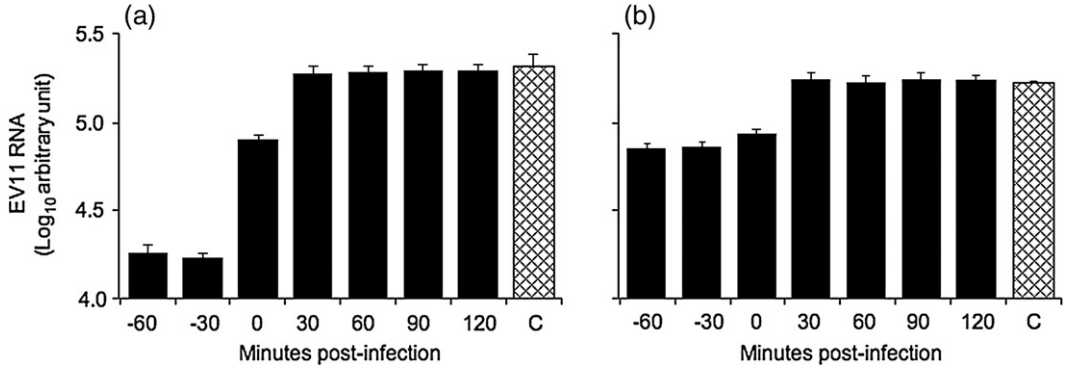


Fig. 3. Effect of the time of mAb treatment on RD cell infection by EV11. RD cell monolayers were treated with fixed concentrations B9.12.1 (a) or MCA1115 (b) mAbs at various times before, concomitantly with, and after EV11 infection. The letter C corresponds to control without mAb at 120 min. Viral RNA was quantified by reverse-transcriptase quantitative PCR 5 h after infection. Results are expressed as means of two independent experiments±SD in µg relative to the standard EV11 dilution panel used for calibration.

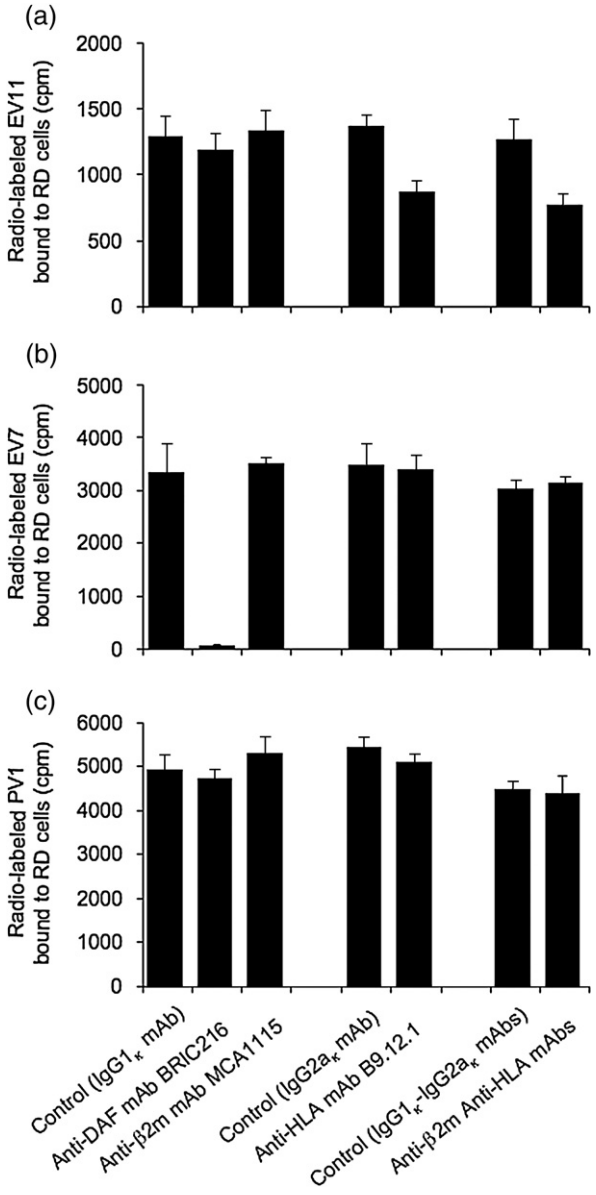


Fig. 5. Inhibition of enterovirus binding to RD cells. RD cell monolayers were incubated with anti-DAF (BRIC216), anti-β2m (MCA1115), and anti-HLA (B9.12.1) mAbs, control isotypes (IgG1κ, IgG2aκ) or DMEM. Cells were then incubated with radiolabeled EV11 (HU-1108/89) (a), EV7 (Wallace) (b) and PV1 (Mahoney) (c). Bound radiolabeled virus levels were determined by liquid scintillation counting. Results are expressed as the means of two experiments ± SD.

the specific anti-DAF mAb BRIC216 (91% inhibition) (Fig. 6b), whereas CHO/Bill, a DAF-deficient cell line, is not able to bind a large quantity of radiolabeled EV11 and this binding is not abrogated by an anti-DAF mAb directed against SCR3 domain (Fig. 7c). Co-expression of β2m-HLA-A*2 was also associated with radiolabeled EV11 binding to CHO-DAF-β2m-HLA-A*2 cells (Fig. 6a). Both anti-DAF mAb and anti-HLA mAb B9.12.1 inhibited EV11 binding to these CHO cells. However, inhibition was stronger with anti-HLA mAb B9.12.1 than with anti-DAF mAb (73% vs 39%, respectively) (Fig. 6a). Used together, the two antibodies appeared to have a partly additive inhibitory effect (90% of binding inhibition). Altogether, these results suggest that, in contrast to RD cells, human DAF may be involved in EV11 HU-1108/89 binding to the surface of CHO cells. However, the stronger inhibition of binding with anti-HLA mAb B9.12.1 when β2m-HLA-A*2 was expressed suggests preferential use of surface HLA molecules by EV11 in this model. This result confirms the role of HLA molecules in EV11 binding.

Expression of both human DAF and class I HLA molecules is not sufficient for EV11 replication and particle production in CHO cells

As rodent cells expressing DAF and class I HLA bound EV11 HU-1108/89 and formed A-particles (data not shown), we investigated whether EV11 was able to replicate into the transfected CHO cells. Confluent monolayers of RD and CHO-DAF-β2m-HLA-A*2 cells were infected with virus (1 to 50 PFU/cell). As expected, a complete cytopathic effect was observed in RD cells 24 h after infection (data not shown). In contrast, no cytopathic effect was seen in CHO-DAF-β2m-HLA-A*2 cells and no viral increase was observed between 0 and 48 h after infection (data not shown).

We looked for intracellular viruses, 6 h after infection, using flow cytometry and a polyclonal rabbit anti-EV11/HU-1108/89 serum. We also investigated whether the lack of viral multiplication was due to a blockade late in the viral cycle by seeking for negative and positive strands of intracellular viral RNA three and 6 h post-infection. EV11 virions and positive strands of intracellular viral RNA were detected 6 h post-infection in the cytoplasm of RD cells but not in CHO-DAF-β2m-HLA-A*2 cells (data not shown) or other rodent cells (CHO/Bill, CHO-DAF/A9, data not shown). Negative strands were only detected 3 h post-infection in the cytoplasm of RD cells (data not shown). Thus, CHO cells expressing human DAF and monochain HLA molecules are not permissive to EV11 infection. However, transfection of CHO-DAF-β2m-HLA-A*2 cells with purified EV11 HU-1108/89 RNA led to efficient viral replication and abundant viral particle production (data not shown). Moreover, following adsorption of radiolabeled EV11 virions onto CHO-DAF-β2m-HLA-A*2 cells and incubation at 37 °C, 135S A-particles were detected associated with cells and free in the supernatant (data not shown).

This suggests that the EV11 infection of CHO-DAF-β2m-HLA-A*2 cells is blocked in an early step of the viral cycle, between A-particles formation and release of the viral genome in the infected cells.

