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Echovirus infection of rhabdomyosarcoma cells is inhibited by antiserum to the complement control protein CD59

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A number of echoviruses use decay accelerating factor (DAF) as a cellular receptor or attachment protein for cell infection. Binding of echovirus 7 to DAF at the cell surface, but not to soluble DAF in solution, triggers the formation of virus particles exhibiting an altered sedimentation coefficient ('A' particles) which are considered indicative of the particle uncoating process. We have previously demonstrated that antibodies to β_2-microglobulin block cell infection at a stage prior to 'A' particle formation and suggested that this reflects the involvement of β_2-microglobulin (or the associated MHC-I) in a virus–receptor complex that forms at the cell surface. We demonstrate here that antiserum to CD59 specifically blocks infection of rhabdomyosarcoma cells by a range of echoviruses, including viruses that bind DAF (e.g. echovirus 7) and those that use currently unidentified receptors other than DAF. The block occurs prior to 'A' particle formation and is cell-type specific. The potential role of CD59 as an active member, or passive participant, in the virus–receptor complex is discussed.

Introduction

Representatives of the picornavirus family, of which the echoviruses are a member of the enterovirus genus, use a range of different cell surface proteins as receptors. At least nine different receptors have been identified, some restricted to individual virus species such as the poliovirus receptor (PVR), and others which are used by several representatives within the family (Evans & Almond, 1998; Evans, 1997). Decay accelerating factor, DAF (CD55), a glycophosphatidylinositol-linked (GPI) 70 kDa protein possessing four extracellular domains exhibiting protein folds characteristic of short consensus repeat proteins, is one receptor that has been implicated in cell binding and infection by the haemagglutinating echoviruses (Powell *et al.*, 1998), enterovirus 70 (Karnauchow *et al.*, 1996), coxsackievirus A21 and the coxsackie B viruses (Shafren *et al.*, 1995). Some of these viruses can also use alternative receptors, though the distinction between dual tropism and receptor switching is complicated by the passage history of the virus isolates (Powell *et al.*, 1998).

The ability of a virus to bind to a target cell does not necessarily result in infection; the latter process may involve secondary events distinct from receptor binding, possibly mediated by additional cellular factors (Alkhatib *et al.*, 1996; Bai *et al.*, 1994; Deng *et al.*, 1996; Feng *et al.*, 1996). Evidence is accumulating for the involvement of secondary factors for cell infection by certain picornaviruses, including the echoviruses that bind DAF (Evans, 1997; Powell *et al.*, 1997) and coxsackievirus A21 (Shafren *et al.*, 1997*a*). Soluble DAF (sDAF) blocks virus binding to the cell surface by steric inhibition, which contrasts with the irreversible conformational changes induced in the poliovirus particle by soluble PVR (Kaplan *et al.*, 1990), or the rhinovirus particle by soluble derivatives of its receptor, ICAM-1 (Greve *et al.*, 1991; Hooverlitty & Greve, 1993). In contrast, echovirus type 7 (EV7) binding of DAF at the cell surface results in the formation of 135S particles (Powell *et al.*, 1997), the altered sedimentation coefficient of

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these 'A' particles reflects conformational changes that include the loss of the internal capsid protein VP4, indicative of an uncoating event involved in the infection process (Yafal *et al.*, 1993). Taken together these results suggest that additional determinants at the cell surface are required for infection by DAF-binding echoviruses. One potential candidate for a secondary factor is β_2-microglobulin, antibodies to which block echovirus infection of rhabdomyosarcoma (RD) cells in a cell-specific manner (Ward *et al.*, 1998). The observed block occurs post-attachment but prior to RNA translation and replication, though the precise mechanism remains unclear. Our studies suggest that EV7 enters a receptor 'complex' at the cell surface that is resistant to proteinase K and sDAF (Powell *et al.*, 1998; Ward *et al.*, 1998). Whether this complex consists solely of virus bound to DAF, or also contains the secondary factor(s) required for infection remains to be determined.

An involvement for β_2-microglobulin was identified by the cloning and characterization of the ligand for an antibody raised to Ohio HeLa cell membrane fractions that blocked echovirus infection (Ward *et al.*, 1998). An alternative approach, used in this communication, is to determine the ability of antisera to cell surface proteins, known to co-localize with DAF, to block virus infection. The identification of such proteins may help in the identification of other components of the sDAF-resistant receptor complex that forms during echovirus infection of permissive cells. We report here that polyclonal antiserum to the complement control protein CD59 blocks infection of RD cells by several echoviruses. This block is not mediated at the level of attachment, but during a post-binding event necessary for the uncoating of the virus and the formation of intracellular 'A' particles.

Methods

■ **Virus strains.** Echo- and coxsackie viruses were a kind gift from Brian Megson, Public Health Laboratory Service, Colindale, London, UK. The viruses were passaged and quantified in RD cells.

