Binding to Decay-Accelerating Factor Is Not Required for Infection of Human Leukocyte Cell Lines by Enterovirus 70

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Enterovirus 70 (EV70) is one of several human enteroviruses that exhibit a propensity for infecting the central nervous system (CNS). The mechanisms by which neurotropic enteroviruses gain access to and invade the CNS are poorly understood. One possibility is that circulating leukocytes become infected and carry neurotropic enteroviruses to the CNS. We examined the ability of EV70 to infect cell lines derived from lymphoid, myeloid, and monocytic lineages. Most leukocyte cell lines tested bound radiolabeled EV70 and were permissive for EV70 replication, suggesting that EV70, in contrast to other enteroviruses, has an in vitro tropism that includes lymphoid, monocytic, and myeloid cell lines. For some of the cell lines, virus binding and infection correlated with surface expression of decay-accelerating factor (DAF), an attachment protein for EV70 on HeLa cells. However, EV70 also adsorbed to and infected cell lines that expressed little or no DAF. In contrast to what was observed for HeLa cells, neither DAF-specific monoclonal antibodies nor phosphatidyl-sialidolositol-specific phospholipase C treatment inhibited EV70 binding to permissive leukocyte cell lines, and antibody blockade of DAF had little or no effect on EV70 replication. We also found that neither the human coxsackievirus-adenovirus receptor nor intercellular adhesion molecule 1, which mediate the entry of coxsackie B viruses and coxsackievirus A21, respectively, functions as a receptor for EV70. EV70 binding to all cell lines was sensitive to sialidase treatment and to inhibition of O-glycosylation by benzyl N-acetyl-α-D-galactosaminide. Taken together, these results suggest that a sialylated molecule(s) other than DAF serves as a receptor for EV70 on permissive human leukocyte cell lines.

Human enteroviruses are the leading recognizable cause of aseptic meningitis, particularly in children, and have also been implicated as agents of encephalitis (29). Although the mechanisms by which enteroviruses actually invade the central nervous system (CNS) are not well understood, two general routes have been recognized, based mainly on studies of poliovirus (27, 29). Enteroviruses typically infect and undergo primary replication in the respiratory tract or the gastrointestinal tract, spread to local lymph nodes, and are disseminated via the bloodstream to secondary target organs or tissues, including the CNS. A more direct route to the CNS is suspected for bulbular poliomyelitis following tonsillectomy, where poliovirus may infect neurons locally and then spread along neural tissue (27).

Enterovirus 70 (EV70), the etiologic agent of acute hemorrhagic conjunctivitis (AHC), has also been linked to neurological complications. Normally, EV70 infections are restricted to the conjunctival and corneal epithelia (41, 51), but acute lumbar radiculomyelopathy, cranial nerve involvement, and facial palsy, as well as a flaccid motor paralysis resembling poliomyelitis, have been reported to follow AHC (19, 49). EV70 is also neurotropic in cynomolgus monkeys (40). Higgins (18) suggested that CNS involvement following AHC is likely to result from direct neural spread of EV70 from the eye. However, the preauricular lymphadenopathy that accompanies EV70 infection (41) indicates that EV70 can reach the local lymph nodes; EV70 could spread from there to the CNS directly, via the blood, or via infected cells in the circulation. The inflammation that occurs in the eye during AHC presents the virus with the opportunity to infect invading white blood cells. Several studies have demonstrated that some enteroviruses can replicate in peripheral blood mononuclear cells (PBMCs) in vitro (11, 14, 15, 46, 47, 48) and that differences in the susceptibility and permissiveness of lymphoid, monocytic, or myeloid cell lines correlate with the expression of viral receptors (14, 15, 46, 47, 48).

