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Infection by Echoviruses 1 and 8 Depends on the α2 Subunit of Human VLA-2

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Anti-VLA-2 antibodies protected HeLa cells from infection by echoviruses 1 and 8 but not from infection by other echovirus serotypes. Echoviruses 1 and 8 bound to and infected nonpermissive hamster cells transfected with the α2 subunit of human VLA-2. These results indicate that the human α2 subunit is critical for infection by echoviruses 1 and 8 but that other echovirus serotypes must bind receptors other than VLA-2.

Echoviruses, nonenveloped RNA viruses belonging to the picornavirus family, are a common cause of human disease, including febrile illness, rash, and aseptic meningitis (5). Neutralizing antisera to echovirus isolates have been used to distinguish at least 30 viral serotypes (15).

A variety of evidence demonstrates that the cell surface receptor for echovirus serotype 1 is the integrin VLA-2, an adhesion receptor mediating cell interactions with collagen and laminin. Monoclonal antibodies (MAbs) that protect susceptible cells from infection and prevent cell surface attachment of radiolabeled echovirus 1 recognize the α2 and β1 subunits of the VLA-2 heterodimer (4). Human RD cells, deficient in α2 expression, gain the capacity to bind virus and become infected when transfected with α2 cDNA (4). In addition, echovirus 1 binds directly to VLA-2 isolated on beads (3). It is not known, however, whether other echoviruses also bind VLA-2.

In this study we examined whether VLA-2 expression is critical for infection by many echovirus serotypes. We present evidence that echoviruses 1 and 8 share a receptor site on VLA-2, whereas other echoviruses appear to bind cells independently of VLA-2. In addition, we now show that nonpermissive rodent cells become susceptible to infection when transfected with the α2 subunit of human VLA-2.

Inhibition of echovirus cytopathic effect by anti-VLA-2 MAbs. MAbs to the α2 and β1 subunits of VLA-2 protect HeLa cells from infection by echovirus 1 (4). We tested the ability of MAbs to the α2 and β1 subunits of VLA-2 to protect HeLa cells from infection by a variety of echovirus serotypes. Prototype echoviruses were obtained from the American Type Culture Collection, and stocks were prepared by passage on HeLa cells. For these experiments, we selected those viruses whose cytopathic effects were evident in a 2-day assay. HeLa cell monolayers were pretreated with anti-α2 (AA10 [4]) and anti-β1 (DE9 [4] and A-1A5 [11]) MAbs (approximately 10 μg/ml), each of which had significant protective activity against echovirus 1 (4) (data not shown); after incubation with antibodies, monolayers were exposed for 1 h to serial dilutions of each echovirus, washed, refed with medium containing antibodies at 1 to 2 μg/ml, and incubated at 37°C for 40 to 44 h. Control monolayers were incubated with a combination of irrelevant isotype-matched antibodies at similar concentrations. Monolayers were scored for the appearance of visible cytopathic changes. Only echovirus serotypes 1 and 8 were definitively inhibited by either AA10 or the combination of antibodies: AA10-treated monolayers showed no cytopathic changes even when exposed to 1,000-fold more virus than was required to destroy control monolayers. For the other viruses tested, at virus concentrations sufficient to produce evident cytopathic changes in control monolayers, antibody-treated monolayers also showed cytopathic effects. With echovirus 22, complete destruction of the monolayer was not seen even at the highest virus concentration; however, cytopathic effects were seen even in the presence of anti-VLA-2 antibodies.

Cell survival was also scored in a colorimetric assay based on the ability of healthy cells to metabolize a combination of tetrazolium dyes, generating a purple color (16). The optical densities generated by surviving cells were plotted against the virus dilution to which the cells were exposed (Fig. 1). For echoviruses 1 and 8, the resulting curves were widely divergent, reflecting the survival of antibody-treated cells at all virus concentrations. In contrast, for other echoviruses, identical (or closely parallel) curves were seen; in some cases, monolayers treated with VLA-2 antibodies generated somewhat higher optical density values than control monolayers. We believe for two reasons that small differences seen in these assays are nonspecific. First, as shown in Fig. 1 (unconnected symbols to the right of most panels), monolayers incubated with anti-VLA-2 antibodies generated a higher optical density than control monolayers even when no virus was added; second, although the largest differences were observed for echovirus 6, we found in plaque assays and in direct binding studies that this virus was not affected by VLA-2 antibodies (see below). We conclude that anti-VLA-2 antibodies protect cells against echoviruses 1 and 8 but not against the other viruses tested.