Expression of HLA class I molecules, but not DAF, is associated with leukocyte cell line permissiveness to EV11 infection

Daudi cells, Raji B cells and the monocytic cell line U-937 were infected with EV11 (HU-1108/89). Daudi cells naturally express DAF, Raji cells express class I HLA molecules and U-937 cells express both molecules at their surface (Haddad et al., 2004; Nilsson et al. 1974; Vuorinen et al., 1999). DAF and class I HLA molecule expression was confirmed in these cell lines by flow cytometry with mAbs (Fig. 8c). As shown in Fig. 8a, only Raji cells and U-937 cells, that both express class I HLA molecules, produced EV11 viral particles in culture. In contrast, Daudi cell infection did not lead to viral particle production, whereas Daudi cell transfection with purified EV11 HU-1108/89 RNA led to efficient viral replication and abundant viral particle production (Fig. 8b). Although we cannot rule out that other factors between the two cell lines could play a role, this result reinforces the implication of the class I HLA molecules in infection by at least certain EV11 strains, via a DAF-independent mechanism. Similar results were obtained when leukocyte cell lines were infected with EV7 (Wallace). Only Raji and U-937 cell lines are able to produce infectious viral EV7 particles, suggesting that the only expression of DAF molecule at the cell surface is not sufficient to supportive EV7 infection (Data not shown). However, Raji cell line a DAF-deficient cell line expression is able to produce infectious virions, suggesting a DAF-independent mechanism for EV7 viral entry in this specific cell line.

Discussion

Enterovirus entry into target cells is a complex, multistep process. Cell attachment and internalization of several enterovirus serotypes have been reported to involve different cell surface receptors and mechanisms of entry, including internalization via clathrin-coated

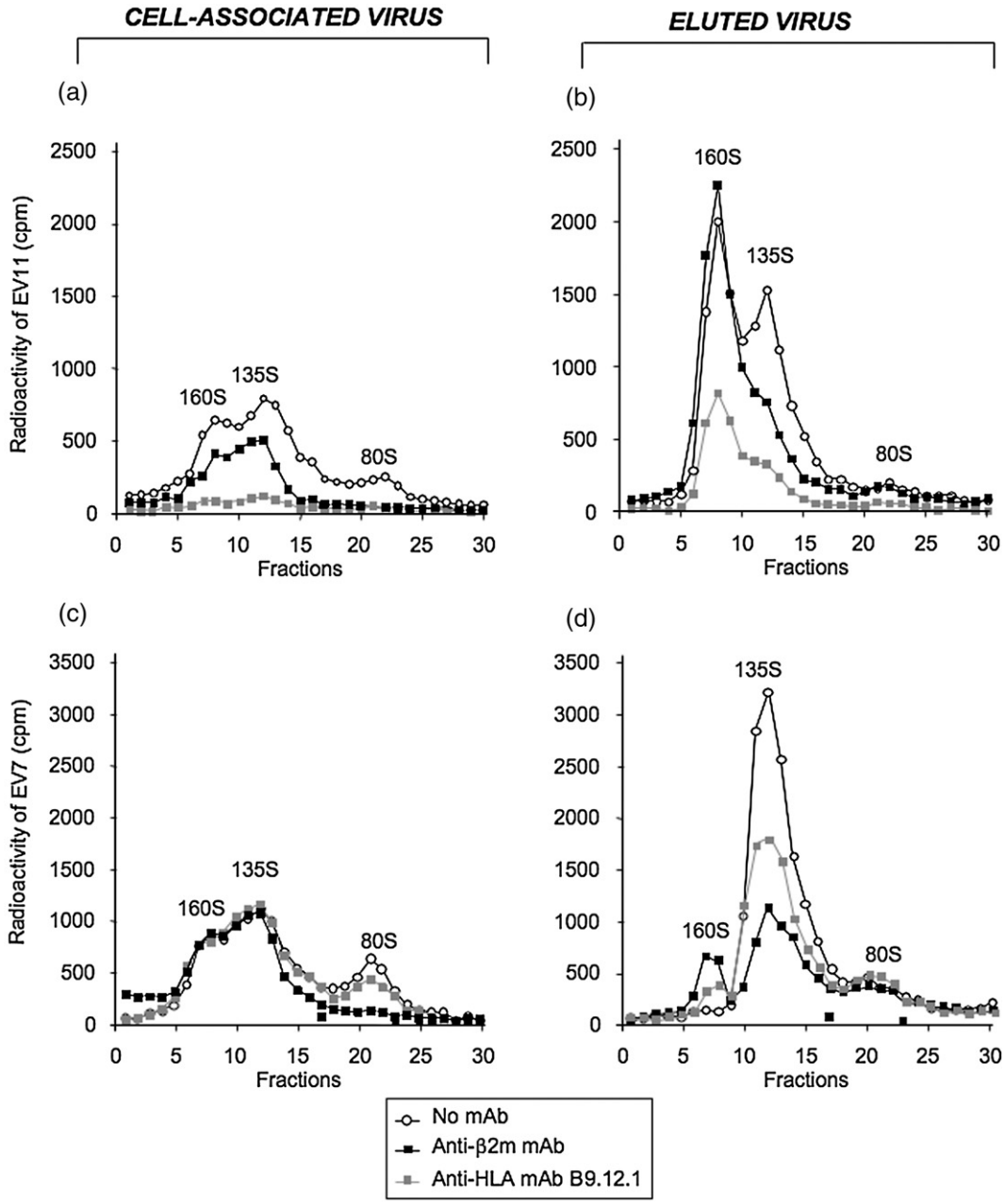


Fig. 6. Sedimentation gradient analysis of viral and subviral particles formed in the presence of anti-β2m and anti-HLA mAbs. RD cell monolayers were incubated with radiolabeled EV11 (HU-1108/89; MOI 50 PFU/cell) or EV7 (Wallace; MOI 50 PFU/cell) in the presence of the anti-β2m mAb MCA1115 (5 μg/ml) in black squares, or the anti-HLA mAb B9.12.1 (1:100) in gray squares or in the presence of DMEM without mAb in empty circles. Unbound virus was removed and cells were incubated in the presence of the same mAbs or medium alone. Cell-associated (a and c) and eluted (b and d) viral and subviral particles were separated by sucrose gradient centrifugation, then fractionated and quantified.

pits, lipid rafts or caveolae (DeTulleo and Kirchhausen, 1998; Gromeier and Wetz, 1990; Marjomaki et al., 2002; Powell et al., 1998; Roivainen et al., 1996; Shafren et al., 1997a,b; Stuart et al., 2002a; Triantafilou et al., 1999). In the search for coreceptor molecules that could be involved, together with DAF, in EV11 entry into cells, we investigated the role of class I HLA molecules in RD cell infection by EV11. Indeed, a role of β2-microglobulin in the infectious cycle of EV7 and other echovirus serotypes has been suggested and our results confirm that β2-m is involved in later steps of the entry process (Ward et al., 1998). Class I HLA molecules have been shown to play a role in post-binding events of the CAV9 infectious cycle (Triantafilou et al., 1999, 2002). In the present study, our results strongly suggest a role for class I HLA molecules in the early steps the EV11 life cycle, including attachment and entry. Indeed, EV11 infection of RD cells was synergistically inhibited by mAbs directed against the class I HLA heavy chain B9.12.1 and human β2m. Moreover, we found that 135S A-particle production was reduced when the cells were treated with anti-HLA mAb B9.12.1 and, to a lesser extent, with anti-β2m MCA1115 mAb, pointing to a role of HLA molecules in A-particle formation. In addition, we found that Daudi cells, a β2m-deficient B cell line (Klein et al., 1968), could not be infected by EV11, whereas Raji cells, which express high levels of class I HLA molecules at their surface, produced infectious viruses. Although we cannot rule out that other factors between the two cell lines could play a role, our results reinforce the hypothesis of an implication of class I HLA molecules in certain EV11 strain infection.

Indeed, in contrast to CAV9 and EV7, class I HLA molecules play a role in EV11 attachment to RD cells. This argues for a physical association between EV11 and the heavy chain of class I HLA molecules spanning the epitope recognized by mAb B9.12.1. However, the blocking effect of B9.12.1 was partial in our experiments,