We previously identified decay-accelerating factor (DAF/CD55) as a receptor for EV70 on HeLa cells (21). DAF is a glycosylphosphatidylinositol (GPI)-linked glycoprotein that also functions as an attachment protein for several echoviruses and coxsackieviruses (7, 8, 31, 50). In addition, we demonstrated that attachment of EV70 to susceptible cells, such as HeLa cells and U-937 cells, is sensitive to treatment with Vibrio cholerae sialidase (1). Here we show that EV70 is able to infect and replicate in cell lines of lymphoid, monocytic, and myeloid lineages expressing DAF and exhibits a broader in vitro tropism than has been reported for other human enteroviruses. However, in contrast to what was observed for HeLa cells, DAF does not appear to function as a receptor for EV70 on susceptible human leukocyte cell lines. Sialidase treatment and incubation of cells with benzyl N-acetyl-α-D-galactosaminide (benzyl-GalNAc), an inhibitor of O-linked glycosylation, inhibited the binding of EV70 to all cell lines tested, consistent with a model in which a sialylated molecule(s) other than DAF serves as a receptor for EV70 on human leukocyte cell lines.
Materials and Methods
Viruses and cell lines. The EV70 prototype strain J-670/71 was obtained from M. Hatch and M. Pallansch (Centers for Disease Control and Prevention, Atlanta, Ga.). Coxsackievirus B3 strain Cg (CVB3) was a gift of T. Martino and P. Liu (Heart and Stroke/Richard Lewer Centre of Excellence, University of Toronto, Toronto, Ontario, Canada). Human rhinovirus 14 (HRV14) was provided by K. Wright (Department of Biochemistry, University of Ottawa, Ottawa, Ontario, Canada). EV70 and CVB3 were propagated, and infectious titers were determined in LLC-MK2, rhesus macaque kidney cells as described previously (21). Jurkat (T-lymphoblastoid) and U-937 (histiocytic/monocytic) cells were provided by L. Filion (Department of Biochemistry, Microbiology and Immunology, University of Ottawa), HL-60 (promyeloctye) and THP-1 (monocytic) cells were provided by J. Angel (Department of Medicine, University of Ottawa), A2.01 (T-lymphoblastoid) cells were provided by L. Poulin (Department of Microbiology, Laval University, Ste-Foy, Quebec, Canada), Raji (B-lymphoblastoid) cells were provided by F. Diaz-Mitoma (Children’s Hospital of Eastern Ontario, Ottawa, Canada), and Daudi (B-lymphoblastoid) cells were provided by M. Freedman (Department of Medicine, University of Ottawa). All of the leukocyte cell lines were maintained in RPMI 1640 (Life Technologies; supplemented with 10% fetal bovine serum; Life Technologies). NIH 3T3 cells were a gift from S. Bennett (Department of Biochemistry, Microbiology and Immunology, University of Ottawa), and were grown in minimal essential medium (MEM) (Earle’s salts; Life Technologies) supplemented with 10% FBS. Medium used for stably transfected NIH 3T3 cells also contained 10 μg of blasticidin (Invitrogen) per ml.

Transfection. For suspension cultures, cells were counted, washed once, and infected at a multiplicity of infection (MOI) of 5 PFU/cell in a final volume of 100 to 200 μl of culture medium for 1 h at 33°C. They were then washed three times with 1 to 2 ml of medium to remove unbound virus and resuspended in a final volume of 1 to 2 ml of culture medium. Duplicate 100 to 200 μl aliquots were withdrawn immediately and at various times after infection and were frozen at –80°C. Adherent cells were seeded in 12-well plates, counted, and infected at a MOI of 5 in a final volume of 300 μl of culture medium for 1 h at 33°C (EV70 and HRV14) or 37°C (CVB3). The cells were then washed and harvested at different times after infection. The amount of infectious virus in each sample was determined by a plaque assay on monolayers of HeLa cells (HRV14) or LLC-MK2 cells (EV70 and CVB3), as described previously (21).