■ **Antibodies.** Polyclonal rabbit (purified Ig) and murine monoclonal anti-human CD59 antibodies (MEM 43, 43/5, YTH53.1, HelC1, HelC2, A35, 2/24) were obtained from B. P. Morgan, University of Wales College of Medicine, Cardiff, UK and P. J. Sims, Blood Research Institute, Milwaukee, USA. Anti-DAF (Ward *et al.*, 1994) monoclonal antibody (MAb) 854 was obtained from P. D. Minor, NIBSC, Potters Bar, UK. Anti-β_2-microglobulin MAb 1350 was obtained from Chemicon International, as was MAb PID6 directed against α_v integrins, and an anti-CD44 MAb. Anti-enterovirus MAb 5-D8/1 was obtained from Dako, and goat anti-mouse-immunoglobulin (Ig) β-galactosidase was obtained from Harlan Sera-lab. MAbs 308 and DF1513, directed against aminopeptidase N and the transferrin receptor, respectively, were obtained from NeoMarkers. MAbs to CD97 and CD66 were obtained from Pharmingen and D. Fox (University of Reading, UK), and the anti-CD46 MAb J4-48 was obtained from Serotec. Polyclonal antiserum to β5 was kindly provided by B. Cushley (IBLS, University of Glasgow, UK) and the polyclonal antiserum to IgE was purchased from Dako.

■ **Inhibition of infection with anti-CD59 antibodies.** Purified polyclonal anti-CD59 antibody was serially diluted twofold in DMEM and used to treat human RD cells in a 96-well format (10^5 cells per well) for 1 h at 37 °C. 10^4 $TCID_{50}$ of virus was added and infection allowed to proceed for 24 h prior to staining. Soluble recombinant CD59 (sCD59), obtained from B. P. Morgan, University of Wales College of Medicine, Cardiff, UK, was incubated with the antibody for 30 min at room temperature prior to addition to the cells to remove CD59-specific antibodies. MAbs directed against CD59 were tested for their ability to block infection using essentially the same assay. Antibodies were diluted in DMEM, incubated with 10^5 RD cells for 1 h at 37 °C and washed prior to the addition of 10^4 $TCID_{50}$ of virus. MAbs were cross-linked, where appropriate, by the addition of a saturating amount of secondary goat anti-mouse antiserum after washing. Incubation was continued for a further 1 h at 37 °C, at which point 10^4 $TCID_{50}$ of virus was added in the presence of a 1/100 or 1/1000 dilution of the original primary MAbs.

■ **Temporal analysis of the anti-CD59 block on infection.** RD cells were infected with EV7 at an m.o.i. of 1. Infection was allowed to proceed for 6 h at 37 °C before the cells were fixed and permeabilized with acetone–methanol. Intracellular virus antigen was detected using an anti-enterovirus VP1-specific MAb (5-D8/1, Dako) at a 1:400 dilution and an anti-mouse β-galactosidase conjugate (Harlan Sera-labs). X-Gal was added and the assay allowed to develop overnight at room temperature. The blue product was solubilized by the addition of SDS–NaOH (1%, 0·2 M), debris removed by centrifugation, and the absorbance of the samples measured at 560 nm. Parallel samples were treated for various lengths of time with CD59 antiserum by the addition of a 1/100 dilution of antiserum at appropriate times in all media, washes and virus preparation.

■ **Radio-labelled virus binding assay.** Approximately 10^4 c.p.m. of purified ^{35}S-labelled EV7 was incubated with 5×10^6 RD cells that had been pre-treated for 1 h with anti-DAF MAb 854 (1:1000 dilution), anti-CD59 polyclonal antiserum (1:100) or the DMEM control. Virus was allowed to bind for 1 h on ice, the cells washed twice with DMEM and the bound radioactivity quantified by scintillation counting. The percentage of bound virus was calculated relative to the mock-treated sample.

■ **Virus entry assay.** Virus entry to RD cells was performed essentially as described previously (Ward *et al.*, 1998). RD cells were treated with polyclonal anti-CD59 antiserum (1:100) or DMEM control and the susceptibility of bound radiolabelled virus to competition by sDAF assessed at various times after incubating cells at 37 °C.

■ **Single-step growth curve.** RD cells (5×10^5) were treated with polyclonal anti-CD59 antiserum (1:100 dilution) or DMEM alone for 1 h at 37 °C. EV7 (m.o.i. of 3) was adsorbed at room temperature for 30 min in the presence or absence of anti-CD59 antiserum, the virus was removed, the cells washed, and either antiserum (1:100) or DMEM added back to the cell monolayers. Samples were removed at various time-points, freeze–thawed three times and the virus quantified by $TCID_{50}$.

■ **Cold synchronized eclipse products.** 1×10^7 cells were either mock-treated with DMEM or treated with anti-CD59 antiserum (1:100) for 1 h at 37 °C with constant rotation (6 revs/min). The cells were pelleted and radiolabelled virus (approx. 2×10^5 c.p.m.) bound for 1 h on ice. Unbound virus was removed by washing and infection was allowed to proceed in the presence or absence of the polyclonal anti-CD59 antiserum for 1 h at 37 °C with constant rotation. Eluted virus was removed and cell-associated virus was released using 0·2% NP-40. Samples were sedimented through a linear 15–45% sucrose gradient

which was harvested in 1·5 ml fractions and the virus particles quantified by scintillation counting.