Inhibition of plaque formation by an anti-α2 MAb. To confirm the results of cytopathic effect assays, we measured the ability of the anti-α2 MAb AA10 to inhibit plaque formation by echoviruses chosen for their ability to form clear plaques in HeLa cells. HeLa cell monolayers in six-well tissue culture plates were washed and then preincubated with 0.4 ml of AA10 hybridoma supernatant or with an isotype-matched control antibody (approximately 20 μg of IgM per ml). After 2 h at room temperature, antibody solution was removed, virus (0.3 ml) was added, and incubation was continued at room temperature with gentle rocking for 60 min. Monolayers were overlaid with 0.75% agar in modified Eagle medium (Gibco) containing

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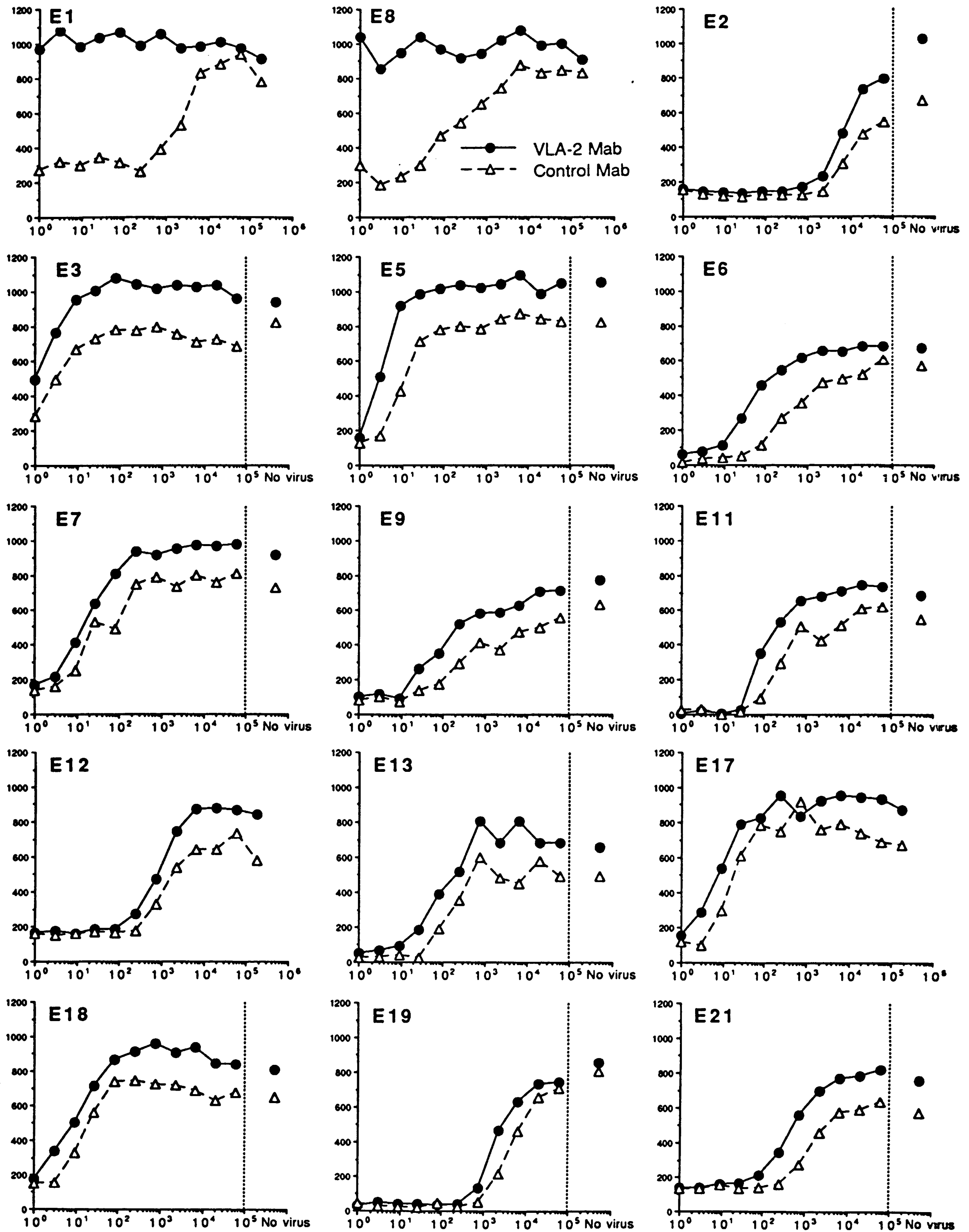
E1
E8
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Control Mab
No virus
Relative cell survival [OD405]
Relative virus dilution