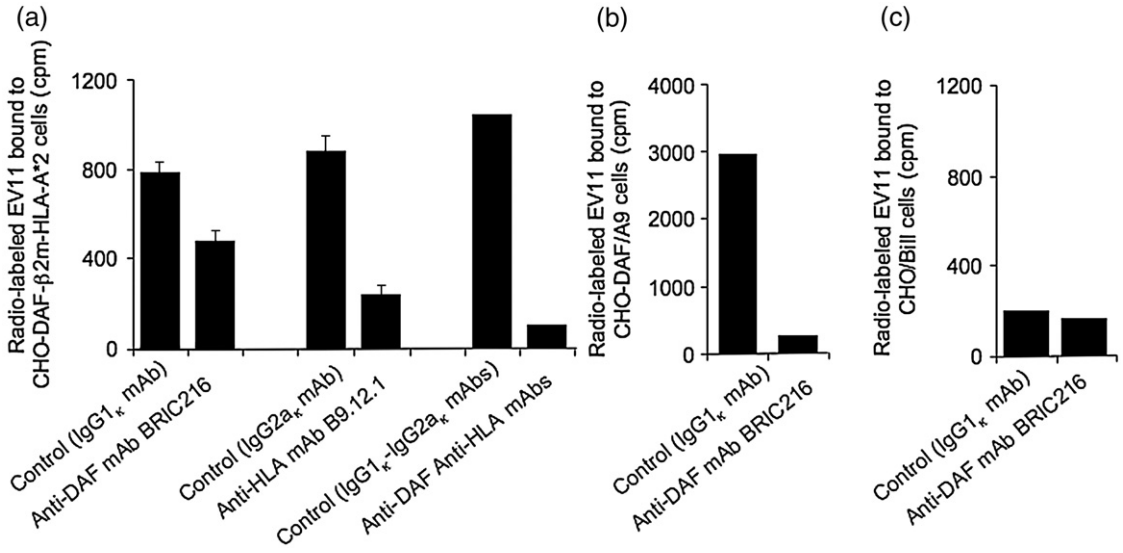


Fig. 7. Inhibition of enterovirus binding to CHO cells transfected with DAF (CHO-DAF/A9 cells) and β2m-HLA-A*2 (CHO-DAF-β2m-HLA-A*2 cells) or an empty vector (CHO/Bill cells). (a) CHO-DAF-β2m-HLA-A*2 cells were incubated in the presence of anti-DAF (BRIC216), anti-β2m (MCA1115), and anti-HLA (B9.12.1) mAbs, control isotypes (IgG1κ, IgG2aκ) or DMEM. (b) CHO-DAF/A9 cells and (c) CHO/Bill cells were incubated in the presence of anti-DAF (BRIC216) or control isotype (IgG1κ). Cells were then incubated with radiolabeled EV11 (HU-1108/89). Bound radiolabeled virus levels were determined by liquid scintillation counting. Results are expressed as the means of two experiments ± SD.

suggesting either that other receptor molecules are used to mediate EV11 entry into RD cells or that the class I HLA molecule domain that interacts with EV11 is only partially masked by mAb B9.12.1. In addition, the data shown in Figs. 1 and 5, which suggest that anti-β2m MCA1115 mAb inhibits EV11 cell cycle but not its binding, that mAb B9.12.1 only partially inhibits EV11 binding but impairs infection, and that both antibodies reduce EV7 infection while not affecting its binding point to a role for these antibodies in host-binding events that are important in the virus cell cycle. The later steps of the virus life cycle were not studied in this work and the underlying mechanisms remain to be determined.

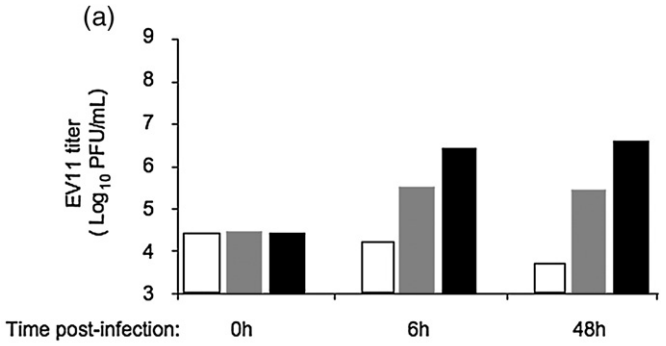
Cross-inhibition studies have shown that mAbs B9.12.1, W6/32 and B1.23.2 target different clusters of epitopes (Layet et al., 1984; Malissen et al., 1982; Rebai and Malissen, 1983). This may explain why only mAbs B9.12.1 is active against EV11 infection. The efficiency of mAb inhibition of EV11 infection could also depend on the heavy chain haplotype, which determines their ability to bind all or only certain HLA molecules. Indeed, B9.12.1 is a panspecific mAb that inhibits EV11 infection, whereas B1.23.2 recognizes only some of the molecules forming complexes with β2m and does not inhibit EV11 infection (Rebai and Malissen, 1983). However, this hypothesis is ruled out by the fact that W6/32, which recognizes all class I HLA-A, -B and -C molecules independently of the haplotype, does not inhibit EV11 infection (Parham et al., 1979). This suggests that the ability of mAb B9.12.1 to affect viral infection does not depend on a certain HLA-haplotype, but on the generic HLA epitope this mAb binds to.

DAF has been reported to play a role within the receptor complex for EV11 in several cell types (Powell et al., 1997; Stuart et al., 2002b). Here we provide evidence that entry into RD cells of (at least some) EV11 strains is a DAF-independent mechanism. Nevertheless, we found that one of the EV11 isolates bound to DAF on CHO cells stably transfected with the human DAF gene, probably in relation with a large expression of human DAF on transfected cells. The difference between CHO and RD cells could be explained by the different levels of expression of DAF at the cell surface, as confirmed by flow cytometry analysis in this study (data not shown). Most of the echovirus strains that have been shown to use DAF as a receptor, including EV11 strains, bind to the SCR3 domain (Lea et al., 1998). This finding confirmed here by the inhibition of EV11 binding to DAF-expressing CHO cells by an anti-DAF mAb specific for the SCR3 domain. However, CAV21 binds DAF via the SCR1 domain (Shafren et al., 1997a). Our experiments showing the lack of inhibition of RD cell infection by anti-DAF mAb directed against SCR3 raise the possibility that EV11 interacts with domains of the DAF molecule not recognized by this mAb. Nevertheless, our findings showing that SCR3 is involved in RD cell infection by other EV11 strains (Lea et al., 1998) points rather to strain-specific differences. In addition, EV11 binding to CHO cells expressing DAF and the HLA monochain was inhibited not only by the anti-DAF mAb, but also and more importantly by the anti-HLA mAb B9.12.1, indicating that both DAF and HLA could be involved in viral binding, depending on the cellular context. Overall, the diversity of molecules and mechanisms involved in enterovirus entry into host cells might ensure optimal permissiveness to virus infection.

Although CHO cells were able to replicate and produce EV11 after transfection with purified viral RNA, infection of rodent cells expressing DAF and the HLA β2m-HLA-A*2 monochain led to A-particle generation but not to a productive infection. We cannot rule out that, although the chimeric HLA monochain has been shown to be functional in terms of antigen presentation (Firat et al., 2001; Pascolo et al., 1997; Snyder et al., 2004), it does not appear to be fully functional for viral infection, in contrast with the natural class I HLA molecules. It is likely that other cellular factors are required for EV11 to complete its replicative cycle in CHO cells.

Our experiments thus suggest that HLA molecules are necessary but not sufficient to mediate EV11 entry into RD cells. Coreceptor molecules or additional receptor molecules have been implicated in the entry of other viruses that use HLA class I molecules. CAV9 uses integrin αvβ3 and GRP78 as receptor molecules, whereas class I HLA molecules are only involved in the internalization step (Triantafilou et al., 1999, 2002). The role of integrins and GRP78 in EV11 infection remains to be tested. Class I HLA heavy chain has been shown to act as a receptor for adenovirus type 5, with human fibronectin type III acting as a coreceptor (Hong et al., 1997). SV40 also binds Class I HLA molecules on the cell surface, but a coreceptor appears to be needed too (Anderson et al., 1998; Tsai et al., 2003). The other molecules involved, together with class I HLA molecules, in the early steps of EV11 infection remain to be identified.

In conclusion, we show here that HLA class I molecules are involved in the early steps of EV11 infection of RD cells. These molecules are involved in viral attachment and might also play a role in A-particle formation. HLA molecules appear to participate in a complex interplay



(c)

	Membrane Expression		
	DAF	Class I HLA	
		β2m	Heavy chain
□ Daudi cells	3.82	1.39	1.37
■ Raji cells	1.21	5.33	4.91
■ U937 cells	3.29	4.98	2.73

Fig. 8. Echovirus replication in leukocytic cell lines. Suspension cultures of Daudi (white), Raji (gray) and U-937 (black) cells were infected with EV11 (HU-1108/89) (a). Daudi cells were also transfected with 1 μg of purified viral EV11 (HU-1108/89) RNA in the absence or in the presence of RNAse (100 μg/mL) using Effectene® reagent and the infection was allowed to proceed for 5 h at 37 °C in 5% CO_2 (b). The infectivity of each sample was assessed by plaque assay on RD cells and the results were expressed in number of PFU per μg of viral RNA ± SD. The infectious titers were determined by plaque assay and are shown at the top. (c) Membrane expression of DAF and β2m-class I HLA molecules measured by flow cytometry is shown in the table at the bottom. The results are expressed as a mean fluorescence intensity (MFI) ratio calculated by dividing the observed MFI in the presence of the antibody by the MFI in the negative control. A ratio ≥ 1.5 was considered positive.

of surface molecules acting as coreceptors, including DAF. The other components and related pathways remain to be determined.