Results

Antibodies to CD59 block echovirus infection of RD cells

The uncoating of the EV7 particle during cell infection appears to require one or more secondary cellular factors after binding to the cell surface by DAF has occurred (Powell *et al*., 1997). In RD cells at least, we have demonstrated that antibodies to β_2-microglobulin, which in association with MHC-I co-localizes with DAF (Stang *et al*., 1997), block the uncoating process (Ward *et al*., 1998). We speculated that other DAF-associated molecules, such as CD59, which being GPI-anchored are also concentrated in cholesterol-rich lipid microdomains (Cerny *et al*., 1996; Lisanti & Rodriguez-Boulan, 1991), may also be involved. We therefore investigated whether antibodies to CD59 blocked DAF-mediated binding or infection of RD cells.

Polyclonal rabbit anti-human CD59 antiserum blocked infection of RD cells by similar titres of a range of echoviruses (Fig. 1*a*). The end-point antiserum titre varied from 1/400 for EV4 to 1/1600 for EV29 and EV6′, and was highest for echoviruses that use DAF alone, or DAF and an unidentified receptor for cell entry. However, echoviruses that are known not to use DAF, such as EV4 and EV9, were also blocked at broadly similar end-point titres. The effect of the anti-CD59 antiserum was not batch-dependent, as antiserum from an independent source was also shown to inhibit infection in a similar fashion (data not shown). The specificity of this block was demonstrated in several ways. Poliovirus type 3 and coxsackievirus B2 and B3, which respectively use PVR, CAR and CAR + DAF as receptors (Bergelson *et al*., 1997; Mendelsohn *et al*., 1989; Shafren *et al*., 1997*b*), were not inhibited from infecting RD cells under similar conditions. To confirm that the block was specific for the anti-CD59 components of the rabbit antiserum, antiserum was pre-incubated with purified soluble CD59 (sCD59). A 1/400 dilution of rabbit antiserum was incubated with varying concentrations of sCD59 and the remaining virus blocking activity tested against EV7 (Fig. 1*b*). As little as 20 ng/ml of sCD59 abrogated the ability of anti-CD59 to block infection. Soluble CD59 alone, at a concentration of 200 ng/ml, had no effect upon EV7 infection of RD cells (Fig. 1*b*). We investigated the nature of the affinity-purified sCD59 used in these assays to confirm that the blocking activity could not be attributed to a contaminating protein (see also Bodian *et al*., 1997). Varying amounts of sCD59 were electrophoresed under reducing conditions and visualized by silver-staining (Fig. 1*c*). Three bands were visible, corresponding to a trace of the dimeric form of CD59, the 20 kDa monomeric CD59 protein, and the slightly smaller deglycosylated form. This preparation of CD59 has previously been analysed by Western blot. Other than a small amount of non-glycosylated material, no contaminating proteins are visible (Bodian *et al*., 1997), supporting our conclusion that the block to echovirus infection we observe is mediated by antibodies in the polyclonal antiserum specific for CD59.

Other polyclonal rabbit antisera that bind to the cell surface of RD cells did not block echovirus infection. Fig. 1(*d*) illustrates that neither anti-CD46 nor anti-β5 integrin exhibited any block to RD cell infection by a range of echoviruses, all of which were blocked by antiserum to CD59. Anti-IgE antiserum, known not to bind to the surface of RD cells was included as a negative control in this assay. Similarly, anti-CD66, anti-CD44 and anti-MHC-I MAbs were tested for their ability to block RD cell infection by EV7 and EV9. Poliovirus, anti-CD59 polyclonal antiserum and the anti-DAF MAb 854 were included as controls (Fig. 1*e*). Infection of RD cells by EV7 (a DAF binding enterovirus) was blocked by the anti-DAF MAb and anti-CD59 antiserum. In contrast, EV9 (which is known not to bind DAF) was only blocked by the anti-CD59 antiserum. Seven epitope-mapped murine MAbs directed against CD59 (see Methods) were screened for their ability to block EV6, -7 or -9 infection of RD cells. No inhibitory activity was observed, either with individual MAbs or combinations, including all seven MAbs together (data not shown). Similarly, anti-CD59 MAbs bound to the cell surface and cross-linked by incubation with a goat anti-mouse secondary antibody did not block echovirus infection of RD cells (data not shown). In contrast to the infection of RD cells, echovirus infection of Ohio HeLa or HT29 cells was not inhibited by anti-CD59 antiserum, despite these lines expressing comparable levels of CD59 as determined by flow cytometry (data not shown).

Antibodies to CD59 do not affect DAF binding by echovirus

Cell surface proteins involved in virus entry may have a role in virus binding or in a post-binding event required for a later stage of the infection process. To determine whether CD59 was implicated in virus binding we investigated the ability of polyclonal anti-CD59 rabbit antiserum to inhibit the binding of radiolabelled EV6 or EV7 to the surface of RD cells. EV6 was not blocked by either the anti-DAF MAb 854 or the anti-CD59 antiserum (Fig. 2); previous studies have demonstrated that this isolate does interact with DAF, as cell binding can be blocked with sDAF (Powell *et al*., 1998). In contrast, although at least 90% of the EV7 binding was blocked by the anti-DAF MAb 854, there was no reduction in binding in the presence of the anti-CD59 antiserum. These results imply that CD59 probably does not function as a secondary attachment molecule.