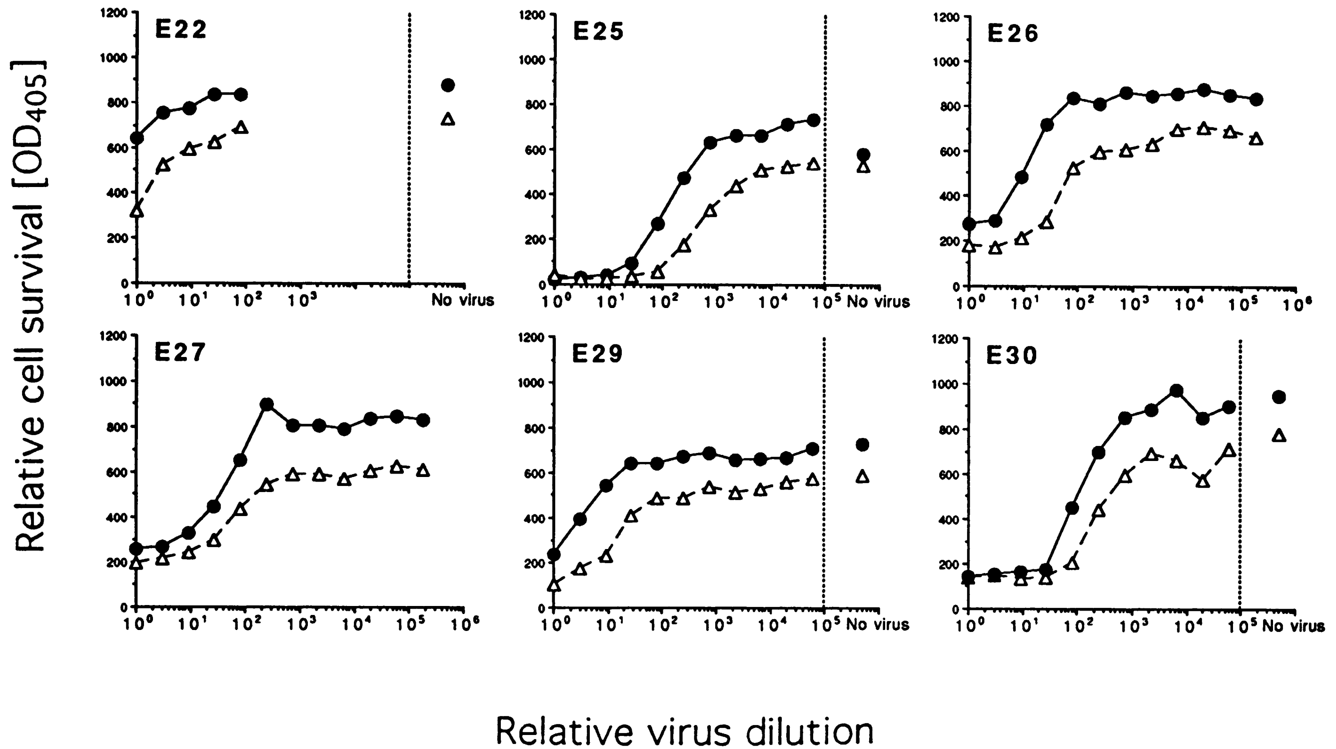

FIG. 1. Inhibition of echovirus cytopathic effect by anti-VLA-2 MAbs. HeLa cell monolayers in 96-well round-bottom plates were pretreated with anti-α2 and anti-β1 MAbs as described in the text, exposed to serial threefold dilutions of echoviruses, and then washed, refed, and incubated for 40 to 44 h. To quantitate cell survival, monolayers were incubated with the nitrotetrazolium dyes MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and INT (*p*-iodonitrotetrazolium violet), and optical densities (405 nm) were measured as described previously (16). Graphs show the optical density (mean of four samples × 1,000) plotted versus virus dilution (10^0 represents an arbitrary dilution of a virus stock). Results obtained for monolayers not exposed to virus are shown in most panels as unconnected symbols to the right of a vertical dotted line.

5% fetal calf serum, and plaques were allowed to develop for 40 to 44 h. Inhibition of plaque formation was calculated as follows: percent inhibition = 100% × [(number of plaques on control monolayers − number of plaques on antibody-treated monolayers)/number of plaques on control monolayers]. As shown in Fig. 2, MAb AA10 prevented plaque formation by echoviruses 1 and 8 but not that by echoviruses 6, 7, and 21.

Echoviruses 1 and 8 bind to a common receptor site. AA10 prevented attachment of radiolabeled echovirus 8 to HeLa cells, confirming that, as for echovirus 1, anti-α2 antibodies acted by blocking access to a virus receptor site (Fig. 3A). To prepare