Antibodies. The monoclonal antibody, EVR1, which is specific for complement control protein 1 (CCP1) of human DAF has been described previously (21) and was provided by E. Altman (National Research Council of Canada, Ottawa, Ontario, Canada). DAF-specific monoclonal antibodies 8D11 (CCP4) and 1H4 (CCP3) were generous gifts of W. Rosse (Department of Medicine, Duke University Medical Center, Durham, N.C.) and D. Lublin (Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, Mo.), respectively. The human coxsackievirus-adenovirus receptor (CAR)-specific monoclonal antibody, RmCB, was provided by J. Bergelson (Division of Immunological and Infectious Diseases, Children’s Hospital of Philadelphia, Philadelphia, Pa.). Human CD54 (intercellular cell adhesion molecule 1 [ICAM-1])-specific monoclonal antibody, 84H10 (Serotech), was purchased from Cedarlane Laboratories (Hornby, Ontario, Canada), and the EV70-specific monoclonal antibody, 74-5G, was purchased from Chemicon (Temecula, Calif.).

Flow cytometry and cell sorting. For flow cytometry of cell surface proteins, cells (3 × 10^5 per sample; monolayers dispersed with trypsin) were washed once in flow cytometry buffer (phosphate-buffered saline [PBS] containing 2% [wt/vol] bovine serum albumin and 0.1% [wt/vol] sodium azide) and then resuspended for 15 min at room temperature in 150 μl of flow cytometry buffer and resuspended for 15 min at room temperature in 150 μl of flow cytometry buffer containing a 1:1,000 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Roche). The cells were then diluted in flow cytometry buffer and analyzed using a Coulter EpicsXL-MCL flow cytometer. A minimum of 5,000 events were counted for each sample in the established gate, and mean fluorescence intensities were determined from single-parameter histograms generated with Epics XL v.1.5 software. Controls for autofluorescence and nonspecific secondary antibody binding were included in each analysis.

When cells were intended for sorting, flow cytometry buffer was replaced with serum-free culture medium. Cells were sorted using a Coulter Epics Elite ESP cell sorter and the Expo v.2.0 software (Applied Cytometry Systems). A total of 1.5 × 10^5 to 2.0 × 10^5 cells were sorted directly into culture medium, pelleted, and transferred immediately to a six-well culture dish.

EV70 protein synthesis was also monitored by intracellular staining and flow cytometry. Infected cells (3 × 10^4 per sample) were washed once in culture medium and once in flow cytometry buffer and then fixed and permeabilized for 15 min in PBS containing 4% paraformaldehyde and 0.1% saponin. They were washed once in flow cytometry buffer containing 0.1% saponin and then incubated for 30 min in flow cytometry buffer containing saponin and 100 μg of Gamimune (Bayer; provided by L. Filion) per ml prior to the addition of monoclonal antibody 74-5G (final concentration, 1 μg/ml) (3). After a 20-min incubation, the cells were washed once with flow cytometry buffer containing 0.1% saponin and once with flow buffer. They were resuspended for 15 min in 150 μl of flow cytometry buffer containing a 1:1,000 dilution of phycoerythrin-conjugated goat anti-mouse immunoglobulin (Molecular Probes), diluted, and analyzed as described above.

Virus binding assays. ^35S-labeled EV70 or CVB3 was harvested from LLC-MK2 cells and purified by sucrose gradient ultracentrifugation as described previously (21). For virus binding assays, suspension cells were washed once and resuspended to a final concentration of 5 × 10^6 cells in 50 μl of OptiMEM (Life Technologies) containing 3 × 10^8 to 6 × 10^9 cpm of radiolabeled EV70 or CVB3. Adherent cells were grown to over 80% confluency in 12-well plates, washed once, and incubated in 300 μl of OptiMEM containing radiolabeled virus. Following a 1-h incubation at 33°C (or 37°C for CVB3), the radioactivity in the bound and unbound fractions was determined by liquid scintillation counting as described previously (21).