Materials and methods

Virus strains

All EV11 strains used in this study, including the prototype strain Gregory, the clinical isolates HU-1108/89 and RO-91/91 and the environmental isolate FI-06666/89 have been described elsewhere (Chevaliez et al., 2004). In addition, control strains, including echovirus 7 (Wallace) and poliovirus type 1 (Mahoney), were propagated in RD and HEp-2C cells, respectively. Viral stocks were titrated by plating appropriate dilutions of infected RD cells and counting plaque-forming units (PFU).

Cell culture

RD and HEp-2C (human epidermoid larynx carcinoma) cells were maintained in DMEM (Dulbecco's modified Eagle's medium) containing 4.5 g/l glucose, 0.02 M tricine and 0.004 g/l D-biotin (BioMedia), supplemented with 10% newborn calf serum (BioMedia). U-937 (human histiocytic lymphoma) cells, Daudi (human Burkitt lymphoma, β2m-deficient) (Klein et al., 1968) and Raji (human Burkitt lymphoma) cells were propagated as non-adherent stationary cultures in RPMI 1640 medium (Life Technologies), containing 25 mM HEPES (Life Technologies) and supplemented with 10% fetal calf serum (FCS) (BioMedia). Chinese hamster ovary (CHO) cell clones stably transfected with the empty expression vector (CHO-Bill) or with a plasmid expressing human DAF (CHO-DAF/A9) were obtained from D.M. Lublin (Faculty of Medicine, Washington) (Clarkson et al., 1995; Coyne et al., 1992). CHO cells were cultured in Ham's F-12 medium (Life Technologies) supplemented with 10% FCS and 0.25 mg/mL Geneticin (Life Technologies). Cells were cultured at 37 °C in an atmosphere containing 5% CO_2.

Antibodies

Monoclonal antibody (mAb) BRIC216 (IgG1κ), that recognizes the SCR3 domain of DAF, was obtained from IBGRL and purified from hybridoma culture supernatant by affinity chromatography on Protein A Sepharose (resuspended in 0.1 M glycine buffer pH7). MAb MCA1115 (IgG1κ), that recognizes human β2m, was used after isolation from tissue culture supernatant (Serotec) and purification by affinity chromatography on Protein A Sepharose (resuspended in PBS, 0.09% sodium azide (NaN_3)). MAb W6/32 (IgG2aκ), that recognizes a monomorphic epitope composed of the heavy chain and β2m of class I HLA molecules (Parham et al., 1979), was obtained from BIODESIGN following purification from hybridoma supernatant by affinity chromatography on Protein A Sepharose (resuspended in PBS, 1 mg/mL BSA, 10 mM NaN3). MAb B9.12.1, that also binds class I HLA-A, HLA-B, and HLA-C molecules, and MAb B1.23.2 (IgG2aκ), that recognizes HLA-A and HLA-B molecules (Malissen et al., 1982; Rebai and Malissen, 1983), were recovered from hybridoma supernatants without further purification or were obtained from BIODESIGN after purification by ion-exchange chromatography and resuspension in water. Polyclonal rabbit anti-EV11/HU-1108/89 antibody was kindly provided by G. Berensci (Department of Virology, "Bela Johan National" Center of Epidemiology, Budapest, Hungary) (El-Sageyer et al., 1998). Anti-mouse and anti-rabbit immunoglobulins conjugated to fluorescein isothiocyanate (FITC) were obtained from Biorad. Purified immunoglobulin isotype controls IgG1κ (MOPC-21) and IgG2aκ (UPC-10), recovered from ascites, were purchased from Sigma. Purified mouse whole IgG fraction was obtained from Pierce.

*Purification of the soluble monochain class I HLA molecule β2m-HLA-A*2*

We used a previously described supernatant of CHO cells expressing the soluble recombinant monochain β2m-HLA-A*2 (SC-A2) (Lone et al., 1998; Motta et al., 1998). Briefly, an SC-A2 construct was engineered by connecting the first three domains of HLA-A*2 heavy chain to human β2m through a 15-amino-acid spacer and transfected into CHO cells. Clones resistant to methotrexate were selected and the level of the secreted soluble monochain was monitored in the culture supernatant by ELISA in microtitration plates coated with an appropriate monoclonal antibody (Abastado et al., 1995; Lone et al., 1998). The recombinant soluble monochain was purified from the supernatant of HLA-A*2 transfected cells by means of affinity chromatography using the anti-β2m mAb MCA1115, concentrated by ultrafiltration using Vivaspin centrifugal concentrators (Sartorius AG, Goettingen, Germany) and eluted in PBS containing protease inhibitors (Complete, Boerhinger-Mannheim, Mannheim, Germany). The concentration of purified molecule β2m-HLA-A*2 in the eluate was measured in mg/L by means of the N Latex β2-

Microglobulin test in a Nephelometer II Analyzer (Behring, Marburg, Germany).

Virus blocking assays

96-well plates of RD cells at 80% confluence were washed with serum-free DMEM and then incubated with 50 µl of the anti-DAF mAb BRIC216 (40 µg/mL), the anti-β2m mAb MCA1115 (5 µg/mL), the anti-HLA mAbs (B9.12.1 from hybridoma supernatants (1:100) or purified (10 µg/mL), B1.23.2 (1:100) or W6/32 (10 µg/mL)), or the negative controls (mouse IgG fraction or DMEM) for 1 h at 37 °C. Cell monolayers were then challenged with 100 µl of 10-fold serial dilutions of viral stocks (10^8 PFU/mL) and the plates were incubated at 37 °C for 40 h. Infected cell monolayers were stained with crystal violet-ethanol solution. We also studied concentration-dependent mAb blockade of EV11 infection in monolayers of RD cells in 96-well plates previously incubated with the anti-β2m mAb MCA1115 at concentrations of 0.16 to 5.00 µg/mL, alone or in combination with the anti-HLA mAb B9.12.1 (hybridoma supernatant) at a dilution of 1:100, 1:500 or 1:1000. The plates were incubated for 1 h at 37 °C before challenge with 10^3 PFU of EV11 (HU-1108/89) per well for 40 h at 37 °C. We quantified cell survival after infection by incubating monolayers with crystal violet-ethanol solution, washing them with distilled water and reading the plates at a wavelength of 560 nm with a 96-well plate reader. The experiments were run in quadriplicate and the results are expressed as means ± standard deviations (SD) of the ratio of infected cell lysis relative to uninfected control cell monolayers.

Assessment of mAb effect on EV11 genomic RNA production in infected cells

RD cells were treated with the anti-β2m mAb MCA1115 (5 µg/mL) or the anti-HLA mAb B9.12.1 (1:100) at various times before or after infection with EV11 (HU-1108/89) at a multiplicity of infection (MOI) of 1 PFU/cell in 12-well plates for 5 h at 37 °C. The cell monolayers were then washed three times with PBS and total RNA was extracted.

RNA isolation and reverse transcription

Total RNA was extracted with the RNable reagent (Eurobio, Montpellier, France), according to the manufacturer's instructions. The RNA pellet was dissolved in 20 µl of DEPC-treated water (Invitrogen, Carlsbad, California) and the absorbance of the resulting solution was determined at 260 nm. RNA was reverse transcribed with 10 pmol of the previously described EUC2a primer (Caro et al., 2001). The reaction mixture (20 µl) contained 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM dNTPs, 2 mM DTT, 20 U of ribonuclease inhibitor (RNasin, Promega, Madison, Wisconsin) and 50 U of Superscript II reverse transcriptase (Invitrogen). The reaction mixture was incubated for 30 min at 50 °C and then for 5 min at 95 °C to inactivate the enzyme. The cDNA products were purified with the QIAquick PCR Purification kit (Qiagen, Valencia, California) and eluted in 50 µl of 10 mM Tris–Cl pH 8.5.

Real-time quantitative PCR

Primers specific for EV11 HU-1108/89 2A genomic region (5′-CACAACGATTGGCAAAACTGTG-3′ and 5′-GAGGATGACGCGATG-GAGCA-3′) were used to amplify a 376-bp DNA fragment. The PCR mixture contained 5 µl each of the forward and reverse primers (final concentration 600 nM), 25 µl of 2X Real-time SYBR® Green I mix (qPCRTMMastermix for SYBR® Green I, Eurogentec, Seraing, Belgium), and 5 µl of cDNA. Real-time PCR was performed with an ABI Prism 7700 automatic sequencer (Applied Biosystems, Foster City, California), using universal cycling conditions (2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C). Cycle threshold (CT) values were determined by automatic threshold analysis with ABI Prism version 1.0 software. Dissociation curves were plotted and the amplified products were visualized by electrophoresis in 2% agarose gels. Quantification was carried out by comparing the results with a standard curve run in parallel, created with serial 10-fold dilutions of EV11 viral RNA (EV11/HU-1108/89).