viruses metabolically labeled with [^{35}S]methionine, HeLa cell monolayers were inoculated with each virus (multiplicity of infection, 40 PFU per cell), incubated for 45 min at room temperature, and then washed and incubated at 37°C in methionine-free medium. After 3 h, the medium was replaced with methionine-free medium containing 100 μCi of [^{35}S]methionine per ml (Express Protein Labeling Mix, NEG-072; New England Nuclear) and incubation continued overnight. Cells were lysed by freezing and thawing three times, and then lysates were made in 0.5% Nonidet P-40 and clarified by centrifugation. Sodium dodecyl sulfate (0.8%) and 2-mercaptoethanol (0.1%) were added, and then the virus was pelleted through a 30% sucrose cushion and purified by sedimentation through 7.5 to 50% sucrose gradients in a modification of procedures described for rhinovirus 14 (17). Fractions corresponding to the approximately 150S peak of infectious virus were chosen. Purity of radiolabeled virus was assessed by electrophoresis in polyacrylamide gels, which revealed labeling

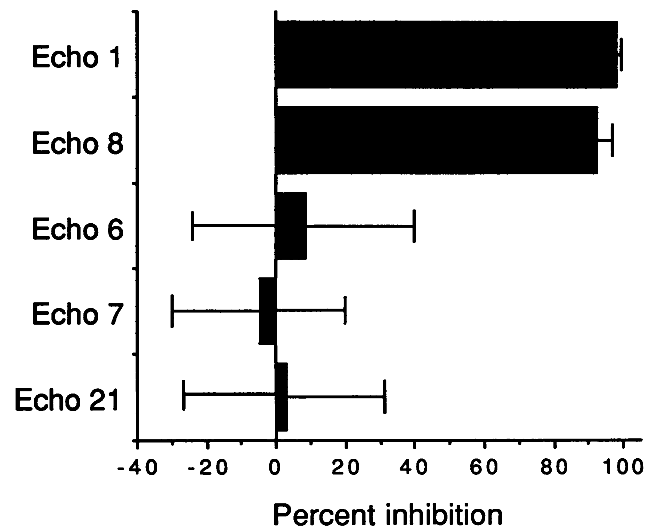

FIG. 2. Inhibition of viral plaque formation by MAb AA10. HeLa cell monolayers were preincubated with AA10 or a control antibody, and then antibody was removed and plaque assays were performed. To visualize plaques, monolayers were stained for 2 h at 37°C with MTT (6 mg/ml)-INT (1.5 mg/ml) dissolved in phosphate-buffered saline solution. The graph shows the mean percent inhibition of plaque formation ±95% confidence interval for triplicate monolayers.