Antibodies to CD59 block at an early stage of EV7 infection

To determine the stage at which anti-CD59 antibodies blocked RD infection by EV7 we investigated the effect of adding antiserum at various times through a 3 h window

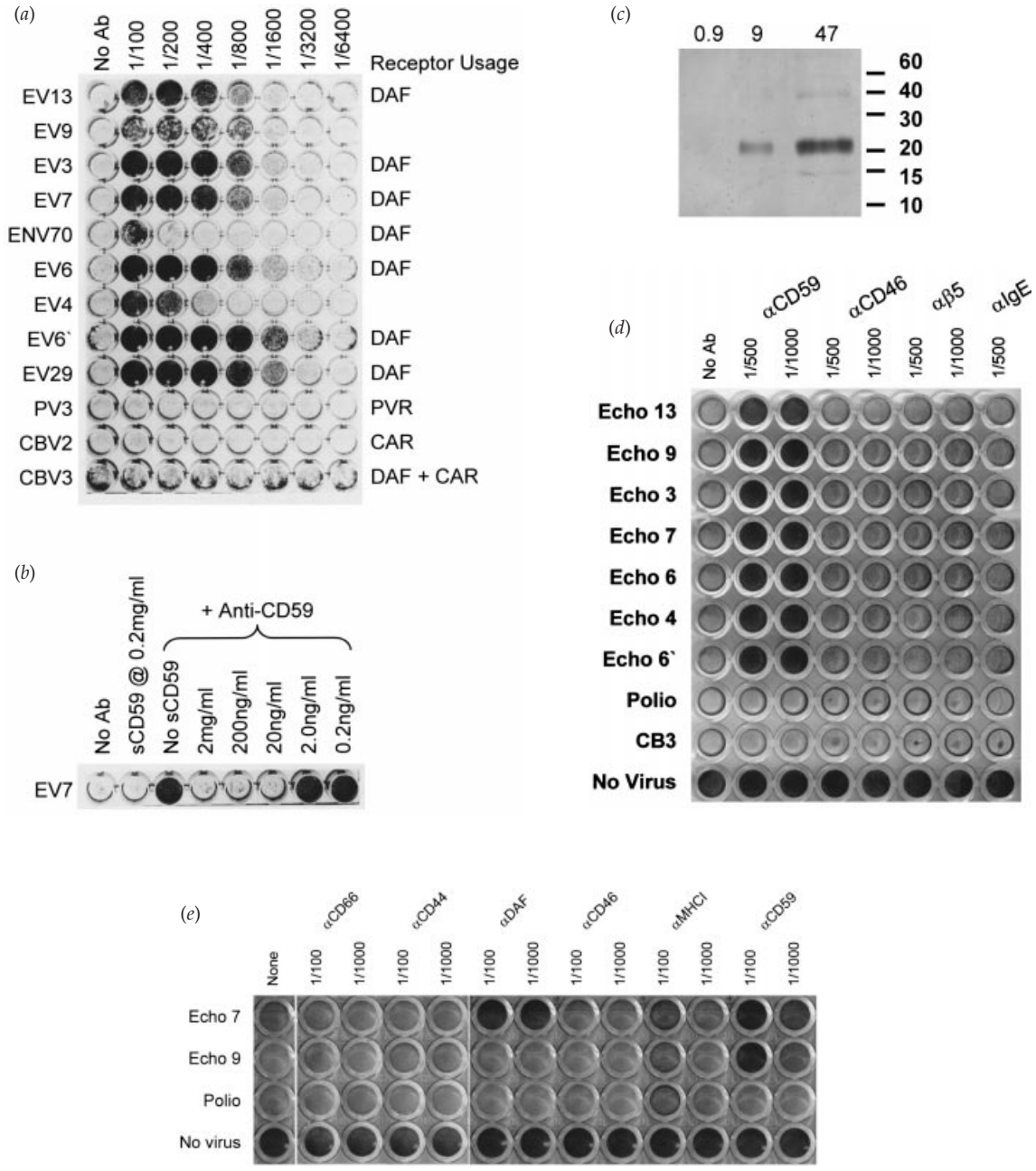


Fig. 1. Inhibition of echovirus infection in RD cells by anti-CD59 antiserum. The ability of polyclonal antiserum to human CD59 to inhibit infection of RD cells by a variety of echoviruses was examined (*a*). RD cells were pre-treated with doubling dilutions of anti-CD59 antiserum or mock-treated with DMEM (No Ab) and infected with 10000 $TCID_{50}$ of virus. Infection was stopped after 24 h by fixing and staining with crystal violet. The known receptor binding interactions of the tested panel of viruses are indicated on the right. DAF, decay accelerating factor; PVR, poliovirus receptor; CAR, coxsackie and adenovirus receptor. EV4 and EV9 are known not to bind to these receptors. (*b*) The specificity of the block mediated by anti-CD59 antiserum was demonstrated by pre-treating RD cells with media alone (No Ab), sCD59 at 200 ng/ml or a 1 : 400 dilution of anti-CD59 with varying dilutions of purified sCD59. (*c*) Characterization of affinity-purified CD59 used in blocking assays. A silver-stained 12·5% SDS–PAGE gel run under reducing conditions with 0·9 ng, 9 ng and 47 ng of sCD59 loaded is shown. The predominant band in the latter lane is monomeric sCD59; small amounts of dimerized protein are also visible, and a trace of unglycosylated protein. Molecular mass markers (kDa) are indicated. (*d*) Polyclonal rabbit antisera to CD46 and β5 integrin, both of which bind RD cells, do not block echovirus infection. Antiserum to IgE does not bind RD cells and is included as a negative control. Antisera were diluted 1/500 or 1/1000 from a starting concentration of 1 mg/ml. (*e*) Analysis of the inhibitory effect of anti-CD66, anti-CD44 and anti-MHC-I on RD cell infection by EV7, EV9 and poliovirus. Antibodies were diluted by the indicated amount from a starting concentration of 1 mg/ml.