Antibody blockade of DAF. Cells (3 × 10^4 to 4 × 10^4 per sample) were washed once and incubated for 1 h at 37°C in 300 μl of medium containing 17.5 μg of monoclonal antibody EVR1 per ml. This concentration of EVR1 saturates binding sites on U-937 and Jurkat cells (data not shown). The cells were then washed and used in virus binding assays or for replication studies.

PipL-C treatment of leukocytes. Cells (2.5 × 10^4) were washed three times with PBS, resuspended in 50 μl of phosphatidylinositol-specific phospholipase C (PipL-C) buffer (RPMI 1640, 0.2% bovine serum albumin, 50 μM 2-mercaptoethanol, 10 mM HEPES, 0.1% sodium azide) containing 0.3 U of Bacillus cereus PipL-C (Sigma), and incubated at 37°C for 90 min. The cells were washed again, and virus binding was assessed as described above. A portion of each sample of cells was analyzed by flow cytometry to monitor the extent of enzymatic removal of DAF from the surface of cells.

Expression of DAF, ICAM-1, and CAR in NIH 3T3 cells. Human DAF, ICAM-1, and CAR coding sequences were amplified by standard PCR and cloned into plasmid pE6E/FisVSTOPO (Invitrogen) to produce pE6E-DAF, pE6E-ICAM, and pE6E-CAR, respectively. The templates for amplification were pDNA3-DAF (21), pEF6OS-ICAM (from D. Shafren, Department of Microbiology, University of Newcastle, Newcastle, Australia), and hCAR-pcDNA1 (from T. Martino and P. Liu). The correct orientation of inserts was determined by restriction endonuclease digestion, and the accuracy of the coding sequences for DAF, CAR, and ICAM-1 was confirmed by nucleotide sequence analysis (University of Ottawa Biotechnology Research Institute). NIH 3T3 cells, grown to 90% confluence in six-well culture dishes, were transfected with 1 μg of pE6E-DAF, pE6E-ICAM, or pE6E-CAR using Lipofectamine PLUS (Life Technologies), in a volume of 1 ml, as recommended by the supplier. The cells were incubated with the transfection complexes at 37°C for 5 h, and then 3 ml of prewarmed MEM containing 10% FBS was added. The culture medium was replaced, at 48 h after transfection, with fresh MEM supplemented with FBS and 10 μg of blasticidin per ml. The medium was changed every 3 or 4 days, and cultures were monitored for DAF, ICAM-1, and CAR expression by flow cytometry. Approximately 3 weeks after transfection, cells expressing DAF, CAR, or ICAM-1 (3T3/DAF, 3T3/CAR, or 3T3/ICAM-1) were collected by cell sorting and maintained in medium containing blasticidin.

Silaladase treatment of leukocytes. Cells (1.5 × 10^5) were pelleted, resuspended in 50 μl of OptiMEM containing 25 μl of V. cholerae silaladase per ml, and incubated at 37°C for 30 min. They were then washed twice with PBS, and virus binding was assessed as described above.

Inhibition of O-linked glycosylation. To inhibit O-linked glycosylation, cells were incubated for 48 h in culture medium containing 3 mM benzy1-GaINAc (Sigma) (1, 17, 24, 26). The cells were washed twice with PBS, cell viability was determined by trypan blue staining, and virus binding was assessed as described above.