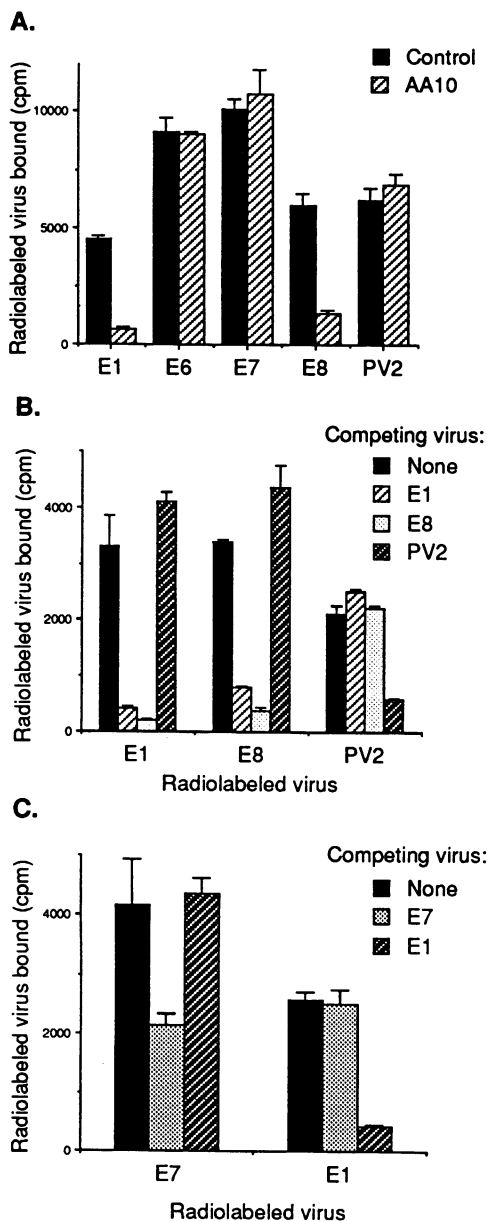

FIG. 3. Inhibition of radiolabeled echovirus attachment to HeLa cells by MAb AA10 and by excess homologous virus. (A) Inhibition by MAb AA10. HeLa cells (500,000) were incubated with either AA10 or an isotype-matched control antibody (23A-5-21S) for 1 h on ice, and then cells were washed with phosphate-buffered saline and suspended in 0.1 ml of each radiolabeled virus (10,000 to 15,000 cpm) in virus binding buffer (Hank's balanced salt solution [Gibco] buffered with 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.0] and containing 10 mM $MgCl_2$, 2 mM $CaCl_2$, and 4% fetal calf serum). After incubation for 2 h at room temperature, cells were washed extensively to remove unbound virus, and cell-associated radioactivity was determined. The graph shows results as mean counts per minute bound ± standard deviation (SD) for triplicate samples. (B) Binding competition between echoviruses 1 and 8. HeLa cell monolayers in 24-well plates were preincubated with 1.5×10^8 PFU of echovirus 1 or 8 or poliovirus 2 in 0.1 ml of virus binding buffer for 1 h at room temperature, and then unattached virus was removed and radiolabeled viruses (approximately 20,000 cpm per well) were added. After 1 h of incubation at room temperature, monolayers were washed and cell-bound radioactivity was determined. Results are shown as mean counts per minute bound ± SD for triplicate monolayers. (C). Binding competition between echoviruses 1 and 7. HeLa cell monolayers were preincubated with 3.5×10^8 PFU of echovirus 1 or 7, and then unattached virus was removed and binding of radiolabeled viruses was determined as described above.

only of virus capsid proteins. Preincubation of HeLa cells with excess echovirus 1 or 8 inhibited subsequent binding of radiolabeled echovirus 1 or 8 but not of poliovirus 2, confirming that these two echovirus serotypes bound to a common receptor site (Fig. 3B). In contrast, excess echovirus 1 did not inhibit binding of radiolabeled echovirus 7, and excess echovirus 7 did not inhibit binding of radiolabeled echovirus 1 (Fig. 3B).