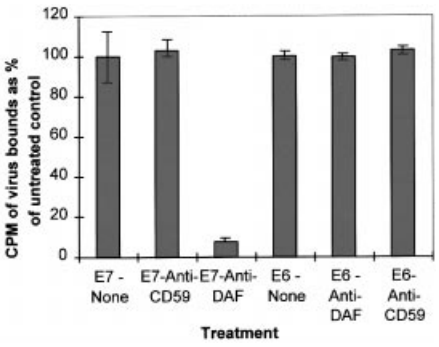


Fig. 2. Anti-CD59 does not prevent EV6 or EV7 binding to RD cells. Radiolabelled virus was bound to RD cells pre-treated with media alone (None), polyclonal rabbit anti-CD59 antiserum at 1:100 (Anti-CD59) or the anti-DAF MAb 854 at 1:1000 (Anti-DAF) for 1 h at 4 °C. Virus was allowed to adsorb for 1 h at 4 °C, the unbound virus removed and the cell-associated virus quantified by scintillation counting. Results are shown as a percent of the mock-treated control.

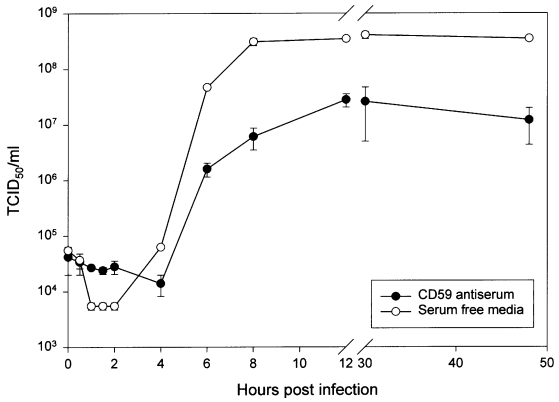


Fig. 4. One-step growth curve analysis of EV7. RD cells were treated with either serum-free media (○) or rabbit CD59 antiserum (●; 1:100 dilution) for 1 h prior to infection with EV7 at an m.o.i. of 3. At various times post-infection samples were frozen and the infectious virus present quantified by $TCID_{50}$ and plaque assay.

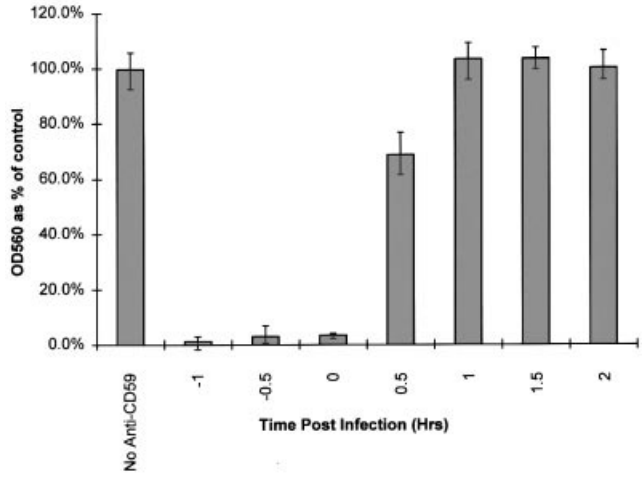


Fig. 3. Temporal analysis of the anti-CD59 block on echovirus infection. RD cell monolayers were treated with anti-CD59 antiserum (1:100 dilution) for various times during cell infection with EV7. Virus infection was detected using a capsid-specific MAb to detect virus antigen production in an immunofocal assay which resulted in the production of a blue product. Antigen production was quantified by absorbance at 560 nm and expressed as a percentage of the level observed when cells were treated with antiserum 2 h post-infection. Preliminary studies had demonstrated that addition of anti-CD59 antiserum at 2 h post-infection yielded similar levels of virus antigen as untreated cells (data not shown).