Results
Leukocytes of different lineages are permissive for EV70. We first compared different leukocyte cell lines for their abilities to support EV70 replication by determining the amount of infectious virus produced by each cell line at different times.
after infection with EV70. As shown in Fig. 1. Jurkat T cells, promyelocytic HL-60 cells, and Raji B cells were permissive for EV70 and substantial amounts of virus were produced by 12 h after infection. Similar results were observed for both the Daudi B-cell line and monocytic U-937 cells, although virus yields were lower. The results for THP-1 cells were equivocal because of the low yields of EV70 at the various time points. A second T-lymphoblastoid cell line, A2.01, did not support EV70 replication. A2.01 cells bound noticeably less virus than did the other cell lines, and the amount of infectious virus detected in A2.01 cells did not increase above the amount of virus present at the zero time point and began to decline between 12 and 24 h after infection. As an alternative method for determining if the various cell lines were infected by EV70, viral protein synthesis in leukocytes exposed to EV70 was assessed by intracellular staining and flow cytometry. EV70 polypeptides were readily detected in U-937, THP-1, HL-60, Daudi, and Jurkat cells 10 h after infection (Table 1), confirming that these leukocyte cell lines were susceptible to EV70. Little or no EV70 protein synthesis was detected in Raji cells, even though the yield of EV70 (>1 log unit 12 h postinfection) is strong evidence that these cells were infected. The lack of correlation between virus yield and the amounts of EV70 protein detected in infected cells may reflect differences in the efficiency of virus assembly in the various cell lines.

**EV70 binding to leukocyte cell lines does not correlate with surface expression of DAF.** The seven different cell lines were also evaluated for their abilities to bind radiolabeled EV70. All of the cell lines except A2.01 cells bound significant amounts of radiolabeled virus (data not shown). For the permissive cell lines, the binding data corresponded reasonably well to the amounts of infectious virus that remained cell associated after the adsorption period in the time course experiments (Fig. 1), although HL-60 cells were an exception. Since DAF is an attachment protein for EV70 on HeLa cells (21), we examined the level of expression of DAF on each of the different leukocyte cell lines by flow cytometry, using monoclonal antibody 8D11. Flow cytometry histograms are presented in Fig. 2, and the data are summarized in Table 1. U-937 cells and Daudi cells expressed the greatest amounts of DAF, and DAF was readily detected on THP-1 cells and Jurkat cells. Lower levels of DAF were present on the surface of Raji cells and A2.01 cells, but little or no DAF could be detected on the surface of HL-60 cells. Flow cytometric analysis with monoclonal antibody IF7, which is specific for DAF CCP2 and which routinely gives a higher mean fluorescence intensity than 8D11, indicated that small amounts of DAF were present on HL-60 cells (data not shown).

**Table 1. Expression of DAF, CAR, ICAM-1, and EV70 proteins in leukocyte cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>DAF</th>
<th>CAR</th>
<th>ICAM-1</th>
<th>EV70</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa T4</td>
<td>4.76</td>
<td>1.47</td>
<td>2.24</td>
<td>5.11</td>
</tr>
<tr>
<td>U-937</td>
<td>5.34</td>
<td>1.85</td>
<td>2.88</td>
<td>3.12</td>
</tr>
<tr>
<td>Jurkat</td>
<td>3.03</td>
<td>1.21</td>
<td>2.32</td>
<td>1.64</td>
</tr>
<tr>
<td>HL-60</td>
<td>1.00</td>
<td>1.06</td>
<td>4.79</td>
<td>2.47</td>
</tr>
<tr>
<td>THP-1</td>
<td>3.29</td>
<td>3.28</td>
<td>2.53</td>
<td>2.50</td>
</tr>
<tr>
<td>Daudi</td>
<td>5.14</td>
<td>1.26</td>
<td>2.56</td>
<td>3.14</td>
</tr>
<tr>
<td>Raji</td>
<td>1.44</td>
<td>1.06</td>
<td>4.74</td>
<td>1.09</td>
</tr>
<tr>
<td>A2.01</td>
<td>1.76</td>
<td>1.11</td>
<td>1.08</td>
<td>NT</td>
</tr>
<tr>
<td>NIH 3T3/DAF</td>
<td>6.05</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>NIH 3T3/CAR</td>
<td>NT</td>
<td>8.49</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>NIH 3T3/pEF6</td>
<td>1.14</td>
<td>1.03</td>
<td>0.97</td>
<td>NT</td>
</tr>
</tbody>
</table>