Virus purification and RNA extraction

RD cells were infected at high multiplicity (25–50 PFU/cell) for 30 min at 37 °C. The cell monolayers were then incubated in DMEM supplemented with 2% FCS at 37 °C until complete cell destruction was observed (i.e. within 24–48 h). Cells were subjected to three freeze–thaw cycles and cell debris was removed by centrifugation (12 000 *g*, 30 min, 4 °C). The resulting viral suspensions were concentrated by ultracentrifugation (150 000 *g*, 3 h, 4 °C). Viral concentrates were purified by centrifugation on a CsCl gradient (45% CsCl w/v in PBS), as previously described (Blondel et al., 1983). Virus-containing fractions were pooled and CsCl was eliminated on PD-10 columns (Pharmacia Biotech, Uppsala, Sweden). RNA was extracted directly from purified virus stocks by treatment with proteinase K (Eurobio) at a final concentration of 0.5 mg/mL for 15 min at 37 °C and 30 min at 50 °C, followed by phenol–chloroform (v/v) extraction at 60 °C and ethanol precipitation. The RNA pellet was dried and resuspended in DEPC-treated water. Viral RNA was quantified by UV spectrophotometry and the quality of the RNA was checked by electrophoresis in agarose gel containing formaldehyde (final concentration 2.2 M).

*Neutralization of EV11 infection with soluble monochain Class I HLA molecule β2m-HLA-A*2*

Soluble monochain Class I HLA molecule β2m-HLA-A*2 was made up to volume at concentrations ranging from 2.5 µg/mL to 321.5 µg/mL in PBS and incubated with 0.1 PFU of EV11 (HU-1108/89) or poliovirus type 1 (PV1/Mahoney), used as negative control. β2m-HLA-A*2 was then incubated at 37 °C for 1 h and applied to 96-well plates of RD cells at 80% confluence. The cells were incubated for 3 h at 37 °C. The monolayers were then washed three times with PBS and total RNA was extracted as previously described. For EV11 and poliovirus RNA amplification, reverse transcription followed by real-time quantitative PCR were performed as described above. The amounts of viral RNA were expressed in arbitrary units for the different concentrations of β2m-HLA-A*2, at the time of infection and 3 h later, and were compared to the negative control.

Transfection of RD cells with viral RNA

RD cell monolayers were incubated in 12-well plates with mAbs or negative controls (PBS 0.09% NaN_3 or glycine 0.1 M pH 7) for 1 h at 37 °C. The cells were then transfected with 1 µg of purified EV11 viral RNA (HU-1108/89, FI-06666/89) or PV1/Mahoney viral RNA, by using Effectene®, according to the manufacturer's instructions (Qiagen). Viral RNA (1 µg) was mixed with Condensation Buffer (Buffer EC) and Enhancer (1:8 ratio) for 5 min at room temperature. We then added the Effectene® transfection reagent (1:10 ratio) and the RNA-Effectene mixtures were incubated for 10 min at room temperature. RD cells were washed once with serum-free DMEM. The cells were then overlaid with the RNA-Effectene mixture in DMEM supplemented with 2% FCS. Cells were incubated for 5 h at 37 °C in an atmosphere containing 5% CO_2. Supernatants and cells were harvested. Cells were subjected to three freeze–thaw cycles, cell debris was removed by centrifugation (12 000 *g*, 30 min, 4 °C) and virus production was determined by plaque assays on RD cells. The results were expressed in PFU per µg of RNA. We checked that the viral progeny resulted from transfection with viral RNA and not from residual infectious virions by treating viral RNA solutions with RNAse A (100 µg/mL) for 20 min at room temperature and using the products of this reaction to transfect RD cells.

Radiolabeled virus binding blocking assays

Confluent RD or HEp-2C cells were shaken for 30 min at 37 °C in the presence of the virus at an MOI of approximately 50 PFU/cell. DMEM supplemented with 2% FCS was added and the cells were incubated for 2 h at 37 °C. The medium was then replaced by methionine-free medium, in which the cells were incubated for 30 min, before adding 600 μCi of 35S-methionine. Cells were lysed 24–48 h after infection, frozen and thawed three times, and cell debris was removed by centrifugation (12000 *g*, 30 min, 4 °C). Viral preparations were clarified and purified by CsCl density gradient centrifugation as previously described (Blondel et al., 1983). Purified viruses were desalted by gel filtration on a PD-10 column (Pharmacia Biotech) and stored at −70 °C until analysis. We then determined the specific activity (CPM/PFU) of each purified radiolabeled virus. Cell suspensions on ice were used to study virus adsorption; 1.5×10^6 to 5×10^6 cells were treated for 1 h at 37 °C with the anti-DAF mAb BRIC216 (40 μg/mL), the anti-β2m mAb MCA1115 (5 μg/mL), the anti-HLA mAbs (B9.12.1 (1:100), B1.23.2 (1:100) or W6/32 (10 μg/mL)) or with the isotype controls (IgG1κ and IgG2aκ). The cells were then infected with purified radiolabeled virus at an MOI of 10 PFU/cell in DMEM supplemented with 2% FCS for 2 h on ice with gentle shaking. The cells were then washed twice with 2% FCS in DMEM. Cell-associated radioactivity was quantified by scintillation counting. Percentages of bound viruses were calculated relative to the mock-treated sample (DMEM without mAbs).

Sedimentation gradient analysis of viral and subviral particles

Suspensions of 1×10^7 RD cells were either mock-treated with DMEM or treated with the anti-DAF mAb BRIC216 (40 μg/mL), the anti-β2m mAb MCA1115 (5 μg/mL) or the anti-HLA mAb B9.12.1 (1:100) for 1 h at 37 °C in a shaker. The cells were pelleted by centrifugation (1000 *g*, 5 min, 4 °C) and radiolabeled virus (MOI of 50 PFU/cell) binding experiments were run for 2 h on ice. Unbound viruses were removed by washing and infection was allowed to proceed in the presence or absence of mAbs for 30 min (EV11/HU-1108/89) or 2 h (EV7/Wallace) at 37 °C in a shaker. The cells were pelleted and the supernatants containing the virus particles were harvested. The cells were resuspended in 1 ml DMEM and lysed by adding NP-40 (0.2% final concentration, Calbiochem, La Jolla, California). Insoluble cell debris was removed by centrifugation. Radiolabeled virus particles present in supernatants or cell lysates were then sedimented by centrifugation through 15–30% (w/v in PBS) sucrose gradients for 2 h at 287000 *g* and 4 °C in a Kontron TST 41.14 rotor. Radioactivity was quantified in the fractions by scintillation counting. Similar binding experiments were performed on CHO cells stably transfected with a gene encoding human DAF or monochain HLA molecules.

Transfection of CHO cells and selection of stable transfectants

CHO-DAF/A9 cells were transfected with a plasmid encoding a monochain Class I HLA molecule (β2m-HLA-A*2) in which the C-terminus of human β2m was covalently linked to the N-terminus of a chimeric human/mouse heavy chain (domains α1-α2 of HLA-A*0201, linked to α3-transmembrane and intracytoplasmic domains of the murine H-2Db molecule) (Firat et al., 2001; Pascolo et al., 1997). The monochain gene was inserted into pBluescript II KS (−), which contains a hygromycin resistance gene. Subconfluent CHO-DAF/A9 cells cultured in 35-mm dishes were transfected with the FuGENE 6 transfection reagent. Hygromycin B (Invitrogen) was added to a final concentration of 300 μg/mL 24 h after transfection to select resistant cells. Transfected CHO cell populations were enriched in hygromycin-resistant cells through two cycles of selection of HLA-A positive clones, using an indirect technique based on Dynabeads M-450 (DYNAL A. S, Oslo, Norway) (Deckert et al., 1992). Hygromycin-resistant CHO cells were incubated with the anti-HLA mAb B9.12.1 on ice for 30 min. They were washed in Ham's F-12 medium supplemented with 2% FCS and incubated with Dynabeads coated with rat anti-mouse IgG2a for 30 min on ice. The selected clones were resuspended in Ham's F-12 medium supplemented with 10% FCS and 300 μg/mL hygromycin B at confluence. The expression of DAF, β2m and HLA class I heavy chain on selected cells was monitored by flow cytometry, using the specific mAbs described above.