Echoviruses 1 and 8 infect CHO cells transfected with human VLA-2. To confirm that the binding site for echoviruses 1 and 8 resides within VLA-2, we examined virus attachment to cells transfected with cDNA encoding the human α2 and β1 subunits. Because RD cells used in previous experiments express low levels of surface VLA-2 (4), it was desirable to introduce α2 cDNA into a rodent cell that expresses no human VLA-2. Dihydrofolate reductase-negative CHO cells (19), which express hamster β1 but no α2 (8), were cotransfected with cDNA encoding the human β1 subunit in the mammalian expression vector pECE (8) (provided by E. Ruoslahti, La Jolla Cancer Research Foundation, La Jolla, Calif.), with cDNA encoding the human VLA α2 subunit (18) inserted in the pECE vector with unique *Sal*I and *Xba* sites, and with the plasmid pMDR901 encoding dihydrofolate reductase (provided by M. Rosa, Biogen, Cambridge, Mass.). Transfectants were selected in nucleoside-free medium and enriched for expression of human α2 and β1 by fluorescence-activated cell sorting as described previously (8). As has been reported for echovirus 1 (4), mock-transfected CHO cells bound none of the echoviruses tested, although each of these viruses bound to HeLa cells (Fig. 4A, left and right panels). CHO cells expressing the α2 and β1 chains of VLA-2 bound radiolabeled echoviruses 1 and 8 but not echoviruses 6 or 7 or poliovirus 2 (Fig. 4A, center panel). Echovirus 1 or 8 binding to VLA-2-transfected CHO cells was prevented by AA10 (data not shown).

Expression of the α and β subunits of human VLA-2 rendered CHO cells susceptible to infection by echoviruses 1 and 8. As shown in Fig. 4B, when exposed to echovirus 1 or 8, VLA-2-transfected CHO cells, but not mock-transfected CHO cells, showed steady virus production well in excess of the virus input.

To determine whether the human β1 subunit is required for virus interaction with human α2, CHO cells were transfected with human α2 cDNA alone (again with pMDR901, to provide a selectable marker). Because the α2 subunit is expressed on the cell surface only in a VLA-2 heterodimer, in these cells human α2 was expressed in association with endogenous hamster β1. CHO cells expressing human α2 in the absence of human β1 bound echovirus 1 (Fig. 5A). In addition, these cells supported replication of echovirus 8 but not of echovirus 6 or 7 (Fig. 5B). We have previously reported that, whereas several MAbs directed against the human α2 subunit abrogate virus attachment to cells, no β1 MAb blocks attachment completely (3, 4). The demonstration that CHO cells expressing human α2 bound virus and became infected is consistent with the hypothesis that virus attaches to the α2 subunit itself. However, because the integrin β1 subunit is highly conserved in evolution (10), the data do not exclude the alternative possibility that hamster β1 contains conserved sequences essential to the virus binding site.

Discussion. The data presented here indicate that echoviruses 1 and 8 bind a common receptor site. Cell surface