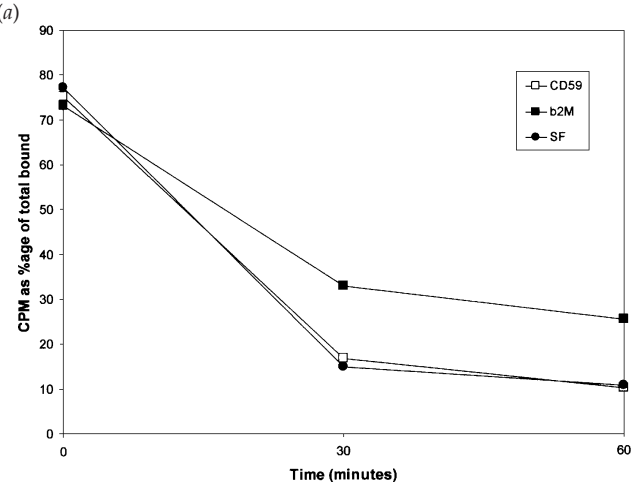
spanning the time of virus addition. RD cells were pre-incubated in the absence of virus, prior to addition of EV7 (m.o.i. of 1), which was allowed to adsorb for 30 min. The cells were thoroughly washed and the media replaced. Where necessary, CD59 antiserum was added at a 1/100 dilution to the pre- or post-infection media, virus preparation and washes. Virus antigen was detected at 6 h post-infection by direct immunofocal staining. Initial addition of anti-CD59 for more than 1 h post-infection produced a result indistinguishable from that seen with mock-treated cells (Fig. 3 and data not shown). The maximum block to infection (> 95%) was achieved by pre-incubation of the cells for 1 h with anti-CD59 serum, though the difference between 1 h pre-treatment and the co-addition with EV7 was not significant. Addition of anti-CD59 antiserum 30 min post-infection reduced infection by about 30%, indicating that the blocking effect observed with anti-CD59 antiserum is due to the inhibition of an early event in the virus replication cycle, similar to that previously reported for β_2-microglobulin (Ward *et al.*, 1998). Addition of CD59 antiserum at times later than 1 h post-infection did not reduce the number of infected cells.

Growth kinetics and early events of EV7 entry in the presence of anti-CD59 antiserum

To determine whether pre-exposure of RD cells to anti-CD59 antiserum resulted in delays in the uncoating or entry of EV7, a one-step growth curve was performed (Fig. 4). Pre-incubation of cells with a 1/100 dilution of rabbit anti-CD59 antiserum resulted in an extension of the lag phase and a reduction in the overall level of virus released, as determined by $TCID_{50}$, of 1·5 $\log_{10}$. The latter was confirmed by determining the end-point titre by plaque assay, which showed a 2 $\log_{10}$ reduction in infectious EV7 produced in the presence of anti-CD59 antiserum (data not shown).

DAF binding by EV7 on RD cells is followed by the formation, at 37 °C but not 0 °C, of a stable virus–receptor complex from which the virus cannot be competed by soluble DAF (Powell *et al.*, 1998; Ward *et al.*, 1998). We investigated the formation of this complex in the presence of antisera to CD59 or β_2-microglobulin, which we have previously shown reduces complex formation (Ward *et al.*, 1998). EV7 bound to DAF at the cell surface in the presence of antiserum to CD59 was as resistant to sDAF competition as virus bound in the presence of serum-free media. In contrast, EV7 bound in the

(*a*)



(*b*)

	0	30	60
CD59 (□)	75.1 ± 1.9	16.6 ± 0.2	9.8 ± 0.1
β2m (■)	73.1 ± 2.5	32.7 ± 0.4	25.0 ± 0.1
SF (●)	77.1 ± 2.6	14.6 ± 0.8	10.4 ± 0.3

Fig. 5. Formation of sDAF-resistant virus–receptor complex at the cell surface. RD cells were treated for 1 h at 37 °C with serum-free media (●), anti-CD59 antiserum (□; 1/100 dilution) or the anti-β_2-microglobulin MAb 1350 (■) and placed on ice. Radiolabelled EV7 was added, allowed to adsorb for 1 h and non-adsorbed virus washed off. Virus entry was induced to proceed by raising the temperature to 37 °C for the time intervals indicated, at which point the samples were chilled at 4 °C, the cells washed and sDAF at 100 μg/ml added for 1 h before quantifying eluted and bound virus remaining. (*a*) Graphical and (*b*) tabulated data from two experiments showing the average and range of duplicate values.

(*a*)

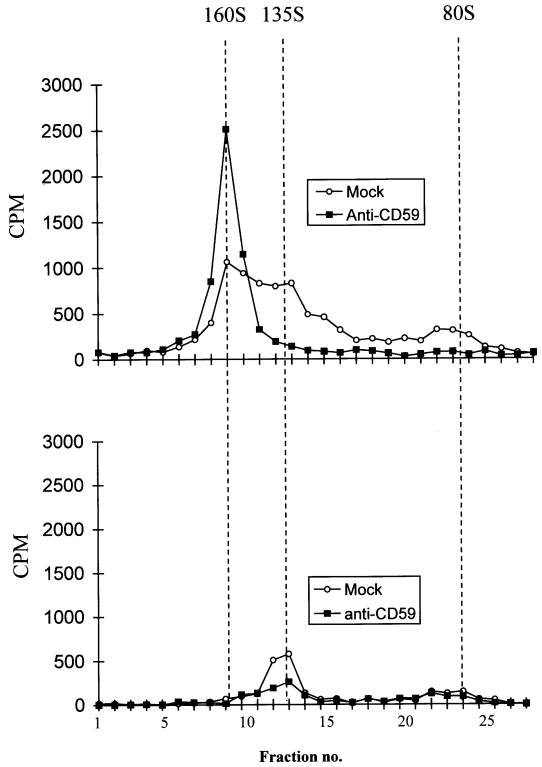


Fig. 6. Sedimentation profiles of cold synchronized eclipse products from EV7. RD cells were treated with media alone (mock) or anti-CD59 antiserum (1:100) for 1 h at 37 °C and radiolabelled virus bound to cells at 4 °C either in the presence or absence of the antiserum. Unbound virus was removed by washing and the virus allowed to enter for 2 h at 37 °C. Eluted virus was removed and both cell-associated virus (*a*) and eluted virus (*b*) were centrifuged on a 15–45% linear sucrose gradient, fractionated and counted by scintillation counting.

presence of anti-β_2-microglobulin was less resistant to competition with soluble receptor (Fig. 5).