Detection of cell membrane protein expression by flow cytometry

Surface expression of DAF, β2m and the HLA class I heavy chain was analyzed by flow cytometry in RD cells, leukocytes (Daudi, Raji, U-937) and transfected CHO cells. Dispersed cells (10^6) were incubated on ice with the appropriate mAbs (10 μg/mL except mAb B9.12.1 (1:100)) for 30 min in staining buffer (PBS (Eurobio) containing 1% bovine serum albumin (wt/v), fraction V and 0.1% NaN_3 (wt/v)). The cells were then washed with staining buffer, pelleted by centrifugation at 900 *g* for 6 min, resuspended in 100 μl FITC-conjugated anti-mouse antibody (diluted 1:100 in staining buffer), and incubated on ice for 30 min. The cells were again washed and pelleted, resuspended in staining buffer, and 10000 events were analyzed for DAF, β2m and HLA class I heavy chain expression with a FACScan cytometer (Becton Dickinson, San Diego, California). Membrane protein expression was quantified on leukocyte lines by determining mean fluorescence intensity (MFI) for each stain and calculating the MFI ratio (MFIR) by dividing the MFI obtained with a specific antibody by the MFI obtained with control IgG1κ (MOPC-21) or IgG2aκ (UPC-10). An MFIR ≥ 1.5 was considered positive.

Virus infectivity assays

Subconfluent cell monolayers were infected with EV11 strains (Gregory or HU-1108/89) at an MOI of 1, 10 and 50 PFU/cell and incubated in DMEM supplemented with 2% FCS at 37 °C for 48 h. For suspension culture, Daudi, Raji and U-937 cells were counted, washed once in Hank's balanced salt solution (Invitrogen), infected in RPMI 1640 supplemented with 2% FCS at an MOI of 10, 50 and 100 PFU/cell and incubated at 37 °C for 6 or 48 h. Supernatants and cells were harvested at various times after infection. The cells were subjected to three freeze–thaw cycles, cell debris was removed, and the infectious virus titer was determined by plaque assay on RD cells.

Detection of positive and negative strands of EV11 genome

Subconfluent CHO-Bill, CHO-DAF/A9, CHO-DAF-β2m-HLA-A*2 and RD cells were infected with EV11 (HU-1108/89) at an MOI of 10 PFU/cell and incubated for 30 min at 37 °C. The inoculum was removed and the cells were washed three times with PBS, covered with 2 ml of medium supplemented with 2% FCS and incubated at 37 °C in an atmosphere containing 5% CO_2. Three and 6 h later the cells were washed three times with PBS and total RNA was extracted with RNable reagent (Eurobio). The RNA pellet was dissolved in 20 μl of DEPC-treated water (Invitrogen), and the absorbance of the resulting solution was determined at 260 nm. Both positive and negative strands of EV11 RNA were sought by RT-PCR. Total RNA (1 μg) was reverse transcribed with the EUC2a primer 5′-TCCAAATGCCGTATT-GAACC-3′ (10 pmol) or the EUG4a primer 5′-TCCACTATCACCACD-CAGGA-3′ (50 pmol) for positive and negative strand amplification, respectively. Reverse transcription was performed as described above, except that the cDNA products were eluted in 30 μl of 10 mM Tris-Cl pH 8.5 during purification with the QIAquick PCR Purification kit (Qiagen). Samples were treated with RNAse A solution (1 μg/μL) for 20 min at 37 °C. The cDNA product (3 μl) was added to a PCR mixture containing 67 mM Tris–HCl pH 8.8, 16 mM $(NH_4)2SO4$, 0.01% Tween 20, 2 mM MgCl2, 10 mM dNTPs, 2 U of Taq DNA polymerase (EurobioTaq, Eurobio) and 10 pmol of the PCR primers for amplification of the 376-bp

DNA fragment of the EV11 genomic region 2A. Amplification was achieved by 29 cycles of denaturation at 95 °C for 20 s, annealing at 45 °C for 1 min and elongation at 72 °C for 1 min, followed by a final cycle of denaturation at 95 °C for 20 s, annealing at 45 °C for 1 min and elongation at 72 °C for 10 min. Amplification products (5 µl) were analyzed by electrophoresis in 1.5% agarose gels. Gels were stained with ethidium bromide and viewed under UV transillumination.

Detection of intracellular viruses by flow cytometry

The procedure for fixing and staining infected cells for flow cytometry is described elsewhere (Laffin and Lehman, 1994; McSharry et al., 1990). Briefly, uninfected cells and cells infected with EV11 (HU-1108/89 strain, MOI 10 PFU/cell) were treated with trypsin 6 h after infection, washed once in a solution containing 10% heat-inactivated goat serum (Invitrogen), 0.002% Triton X-100, and 0.1% NaN_3 in PBS, and fixed by adding cold methanol to a final concentration of 90%. The fixed cells were counted in a hemocytometer and stored at −70 °C at a density of 10^6 cells per ml in 90% methanol. The fixative solution was removed and the cells were washed once in PBS and resuspended in polyclonal rabbit anti-EV11 (HU-1108/89) serum diluted (1:100) in the same washing solution then incubated at 4 °C for 1 h. The cells were pelleted by centrifugation, washed twice, resuspended in FITC-conjugated goat anti-rabbit antibody diluted (1:100) in the washing solution, and incubated at 4 °C for 45 min. The cells were washed twice, pelleted by centrifugation and resuspended in staining buffer. Ten thousand events were then analyzed for virus detection with a FACScan cytometer (Becton Dickinson).

Acknowledgments

We thank D.M. Lublin, L.M. Houdebine, G. Berencsi and C. Le Bouguenec for providing us with biological materials. We thank Bruno Blondel and Florence Colbere-Garapin for critical reading of the manuscript. This work was supported by grants to F.D. from the World Health Organization (WHO B3/181/125) from the Direction of the Transverse Research Programs of the Pasteur Institute (PTR 120) and from the French Ministry of Foreign Affairs (FSP 2001-168).

S. Chevaliez is grateful to Prof. J.-M. Pawlotsky for in-depth discussions, helpful comments on the manuscript, and his continuous support.

References

Abastado, J.P., Lone, Y.C., Casrouge, A., Boulot, G., Kourilsky, P., 1995. Dimerization of soluble major histocompatibility complex-peptide complexes is sufficient for activation of T cell hybridoma and induction of unresponsiveness. J. Exp. Med. 182 (2), 439–447.

Anderson, H.A., Chen, Y., Norkin, L.C., 1998. MHC class I molecules are enriched in caveolae but do not enter with simian virus 40. J. Gen. Virol. 79 (6), 1469–1477.

Bergelson, J.M., Shepley, M.P., Chan, B.M., Hemler, M.E., Finberg, R.W., 1992. Identification of the integrin VLA-2 as a receptor for echovirus 1. Science 255 (5052), 1718–1720.

Bergelson, J.M., St John, N., Kawaguchi, S., Chan, M., Stubdal, H., Modlin, J., Finberg, R.W., 1993. Infection by echoviruses 1 and 8 depends on the alpha 2 subunit of human VLA-2. J. Virol. 67 (11), 6847–6852.

Bergelson, J.M., Chan, M., Solomon, K.R., St John, N.F., Lin, H., Finberg, R.W., 1994. Decay-accelerating factor (CD55), a glycosylphosphatidylinositol-anchored complement regulatory protein, is a receptor for several echoviruses. Proc. Natl. Acad. Sci. U. S. A. 91 (13), 6245–6248.

Berinstein, A., Roivainen, M., Hovi, T., Mason, P.W., Baxt, B., 1995. Antibodies to the vitronectin receptor (integrin alpha V beta 3) inhibit binding and infection of foot-and-mouth disease virus to cultured cells. J. Virol. 69 (4), 2664–2666.

Blondel, B., Akacem, O., Crainic, R., Couillin, P., Horodniceanu, F., 1983. Detection by monoclonal antibodies of an antigenic determinant critical for poliovirus neutralization present on VP1 and on heat inactivated virions. Virology 126, 707–710.

Caro, V., Guillot, S., Delpeyroux, F., Crainic, R., 2001. Molecular strategy for 'serotyping' of human enteroviruses. J. Gen. Virol. 82 (1), 79–91.

Casasnovas, J.M., Springer, T.A., 1994. Pathway of rhinovirus disruption by soluble intercellular adhesion molecule 1 (ICAM-1): an intermediate in which ICAM-1 is bound and RNA is released. J. Virol. 68 (9), 5882–5889.