- Cells were incubated in the presence or absence of the appropriate monoclonal antibody (8D11 for DAF, RmCB for CAR, and 84H10 for ICAM-1) and then with secondary antibody (FITC-conjugated goat anti-mouse immunoglobulin). They were then analyzed by flow cytometry, and mean fluorescence intensities (MFI) were determined. Values are ratios calculated as follows: (MFI of sample)/(MFI of secondary control). In the sample, cells were incubated with both primary and secondary antibodies; in the secondary controls, cells were incubated with secondary antibody alone. A ratio of >1.5 was considered positive.
- Immediately and at 12 h after infection with EV70 (MOI, 5 to 10), cells were fixed, permeabilized, and incubated in the presence or absence of monoclonal antibody 74-5G, and then with secondary antibody (phycoerythrin-conjugated goat anti-mouse immunoglobulin). The cells were analyzed by flow cytometry and MFI ratios were calculated as described above. Differences between the MFI of samples and secondary controls at the zero time point were 0.2 U.
- NT, not tested.
- NIH 3T3 cells transformed with the vector pEF6/HisV5-TOPO.
was little or no inhibitory effect on EV70 replication in U-937 cells (Fig. 3). These data indicate that DAF expressed on leukocyte cell lines does not function in EV70 attachment or entry and that some other molecule(s) must serve as a receptor for the virus on these cells.

PiPL-C treatment of leukocytes does not diminish EV70 binding. As an alternative approach to assessing a potential role for DAF in EV70 binding to leukocyte cell lines, the ability of PiPL-C to remove EV70 binding activity from cells was examined. PiPL-C releases GPI-anchored proteins such as DAF from the surface of cells by cleaving the GPI anchor between the phosphate moiety and the glycerol which is attached to the fatty acids embedded in the plasma membrane (28, 42). Cells were incubated in the presence or absence of PiPL-C and then tested for radiolabeled EV70 binding. The amount of DAF remaining on the surface of PiPL-C-treated cells was monitored by flow cytometry. The flow cytometry and virus binding data summarized in Fig. 2 and 4 show that while incubation of cells with PiPL-C removed the majority (60 to 88%) of DAF molecules from the surface of all of the leukocyte cell lines tested, binding of radiolabeled EV70 to Daudi, Jurkat, THP-1, and U-937 cell lines was reduced by only very small amounts following PiPL-C treatment. This was in contrast to what was observed for HeLa cells, where PiPL-C treatment reduced virus binding and the quantity of DAF on the cell surface by similar amounts. PiPL-C treatment of cells expressing low levels of DAF (Raji, HL-60, and A2.01 cells) also had little or no inhibitory effect on EV70 binding (data not shown). Extending the incubation time with PiPL-C resulted in a >90% reduction in the amount of DAF present on the surface of HeLa cells and almost complete abolition of virus binding but had no additional effect on EV70 binding to leukocytes (data not shown). These results strongly support the idea that DAF is not involved in the attachment of EV70 to the leukocyte cell lines tested.
ICAM-1 and CAR are not involved in EV70 binding to leukocytes. DAF also functions as an attachment protein for other human enteroviruses such as coxsackievirus A21 (CVA21) and CVB. Interactions between these viruses and DAF are not essential for infection of cultured cells, and it has been proposed that DAF is a "sequestration" receptor that serves to enhance binding of CVA21 and CVB (32, 33). Expression of ICAM-1 and CAR is required for infection of cultured cells by CVA21 and CVB (9, 35, 39), respectively, and DAF and ICAM-1 have been shown to associate with each other (34). Therefore, we tested the possibility that either ICAM-1 or CAR is necessary for EV70 infection of leukocytes. We first examined the level of expression of ICAM-1 and CAR on the different leukocyte cell lines. As shown in Table 1, HL-60 cells and Raji cells expressed the highest levels of ICAM-1 and only A2.01 cells did not express detectable amounts of ICAM-1. CAR was readily detected on THP-1 cells and U-937 cells, but was not detected on the surface of HL-60 cells, A2.01 cells or Raji cells.