Anti-CD59 antiserum inhibits 'A' particle formation by EV7

To better identify the stage of infection at which the block by anti-CD59 antiserum occurs we investigated the production of 'A' particles with an altered sedimentation coefficient. Radiolabelled EV7 pre-bound to RD cells at 4 °C was incubated at 37 °C for 2 h, at which point the cell-associated and eluted virus was characterized by sucrose gradient sedimentation (Fig. 6). The effect of antiserum to CD59 was investigated by adding sera diluted 1/100 at 1 h post-infection. Cell-associated EV7 treated with serum-free media exhibited the characteristic shift (Fig. 5*a*, open circles) from infectious 160S peak to the partially uncoated 135S and fully uncoated 80S particles, as we have previously described (Powell *et al.*, 1998). In contrast, in the presence of anti-CD59 antiserum, the cell-associated EV7 contained no detectable 35S or 80S particles, indicating that this stage of the infection process was blocked (Fig. 6*a*, filled squares). Analysis of the very low level of virus eluted from the cell surface during infection demonstrates the presence of 135S particles in both anti-CD59-treated and untreated cells. This demonstrates that the CD59 antiserum prevents the formation of 'A' particles, and those that do form are shed at low levels from the cell, rather than being retained within the receptor complex at the cell surface.

Discussion

It is increasingly evident that in many viruses the process of cell infection involves at least two distinct stages: an initial interaction with an attachment protein, followed by secondary events mediated by one or more cellular factors (secondary factors) which are required during the post-binding events involved in virus entry. However, for the picornaviruses poliovirus and rhinovirus, the process of particle uncoating appears to involve the virus receptor alone, and can be triggered by soluble receptors as well as cell membrane fractions (Greve *et al.*, 1991; Hooverlitty & Greve, 1993; Kaplan *et al.*, 1990), resulting in irreversible conformational changes to the virus particle that, in the case of poliovirus at

least, involves externalization of the amino terminus of VP1 (Fricks & Hogle, 1990; Holland, 1962). We have previously identified DAF as an echovirus receptor (Ward *et al*., 1994) and demonstrated that, at least for EV7, secondary cellular factors are implicated in cell infection (Powell *et al*., 1997). Binding of EV7 to DAF at the cell surface results in the virus undergoing the conformational changes resulting in 'A' particle formation, whereas sDAF does not induce irreversible structural changes in the capsid. We have speculated that this significant difference may reflect the relative abundance of virus receptor on susceptible cells *in vivo*, or the levels of free circulating DAF released by endogenous phospholipases (Davitz *et al*., 1986; Powell *et al*., 1997). We have extended these studies by investigating the potential involvement of CD59, a protein that, along with DAF, is located within sphingolipid-rich microdomains at the cell surface.

CD59 is an 18–20 kDa protein, widely expressed on a range of haematopoietic and non-haematopoietic cells. Like DAF, CD59 is anchored to the cell surface by a GPI tail and functions to regulate the complement cascade, albeit at a later stage than DAF, by interacting with C5b-8 and C5b-9 to block the incorporation and subsequent polymerization of C9 into functional C5b-9 complexes (Morgan & Meri, 1994), thereby preventing the formation of the membrane attack complex. The GPI anchor and functional similarity between DAF and CD59 possibly account for the observed similarity in the distribution patterns of the two molecules at the cell surface (Cerny *et al*., 1996; Lisanti & Rodriguez-Boulan, 1991), and prompted us to investigate whether CD59 has a role in echovirus infection.

We demonstrate that polyclonal antiserum to CD59 blocks infection of RD cells by a range of echoviruses, including representatives that use DAF alone for cell binding (e.g. EV7), those that bind DAF and an as yet an unidentified molecule (e.g. EV3, EV6, EV6′) and some that do not bind DAF (e.g. EV9, EV4). We have not completed an extensive screen of all echoviruses, but preliminary experiments have shown that EV2, -17, -18 and -20 (all of which bind DAF with the exception of EV2; I. Goodfellow, unpublished data; Bergelson *et al*., 1994) are also blocked by CD59 antiserum (data not shown). Our results indicate that the block does not act by inhibiting virus binding to the cell surface. Neither EV6 or EV7 were inhibited from binding to RD cells in the presence of anti-CD59 antiserum, suggesting that CD59 is probably not involved in virus attachment. Of the viruses tested, the block to RD infection by EV9 always produced a characteristic patchy cytopathic effect, suggesting that although the monolayer was protected from direct infection, virus could still spread by cell-to-cell contact. This phenotype was not observed with the other non-DAF-binding virus tested (EV4) and is currently being investigated.