Chevaliez, S., Szendroi, A., Caro, V., Balanant, J., Guillot, S., Berencsi, G., Delpeyroux, F., 2004. Molecular comparison of echovirus 11 strains circulating in Europe during an epidemic of multisystem hemorrhagic disease of infants indicates that evolution generally occurs by recombination. Virology 325 (1), 56–70.

Clarkson, N.A., Kaufman, R., Lublin, D.M., Ward, T., Pipkin, P.A., Minor, P.D., Evans, D.J., Almond, J.W., 1995. Characterization of the echovirus 7 receptor: domains of CD55 critical for virus binding. J. Virol. 69 (9), 5497–5501.

Coyne, K.E., Hall, S.E., Thompson, S., Arce, M.A., Kinoshita, T., Fujita, T., Anstee, D.J., Rosse, W., Lublin, D.M., 1992. Mapping of epitopes, glycosylation sites, and complement regulatory domains in human decay accelerating factor. J. Immunol. 149 (9), 2906–2913.

Deckert, M., Kubar, J., Bernard, A., 1992. CD58 and CD59 molecules exhibit potentializing effects in T cell adhesion and activation. J. Immunol. 148 (3), 672–677.

DeTulleo, L., Kirchhausen, T., 1998. The clathrin endocytic pathway in viral infection. EMBO. J. 17 (16), 4585–4593.

El-Sageyer, M.M., Szendroi, A., Hutter, E., Uj, M., Szucs, G., Mezey, I., Toth, I., Katai, A., Kapiller, Z., Pall, G., Petras, G., Szalay, E., Mihaly, I., Gourova, S., Berencsi, G., 1998. Characterisation of an echovirus type 11′ (prime) epidemic strain causing haemorrhagic syndrome in newborn babies in Hungary. Acta. Virol. 42 (3), 157–166.

Firat, H., Tourdot, S., Ureta-Vidal, A., Scardino, A., Suhrbier, A., Buseyne, F., Riviere, Y., Danos, O., Michel, M.L., Kosmatopoulos, K., Lemonnier, F.A., 2001. Design of a polyepitope construct for the induction of HLA-A0201-restricted HIV 1-specific CTL responses using HLA-A*0201 transgenic, H-2 class I KO mice. Eur. J. Immunol. 31 (10), 3064–3074.

Gomez Yafal, A., Kaplan, G., Racaniello, V.R., Hogle, J.M., 1993. Characterization of poliovirus conformational alteration mediated by soluble cell receptors. Virology 197 (1), 501–505.

Goodfellow, I.G., Powell, R.M., Ward, T., Spiller, O.B., Almond, J.W., Evans, D.J., 2000. Echovirus infection of rhabdomyosarcoma cells is inhibited by antiserum to the complement control protein CD59. J. Gen. Virol. 81 (5), 1393–1401.

Goodfellow, I.G., Sioofy, A.B., Powell, R.M., Evans, D.J., 2001. Echoviruses bind heparan sulfate at the cell surface. J. Virol. 75 (10), 4918–4921.

Greve, J.M., Davis, G., Meyer, A.M., Forte, C.P., Yost, S.C., Marlor, C.W., Kamarck, M.E., McClelland, A., 1989. The major human rhinovirus receptor is ICAM-1. Cell 56 (5), 839–847.

Gromeier, M., Wetz, K., 1990. Kinetics of poliovirus uncoating in HeLa cells in a nonacidic environment. J. Virol. 64 (8), 3590–3597.

Haddad, A., Nokhbeh, M.R., Alexander, D.A., Dawe, S.J., Grise, C., Gulzar, N., Dimock, K., 2004. Binding to decay-accelerating factor is not required for infection of human leukocyte cell lines by enterovirus 70. J. Virol. 78 (6), 2674–2681.

Hong, S.S., Karayan, L., Tournier, J., Curiel, D.T., Boulanger, P.A., 1997. Adenovirus type 5 fiber knob binds to MHC class I alpha2 domain at the surface of human epithelial and B lymphoblastoid cells. EMBO J. 16 (9), 2294–2306.

Huttunen, P., Santti, J., Pulli, T., Hyypia, T., 1996. The major echovirus group is genetically coherent and related to coxsackie B viruses. J. Gen. Virol. 77, 715–725.

Karnauchow, T.M., Tolson, D.L., Harrison, B.A., Altman, E., Lublin, D.M., Dimock, K., 1996. The HeLa cell receptor for enterovirus 70 is decay-accelerating factor (CD55). J. Virol. 70 (8), 5143–5152.

Kim, M.S., Racaniello, V.R., 2007. Enterovirus 70 receptor utilization is controlled by capsid residues that also regulate host range and cytopathogenicity. J. Virol. 81 (16), 8648–8655.

Klein, E., Klein, G., Nadkarni, J.S., Nadkarni, J.J., Wigzell, H., Clifford, P., 1968. Surface IgM-kappa specificity on a Burkitt lymphoma cell in vivo and in derived culture lines. Cancer. Res. 28 (7), 1300–1310.

Kopecka, H., Brown, B., Pallansch, M., 1995. Genotypic variation in coxsackievirus B5 isolates from three different outbreaks in the United States. Virus Res. 38 (2–3), 125–136.

Laffin, J., Lehman, J.M., 1994. Detection of intracellular virus and viral products. Methods Cell Biol. 41, 543–557.

Layet, C., Le Bouteiller, P.P., N'Guyen, C., Mercier, P., Rosa, F., Fellous, M., Caillol, D.H., Jordan, B.R., Lemonnier, F.A., 1984. Transformation of LMTK-cells with purified HLA class I gene. VI. Serological characterization of HLA-B7 and AW24 molecules. Hum. Immunol. 11 (1), 31–45.

Lea, S.M., Powell, R.M., McKee, T., Evans, D.J., Brown, D., Stuart, D.I., van der Merwe, P.A., 1998. Determination of the affinity and kinetic constants for the interaction between the human virus echovirus 11 and its cellular receptor, CD55. J. Biol. Chem. 273 (46), 30443–30447.

Lone, Y.C., Motta, I., Mottez, E., Guilloux, Y., Lim, A., Demay, F., Levraud, J.P., Kourilsky, P., Abastado, J.P., 1998. In vitro induction of specific cytotoxic T lymphocytes using recombinant single-chain MHC class I/peptide complexes. J. Immunother. 21 (4), 283–294.

Lublin, D.M., Atkinson, J.P., 1989. Decay-accelerating factor: biochemistry, molecular biology, and function. Annu. Rev. Immunol. 7, 35–58.

Lukashev, A.N., Lashkevich, V.A., Koroleva, G.A., Ilonen, J., Karganova, G.G., Reznik, V.I., Hinkkanen, A.E., 2003. Molecular epidemiology of enteroviruses causing uveitis and multisystem hemorrhagic disease of infants. Virology 307 (1), 45–53.

Malissen, B., Rebai, N., Liabeuf, A., Mawas, C., 1982. Human cytotoxic T cell structures associated with expression of cytolysis. I. Analysis at the clonal cell level of the cytolysis-inhibiting effect of 7 monoclonal antibodies. Eur. J. Immunol. 12 (9), 739–747.

Marjomaki, V., Pietiainen, V., Matilainen, H., Upla, P., Ivaska, J., Nissinen, L., Reunanen, H., Huttunen, P., Hyypia, T., Heino, J., 2002. Internalization of echovirus 1 in caveolae. J. Virol. 76 (4), 1856–1865.

McSharry, J.J., Costantino, R., Robbiano, E., Echols, R., Stevens, R., Lehman, J.M., 1990. Detection and quantitation of human immunodeficiency virus-infected peripheral blood mononuclear cells by flow cytometry. J. Clin. Microbiol. 28 (4), 724–733.

Melnick, J.L., 1996. Enteroviruses : polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses, In: Fields, D.M.K.B.N., Howley, P.M., Channock, R.M., Melnick, J.L., Monath, T.P., Roizman, B., Straus, S.E. (Eds.), Fields Virology, third edition. Lippincott-Raven Publishers, Philadelphia, pp. 655–712.

Mendelsohn, C.L., Wimmer, E., Racaniello, V.R., 1989. Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. Cell 56 (5), 855–865.

Motta, I., Lone, Y.C., Kourilsky, P., 1998. In vitro induction of naive cytotoxic T lymphocytes with complexes of peptide and recombinant MHC class I molecules coated onto beads: role of TCR/ligand density. Eur. J. Immunol. 28 (11), 3685–3695.

Mullins, J.A., Khetsuriani, N., Nix, W.A., Oberste, M.S., LaMonte, A., Kilpatrick, D.R., Dunn, J., Langer, J., McMinn, P., Huang, Q.S., Grimwood, K., Huang, C., Pallansch, M.A., 2004. Emergence of echovirus type 13 as a prominent enterovirus. Clin. Infect. Dis. 38 (1), 70–77.