Because there appeared to be a correlation between surface expression of ICAM-1 and susceptibility to EV70 infection (Fig. 1, Table 1), mouse NIH 3T3 cells were transfected with pEF6-ICAM and selected for cells stably expressing ICAM-1 at the cell surface, as confirmed by flow cytometry (Table 1). NIH 3T3 cells lack a receptor for EV70 but are permissive for the virus and will support EV70 replication when human DAF is expressed on the cell surface (Fig. 5) (21). NIH 3T3 cells expressing ICAM-1 were not susceptible to EV70 infection (Fig. 5), and binding of radiolabeled virus to these cells was not significantly higher than background (data not shown). Although no correlation was observed between expression of CAR by the leukocyte cell lines and susceptibility to EV70 infection, the possibility that CAR functions as a receptor for EV70 was also investigated. NIH 3T3 cells stably expressing CAR were produced (Table 1); however, these cells did not support EV70 infection (Fig. 5) and were also unable to bind radiolabeled EV70 (data not shown).

To verify that CAR and ICAM-1 expressed on NIH 3T3 cells were functioning as viral receptors, we performed infection and virus binding assays with CVB3 and HRV14. CVB3 utilizes CAR as a receptor, and HRV14, like CVA21, utilizes ICAM-1 as a receptor (16, 37, 38). NIH 3T3 cells expressing CAR bound three times more radiolabeled CVB3 and produced 100 times as much infectious virus following infection with CVB3 than did control NIH 3T3 cells that did not express CAR (data not shown), confirming observations made previously by Tomko et al. (39). Similarly, ICAM-1-expressing NIH 3T3 cells, although not permissive for HRV14 replication, bound 15 times more HRV14 than did control NIH 3T3 cells that did not express ICAM-1 (data not shown).

EV70 binding to leukocytes is sialidase sensitive. It was reported previously that the receptor for EV70 on erythrocytes

FIG. 3. DAF-specific antibodies do not inhibit EV70 replication in U-937 cells. HeLa T4 cells in 12-well dishes (A) and U-937 cells in suspension (B) were washed, counted, and incubated (1.0 × 10^6 to 1.25 × 10^6 cells/ml) for 1 h at 37°C in the presence or absence of monoclonal antibody EVR1 (17.5 μg/ml). The cells were then infected with EV70 at a MOI of 5 for 1 h at 33°C and washed three times with culture medium. HeLa cells were frozen immediately and at the times indicated. Similarly, aliquots of U-937 cells were withdrawn immediately and at various times after infection. The cells were subjected to two cycles of freezing and thawing to release virus, and virus production at each time point was determined by plaque assay on LLC-MK2 cells in triplicate. This experiment was repeated three times for HeLa cells and four times for U-937 cells. The data presented in the figure are from one representative experiment.