The specificity of the block by anti-CD59 antiserum was demonstrated by the failure to inhibit polio or coxsackievirus infection of RD cells, and by the ability of competing sCD59 to release the block. Furthermore, independently raised antiserum to CD59 also blocked infection by a range of echoviruses, suggesting that the inhibition was not a consequence of a minor contaminant of the immunogen used to generate the antiserum. Rabbit polyclonal antisera to other proteins expressed on the surface of RD cells, such as CD46 (MCP, a cellular receptor for measles virus) and β5 integrin, did not inhibit echovirus infection. Although polyclonal antiserum to CD59 efficiently blocked infection, none of the panel of anti-CD59 MAbs alone, in combination, or cross-linked, mediated the same effect. However, the MAbs screened were selected as blocking CD59 complement regulation, and are therefore directed against a limited region of CD59, all but MEM43/5 map to the active site of CD59 (Bodian *et al*., 1997). It is probable that the polyclonal antiserum recognizes epitopes on CD59 outwith the active site that account for the block in infection. MAbs directed against a limited range of other cell surface markers, including CD44, CD46, CD66, the α_2 and α_v integrins, MHC-I, the transferrin receptor and aminopeptidase N all failed to block infection of RD cells by EV6, EV7 and EV9 (data not shown).

β_2-microglobulin has recently been implicated in echovirus infection of RD cells (Ward *et al*., 1998) at a post-binding, pre-entry stage, involving the formation of a multi-component complex. Like β_2-microglobulin, anti-CD59 antiserum blocked infection in a cell-specific manner, being restricted to RD cells, and having no effect on EV7 infection of Ohio Hela or HT29 cells. Both the latter express comparable levels of CD59 to RD cells, and we would speculate that the failure to block is a consequence of the significantly higher levels of DAF that these two cell lines express (data not shown). This could result in the virus using a route for cell infection that bypasses the requirement for CD59 or β_2-microglobulin, thereby masking the blocking effect clearly demonstrable in RD cells.

Although not formally tested, the failure to inhibit EV7 cell infection with sCD59 alone suggests that, in solution at least, the virus does not irreversibly interact with CD59. We have also been unable to demonstrate an interaction between sCD59 and EV7 by surface plasmon resonance, or the binding of radiolabelled EV7 or EV12 to transfected murine cells expressing high levels of human CD59 (I. Goodfellow & B. Spiller, unpublished results). It further suggests that, if EV7 does interact with CD59 within a cell surface receptor complex, either the virus is inaccessible to sCD59 within the complex or sCD59 cannot precisely mimic the function(s) of the GPI-anchored protein, as has been suggested for sDAF (Medof *et al*., 1984; Moran *et al*., 1992).

Anti-CD59 blocks EV7 infection of RD cells at an early stage, but does not inhibit the binding of EV7 to DAF. The precise stage at which the inhibition is effective remains to be determined, but our results demonstrate that the bound virus does not undergo the conformational changes that are associated with particle uncoating (Fig. 6). The absence of significant amounts of 'A' particles in the eluted virus fraction

(Fig. 6*b*) suggests that such particles are not formed in the presence of anti-CD59 antiserum, rather than forming but not being retained at or within the cell. The inability to form 'A' particles in the presence of anti-CD59 antiserum is similar to the inhibitory effect of anti-β_2-microglobulin MAbs (T. Ward, unpublished results), though there are qualitative differences between the inhibition observed. In particular, the formation of an sDAF-resistant virus–receptor complex was not inhibited by anti-CD59, whereas we show here and previously that anti-β_2-microglobulin retards the formation of this complex which remains partially sensitive to competing sDAF (Fig. 5; Ward *et al.*, 1998). Whether this reflects qualitative differences in the reagents used for these experiments, or a true difference in the state of the virus–receptor complex is currently under investigation.

The mechanism by which anti-CD59 antiserum blocks echovirus infection of RD cells remains unclear. Identification of the cellular location of the blocked virus–receptor complexes, which is also unknown, may help determine how antiserum to CD59, and possibly also anti-β_2-microglobulin, blocks infection. The physical characteristics of the blocked particles suggest that this location occurs at, or before, the site at which 'A' particles form. The resistance of the virus–receptor complexes to high levels of sDAF or protease digestion suggests that they are possibly located in cell surface endocytic compartments, such as clathrin-coated pits or caveolae, or are otherwise not exposed at the cell surface. However, we have previously demonstrated that inhibitors of pit or caveolae function do not prevent EV7 infection of RD cells and suggested that such compartments may not allow virus entry (Ward *et al.*, 1998). An alternative cellular location, with which both DAF and CD59 associate through the possession of GPI anchors, are lipid rafts. The latter consist of sphingolipid and cholesterol-rich microdomains that can be purified by resistance to non-ionic detergents such as Triton X-100 (Simons & Ikonen, 1997) and which, although distinct from caveolae, can co-associate under certain conditions (Brown & London, 1998). In particular, GPI-anchored proteins and glycosphingolipids associate in or near caveolae when cross-linked or clustered (Brown & London, 1998; Mayor *et al.*, 1994; Schnitzer *et al.*, 1995; Wu *et al.*, 1997), a situation that could arise upon multi-valent binding to the icosahedral virus particle. We are currently investigating the cellular location of DAF and CD59 in the presence or absence of EV7 to determine whether there are distinct modifications in localization following virus binding. We are also screening antibodies to other GPI-anchored proteins to investigate whether these also co-localize with DAF and block infection by both DAF-binding and other enteroviruses. These studies may help determine whether the observed block to virus infection reported here is due to the direct inhibition of a critical stage in the infection pathway, or to a non-specific steric event that occurs due to the similar location of DAF and CD59 at the cell surface.

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