Neff, S., Sa-Carvalho, D., Rieder, E., Mason, P.W., Blystone, S.D., Brown, E.J., Baxt, B., 1998. Foot-and-mouth disease virus virulent for cattle utilizes the integrin alpha(v)beta3 as its receptor. J. Virol. 72 (5), 3587–3594.

Newcombe, N.G., Andersson, P., Johansson, E.S., Au, G.G., Lindberg, A.M., Barry, R.D., Shafren, D.R., 2003. Cellular receptor interactions of C-cluster human group A coxsackieviruses. J. Gen. Virol. 84 (11), 3041–3050.

Newcombe, N.G., Johansson, E.S., Au, G., Lindberg, A.M., Barry, R.D., Shafren, D.R., 2004. Enterovirus capsid interactions with decay-accelerating factor mediate lytic cell infection. J. Virol. 78 (3), 1431–1439.

Nilsson, K., Evrin, P.E., Welsh, K.I., 1974. Production of beta 2-microglobulin by normal and malignant human cell lines and peripheral lymphocytes. Transplant. Rev. 21, 53–84.

Oberste, M.S., Nix, W.A., Kilpatrick, D.R., Flemister, M.R., Pallansch, M.A., 2003. Molecular epidemiology and type-specific detection of echovirus 11 isolates from the Americas, Europe, Africa, Australia, southern Asia and the Middle East. Virus Res. 91 (2), 241–248.

Pallansch, M., Roos, R., 2001. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. In: Knipe, D., Howley, P. (Eds.), Fields Virology, Vol. 1. Lippincott Williams and Wilkins, Philadelphia, pp. 723–775.

Parham, P., Barnstable, C.J., Bodmer, W.F., 1979. Use of a monoclonal antibody (W6/32) in structural studies of HLA-A,B,C, antigens. J. Immunol. 123 (1), 342–349.

Pasch, A., Kupper, J.H., Wolde, A., Kandolf, R., Selinka, H.C., 1999. Comparative analysis of virus-host cell interactions of haemagglutinating and non-haemagglutinating strains of coxsackievirus B3. J. Gen. Virol. 80 (12), 3153–3158.

Pascolo, S., Bervas, N., Ure, J.M., Smith, A.G., Lemonnier, F.A., Perarnau, B., 1997. HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from beta2 microglobulin (beta2 m) HLA-A2.1 monochain transgenic H-2Db beta2 m double knockout mice. J. Exp. Med. 185 (12), 2043–2051.

Pettigrew, D.M., Williams, D.T., Kerrigan, D., Evans, D.J., Lea, S.M., Bhella, D., 2006. Structural and functional insights into the interaction of echoviruses and decay-accelerating factor. J. Biol. Chem. 281 (8), 5169–5177.

Powell, R.M., Ward, T., Evans, D.J., Almond, J.W., 1997. Interaction between echovirus 7 and its receptor, decay-accelerating factor (CD55): evidence for a secondary cellular factor in A-particle formation. J. Virol. 71 (12), 9306–9312.

Powell, R.M., Schmitt, V., Ward, T., Goodfellow, I., Evans, D.J., Almond, J.W., 1998. Characterization of echoviruses that bind decay accelerating factor (CD55): evidence that some haemagglutinating strains use more than one cellular receptor. J. Gen. Virol. 79 (7), 1707–1713.

Rebai, N., Malissen, B., 1983. Structural and genetic analyses of HLA class I molecules using monoclonal xenoantibodies. Tissue Antigens 22 (2), 107–117.

Roivainen, M., Piirainen, L., Hovi, T., Virtanen, I., Riikonen, T., Heino, J., Hyypia, T., 1994. Entry of coxsackievirus A9 into host cells: specific interactions with alpha v beta 3 integrin, the vitronectin receptor. Virology 203 (2), 357–365.

Roivainen, M., Piirainen, L., Hovi, T., 1996. Efficient RGD-independent entry process of coxsackievirus A9. Arch. Virol. 141 (10), 1909–1919.

Schmidtke, M., Selinka, H.C., Heim, A., Jahn, B., Tonew, M., Kandolf, R., Stelzner, A., Zell, R., 2000. Attachment of coxsackievirus B3 variants to various cell lines: mapping of phenotypic differences to capsid protein VP1. Virology 275 (1), 77–88.

Shafren, D.R., Bates, R.C., Agrez, M.V., Herd, R.L., Burns, G.F., Barry, R.D., 1995. Coxsackieviruses B1, B3, and B5 use decay accelerating factor as a receptor for cell attachment. J. Virol. 69 (6), 3873–3877.

Shafren, D.R., Dorahy, D.J., Ingham, R.A., Burns, G.F., Barry, R.D., 1997a. Coxsackievirus A21 binds to decay-accelerating factor but requires intercellular adhesion molecule 1 for cell entry. J. Virol. 71 (6), 4736–4743.

Shafren, D.R., Williams, D.T., Barry, R.D., 1997b. A decay-accelerating factor-binding strain of coxsackievirus B3 requires the coxsackievirus-adenovirus receptor protein to mediate lytic infection of rhabdomyosarcoma cells. J. Virol. 71 (12), 9844–9848.

Snyder, J.T., Belyakov, I.M., Dzutsev, A., Lemonnier, F., Berzofsky, J.A., 2004. Protection against lethal vaccinia virus challenge in HLA-A2 transgenic mice by immunization with a single CD8+T-cell peptide epitope of vaccinia and variola viruses. J. Virol. 78 (13), 7052–7060.

Stuart, A.D., Eustace, H.E., McKee, T.A., Brown, T.D., 2002a. A novel cell entry pathway for a DAF-using human enterovirus is dependent on lipid rafts. J. Virol. 76 (18), 9307–9322.

Stuart, A.D., McKee, T.A., Williams, P.A., Harley, C., Shen, S., Stuart, D.I., Brown, T.D., Lea, S.M., 2002b. Determination of the structure of a decay accelerating factor-binding clinical isolate of echovirus 11 allows mapping of mutants with altered receptor requirements for infection. J. Virol. 76 (15), 7694–7704.

Triantafilou, M., Triantafilou, K., Wilson, K.M., Takada, Y., Fernandez, N., Stanway, G., 1999. Involvement of beta2-microglobulin and integrin alphavbeta3 molecules in the coxsackievirus A9 infectious cycle. J. Gen. Virol. 80 (10), 2591–2600.

Triantafilou, K., Fradelizi, D., Wilson, K., Triantafilou, M., 2002. GRP78, a coreceptor for coxsackievirus A9, interacts with major histocompatibility complex class I molecules which mediate virus internalization. J. Virol. 76 (2), 633–643.

Tsai, B., Gilbert, J.M., Stehle, T., Lencer, W., Benjamin, T.L., Rapoport, T.A., 2003. Gangliosides are receptors for murine polyoma virus and SV40. EMBO J. 22 (17), 4346–4355.

Vuorinen, T., Vainionpaa, R., Heino, J., Hyypia, T., 1999. Enterovirus receptors and virus replication in human leukocytes. J. Gen. Virol. 80 (4), 921–927.

Ward, T., Pipkin, P.A., Clarkson, N.A., Stone, D.M., Minor, P.D., Almond, J.W., 1994. Decay-accelerating factor CD55 is identified as the receptor for echovirus 7 using CELICS, a rapid immuno-focal cloning method. EMBO J. 13 (21), 5070–5074.

Ward, T., Powell, R.M., Pipkin, P.A., Evans, D.J., Minor, P.D., Almond, J.W., 1998. Role for beta2-microglobulin in echovirus infection of rhabdomyosarcoma cells. J. Virol. 72 (7), 5360–5365.

Williams, C.H., Kajander, T., Hyypia, T., Jackson, T., Sheppard, D., Stanway, G., 2004. Integrin alpha v beta 6 is an RGD-dependent receptor for coxsackievirus A9. J. Virol. 78 (13), 6967–6973.

Zautner, A.E., Korner, U., Henke, A., Badorff, C., Schmidtke, M., 2003. Heparan sulfates and coxsackievirus-adenovirus receptor: each one mediates coxsackievirus B3 PD infection. J. Virol. 77 (18), 10071–10077.

Zautner, A.E., Jahn, B., Hammerschmidt, E., Wutzler, P., Schmidtke, M., 2006. *N*- and 6-*O*-sulfated heparan sulfates mediate internalization of coxsackievirus B3 variant PD into CHO-K1 cells. J. Virol. 80 (13), 6629–6636.