FIG. 4. PiPL-C treatment does not inhibit EV70 binding to leukocytes. Cells were washed and counted, and 2.5 × 10^6 cells were incubated in the presence or absence of PiPL-C (6 U/ml) for 90 min at 37°C before being subjected to virus binding assays. The results are presented as mean percentages of virus binding to cells incubated in the presence of PiPL-C compared to untreated cells, along with the standard deviation for two or three independent experiments for each cell line, each performed in triplicate. The amount of DAF remaining on the surface of leukocytes after PiPL-C treatment, as determined by flow cytometry and as compared to untreated controls, is also shown (see Fig. 2).
The initial objective of this study was to assess the capacity of cell lines derived from different human mononuclear cell lineages to support EV70 replication. Our data show that EV70 can infect and replicate in B-lymphocytic (Raji and Daudi), T-lymphocytic (Jurkat), promyelocytic (HL-60), and monocytic (U-937) cell lines. While the available data on replication of enteroviruses in mononuclear cells or cell lines are rather fragmentary, EV70 appears to have a broader in vitro tropism than other enteroviruses that have been studied. CVB3 replicated in B cells (Raji) and in T cells (Molt 4) but not in promyelocytic or monocytic cells (HL-60, U-937, and THP-1) (48). Conversely, U-937 cells were permissive for echovirus 1, but lymphocytes (Raji and Molt 4) were not (46), which is similar to what has been observed for poliovirus (15, 46). While the in vivo significance of this tropism is unclear, our results are consistent with the possibility that circulating mononuclear cells could transport EV70 to other parts of the body and provide access to the CNS. The capacity for enteroviruses to replicate in PBMCs varies considerably. For example, echoviruses 5 and 11 replicate efficiently in PBMCs, and PBMCs also support echovirus 9 and CVA9 multiplication, although weakly (11). Results with poliovirus have been variable (14, 25, 46), and the CBV strains that have been examined do not appear to replicate in PBMCs (46, 47, 48). It will be of interest to determine if EV70 replicates in specific subsets of human PBMCs.

The ability of EV70 to infect a variety of human leukocyte cell lines and its wide host range in nonprimate mammalian cells (52) may well be related to receptor usage. Our results indicate that there are at least two different ways for EV70 to interact productively with the surfaces of susceptible cells: a DAF-dependent mechanism and a DAF-independent mechanism. Expression of human DAF on NIH 3T3 cells was necessary for virus binding and infection to occur (1, 21), providing compelling evidence that DAF functions on these cells as an obligatory attachment molecule for EV70. For HeLa cells, although EV70-DAF interactions may not be absolutely required, DAF is a major component of virus adsorption leading to productive infection, as evidenced by the sensitivity of EV70 binding and infection to DAF-specific antibody blockade. In contrast, interactions between EV70 and DAF do not appear to be necessary for virus attachment to leukocytes, since attempts to remove or block DAF on the surface of leukocytes had little or no effect on EV70 binding. Antibody blockade of DAF also had no effect on the infection of U-937 cells. EV70, therefore, must utilize as yet unidentified molecules for attachment to leukocytes. Both ICAM-1 and CAR were ruled out as candidate receptors for EV70.

EV70 binding to all of the cell lines tested, including HeLa cells and NIH 3T3 cells expressing DAF (1), was inhibited by sialidase and by benzyl-GalNAc treatment. Infection of HeLa cells and U-937 cells was also sialidase sensitive (1). The simplest model that accounts for these observations, irrespective of whether virus binding is DAF dependent, is one in which EV70 recognizes an O-glycosylated and sialylated receptor, other than DAF, that is common to all of the cell lines examined. On leukocytes, the amount or the nature of the sialic acid-containing receptor may be such that interaction of EV70 with DAF is circumvented, even if DAF is present. Glycoconjugates containing O-linked glycans and sialic acid are ubiquitous cell surface molecules and include glycoproteins, glycosphingolipids, such as the gangliosides, and the proteoglycan keratan sulfate (discussed in references 2 and 45), and many different viruses hijack sialic acid as an attachment or entry ligand. For several nonenveloped viruses, including reovirus type 3 (5, 6), certain animal rotaviruses (4, 12, 13), and low-
neurovirulence Theiler’s murine encephalomyelitis virus (36), sialic acid is predicted to function as an attachment receptor in the initial stages of virus adsorption to cells, facilitating subsequent interactions with receptor molecules responsible for virus entry. Similarly, DAF, which has been ruled out as the sialylated EV70 receptor (1), is also exploited as an attachment receptor for several coxsackieviruses and echoviruses, facilitating their interaction with ICAM-1 (CVA21) or CAR (certain receptor for several coxsackieviruses and echoviruses, facilitating sialylated EV70 receptor (1), is also exploited as an attachment virus entry. Similarly, DAF, which has been ruled out as the neurovirulence Theiler


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