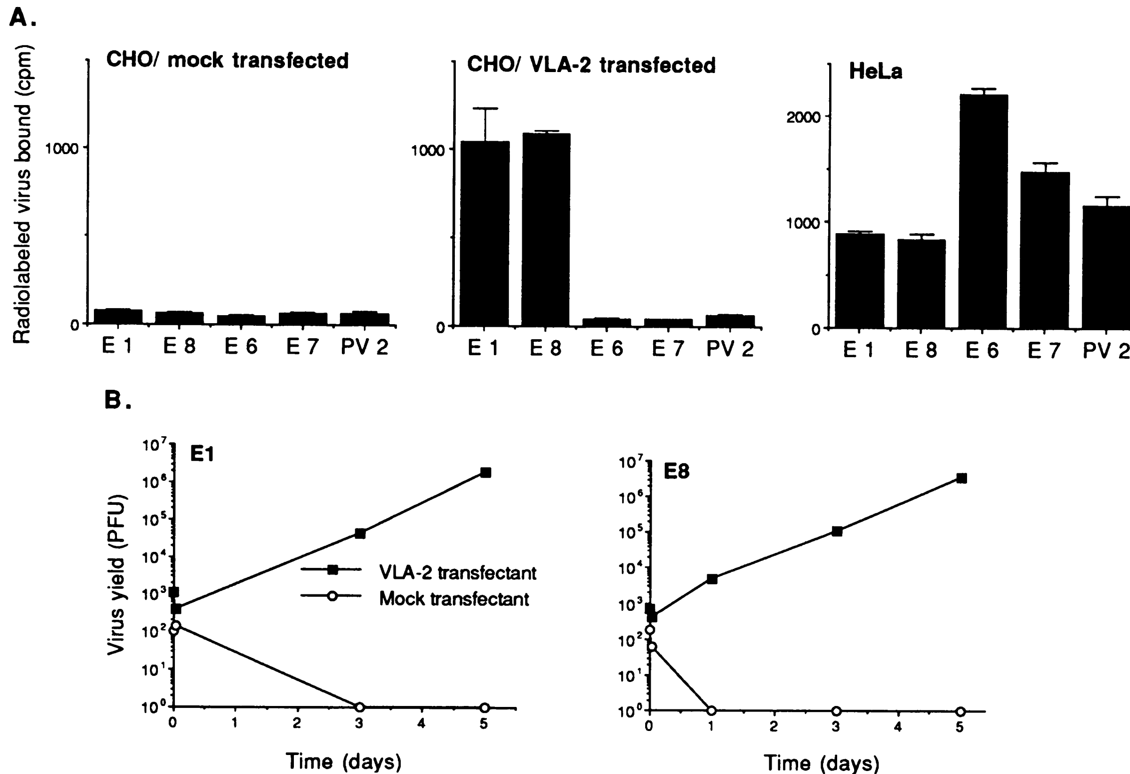

FIG. 4. Echovirus binding and replication by CHO cells transfected with human VLA-2. (A) Radiolabeled virus binding. CHO cells transfected with the α2 and β1 subunits of VLA-2 (center panel), mock-transfected CHO cells (left panel), and HeLa cells (right panel) were plated in 24-well culture plates. Confluent cell monolayers were incubated with radiolabeled viruses (6,000 to 10,000 cpm) for 1 h at room temperature and then washed and dissolved for scintillation counting. Results are shown as counts per minute bound per monolayer ± SD for triplicate samples. (B) Virus production. CHO cells (50,000) transfected with human α2 and β1 and sorted for α2 and β1 expression or 50,000 mock-transfected CHO cells were plated in 12-well tissue culture plates, exposed for 1 h at room temperature to echovirus 1 (left panel: 500,000 PFU per well added; 250,000 recovered in wash) or echovirus 8 (right panel: 19,200 PFU per well added; 18,000 recovered in wash), and then washed extensively and incubated at 37°C. Aliquots of input virus and virus removed by washing were saved. At 0 and 2 h and on days 1, 3, and 5, cultures were frozen and thawed three or four times to release cell-associated virus particles and virus titers were determined by plaque assay. Results are expressed as the mean total virus content (PFU) for duplicate 3-ml cultures.

attachment of both viruses depends critically on cell surface expression of the α2 subunit of human VLA-2. In contrast, other echovirus serotypes examined bind cells independently of VLA-2.

Other investigators have reported that attachment of biotinylated echovirus 11 to primate cells is inhibited by preincubation with a variety of echovirus serotypes, including echoviruses 1 and 8, and concluded that, except for serotypes 22 and 23, all echoviruses bind a common receptor site (14). Their observations are difficult to reconcile with the data presented here but may be related to their use of biotinylated virus and a cytofluorometric assay for virus binding or to their use of cell lines other than HeLa. In the experiments reported here, we found that echoviruses 1 and 8 were distinct from other echovirus serotypes in that (i) infection by these two viruses was uniquely prevented by VLA-2 antibodies, (ii) cell surface attachment of radiolabeled echoviruses 1 and 8, but not of other echoviruses, was prevented by VLA-2 antibodies, and (iii) CHO cells, when transfected with the α2 subunit of VLA-2, gained the capacity to bind and become infected by echoviruses 1 and 8 but not by other echoviruses. In complementary experiments, we found that echoviruses 1 and 8, but not echoviruses 1 and 7, competed for a common cell surface binding site.

In these experiments we have demonstrated that rodent cells, which express no VLA-2 and which are totally nonpermissive, became susceptible to infection when transfected with only the α2 subunit of human VLA-2. The results presented here confirm our previous findings (4) and extend them—first, by demonstrating that binding and infection by echoviruses 1 and 8, but not by other echoviruses, are dependent on VLA-2 expression, and second, by showing that species-restricted binding and infection are determined solely by the human α2 subunit and do not require expression of human β1.

Echoviruses 1 and 8 were among the original echovirus isolates reported, and their antigenic relationship is well recognized. Although antisera raised against prototype isolates show little or no cross-neutralizing activity, intermediate strains exist which cannot be distinguished serologically (1, 6, 9); in addition, both serotypes share a common antigen demonstrable by double-diffusion gel precipitation (9). As a result, in many enterovirus classifications these two viruses have been grouped together (7). Whether the prototype strains used in these studies are different viruses or divergent strains of the same virus is uncertain. In either case, they are distinct from other echoviruses by virtue of their interaction with VLA-2. A minor serologic relationship between echoviruses 1 and 8 and echovirus 12 has also been reported (1, 6). We found that antibodies to VLA-2 did not inhibit infection of HeLa cells by echovirus 12 (Fig. 1).

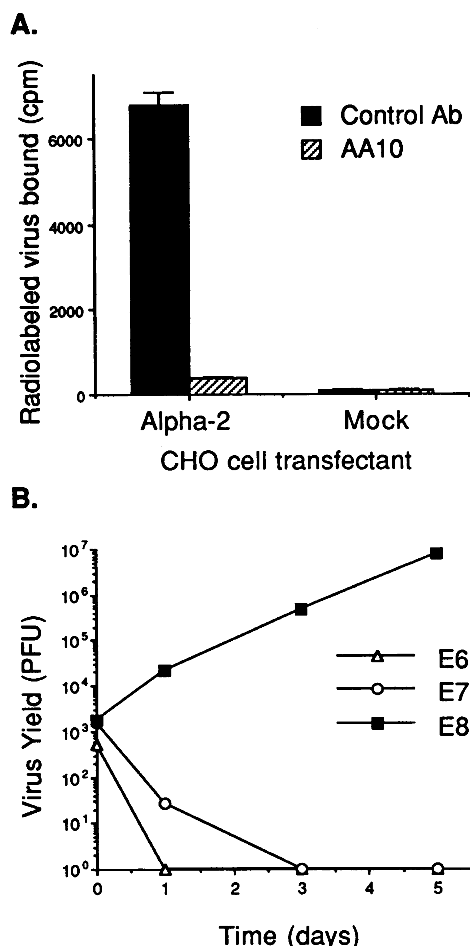

FIG. 5. Echovirus binding and replication by CHO cells transfected with the α2 subunit of human VLA-2. (A) Radiolabeled virus binding. Confluent monolayers of CHO cells transfected with α2 cDNA or mock-transfected CHO cells were treated for 1 h with AA10 or an isotype-matched control antibody, washed, and exposed to radiolabeled echovirus 1 (26,000 cpm) for 2.5 h at room temperature. Monolayers were then washed and dissolved for determination of cell-bound radioactivity. Results are shown as mean counts per minute bound ± SD for triplicate monolayers. (B) Virus production. CHO cells (50,000) transfected with human α2 were plated in 12-well tissue culture plates, exposed for 1 h at room temperature to echovirus 8 (64,000 PFU per well added; 80,000 recovered in wash), echovirus 6 (160,000 PFU per well added, 133,000 recovered), or echovirus 7 (400,000 PFU per well added; 266,000 recovered), and then washed extensively and incubated at 37°C. At 0 h and on days 1, 3, and 5, cultures were frozen and thawed three times and virus titers were determined by plaque assay. Results are expressed as the mean total virus content (PFU) for duplicate 4-ml cultures.

What molecules serve as receptors for other echoviruses? Other investigators have reported that an MAb to a 44-kDa cell surface protein protects susceptible cells from infection by many echovirus serotypes, including 1, as well as 6 and 7 (12, 13). However, no direct evidence has been presented to support a role for this protein in virus attachment to cells. We have obtained an MAb that protects HeLa cells from infection by a variety of echoviruses and that prevents cell attachment of radiolabeled echoviruses 6 and 7 (2); this antibody has no effect on infection or cell surface attachment by echovirus 1 or 8. Whether either of these antibodies will identify a receptor molecule for many echovirus serotypes remains to be determined.

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