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Mouse Cells Expressing Human Intercellular Adhesion Molecule-1 Are Susceptible to Infection by Coxsackievirus A21

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Competitive viral binding assays have revealed previously that coxsackievirus A21 (CAV21) and human rhinovirus 14 (HRV14) share a common cell surface receptor. More recently, intercellular adhesion molecule-1 (ICAM-1) has been identified as the cellular receptor for HRV-14. Also, anti-ICAM-1 monoclonal antibodies (MAbs) blocked infection by HRV14, CAV13, CAV18, and CAV21, suggesting that these viruses share this receptor; however, this has never been established by more direct methods. In this study we show conclusively that CAV21 binds to ICAM-1 and that MAbs directed against the N-terminal domain of the molecule inhibit this attachment. Furthermore, we show that the specific interaction between ICAM-1 and 160S CAV21 virions induces formation of 135S A particles. Finally, we show transfection of normally nonsusceptible mouse L cells with human ICAM-1 cDNA renders them susceptible to infection by CAV21.

The human picornavirus coxsackievirus A21 (CAV21) is one of 23 serotypes of coxsackie A-group viruses. CAV21 and human rhinoviruses (HRVs) constitute one of the major groups of causal agents of upper respiratory infections. The single-stranded CAV21 genome is 7,401 nucleotides long, encoding an open reading frame of 2,206 codons, possessing a 711-nucleotide 5′ noncoding region and a 3′ noncoding region of 72 nucleotides with a poly(A) tract (19). The length of the 5′ noncoding region and the small --U/--A imbalance in codon usage are rhinovirus-like characteristics. While in the 3′ part of the genome there is >90% amino acid homology with poliovirus type 1, the rest of the genome is less homologous (19), suggesting that CAV21 is a recombinant virus, possibly between the poliovirus and rhinovirus genomes.

Cellular receptors for coxsackie A-group viruses have been relatively little studied compared to other members of the *Picornaviridae* family, i.e., polioviruses (12, 20, 26), rhinoviruses (14, 34, 36), echoviruses (2, 3), and coxsackie B group viruses (8, 9, 32). However, recently a cellular attachment receptor for CAV9 was identified as integrin αvβ3, with CAV9 attaching to αvβ3 through RGD motifs located in the viral capsid (29). Little information on the identity of the CAV21 cellular attachment receptor is available, apart from data obtained from relatively crude virus competition binding assays indicating that CAV21 and HRV14 share a common cell surface receptor (22). This conclusion was reinforced by the finding that monoclonal antibodies (MAbs) raised against lysates of HRV14-susceptible cells inhibited infection by HRV14, CAV13, CAV18, and CAV21 (6); blocking MAbs were found to recognize intercellular adhesion molecule-1 (ICAM-1) (34), a member of the immunoglobulin supergene family. ICAM-1 is a counterreceptor for the integrins leukocyte function antigen 1 (CD11a) and Mac-1 (CD11b) (10, 34). Site-directed mutagenesis and domain deletion studies identified the N-terminal extracellular domain of ICAM-1 as the HRV14 attachment site (25). A possible interaction between the ICAM-1 N-terminal domain and the floor of the rhinovirus capsid canyon has been postulated by using data obtained from molecular modelling studies (7, 28, 30). Soluble forms of ICAM-1 (sICAM-1) corresponding to the entire extracellular portion or to the two N-terminal immunoglobulin-like domains were able to reduce HRV14 infectivity and to induce conformational changes in virion structure (5, 15). Transfection of normally nonpermissive monkey kidney cells with ICAM-1 cDNA rendered them susceptible to HRV14 infection (13, 35), although ICAM-1-expressing rodent cells were refractory to infection by HRV14 (13, 16). Despite preliminary data suggesting that CAV21 uses ICAM-1 as its cellular receptor, it has also been reported that CAV21 replicates in human rhabdomyosarcoma cells (31) and tissues of suckling mice, both of which lack human ICAM-1 expression (6).

The recent boom in characterization of cellular receptors has revealed that virus attachment and entry can be a complex multistep process possibly involving numerous cell surface molecules. Conclusions made from data based solely on the ability of MAbs directed against cell surface molecules to inhibit virus attachment can be misleading. For example, a MAb recognizing CD44 (lymphocyte homing receptor) inhibited the attachment of polioviruses to susceptible cells, even though polioviruses do not bind CD44 when this molecule is expressed on the surface of murine cells (33). The complexity of the cell attachment-entry mechanism of viruses has further been highlighted by the finding that MAbs directed against particular cell surface molecules inhibited replication of some viruses without inhibiting viral attachment. For example, anti-αvβ3 MAbs blocked adenovirus type 2 replication (38), anti-CD26 inhibited human immunodeficiency virus (4), and anti-β2 microglobulin MAbs have recently been reported to block many echoviruses and CAV9 (37).

Against this background, the focus of the present study was to determine whether CAV21 binds directly to surface-expressed ICAM-1 and to establish whether the reported inhibition of its replication by anti-ICAM-1 MAbs is due to direct receptor competition. In addition, we sought to determine whether ICAM-1 expression alone is sufficient to mediate CAV21 cell entry and subsequent replication in murine cells.

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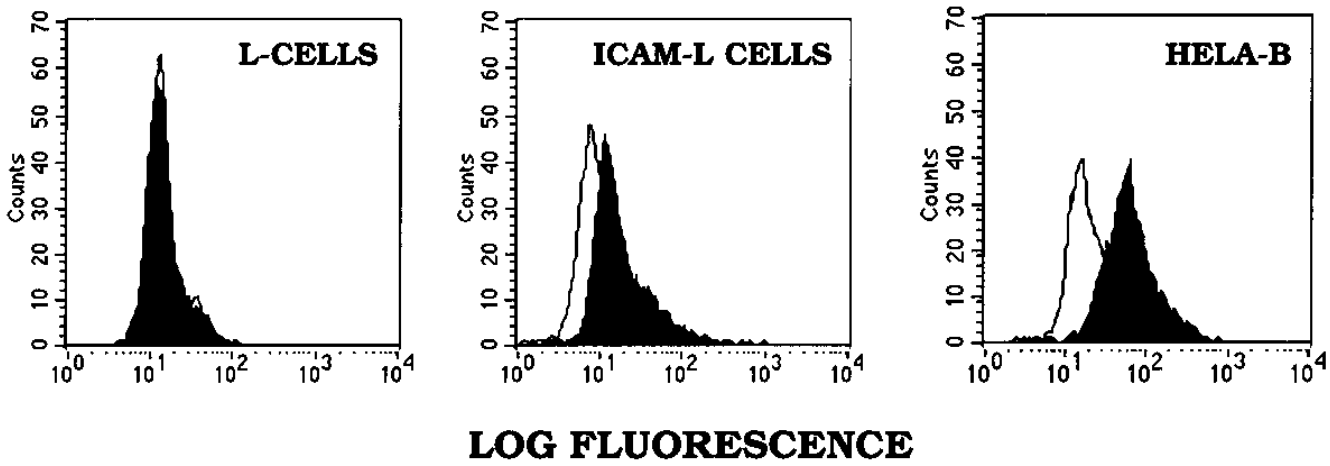

FIG. 1. Relative levels of ICAM-1 expression on ICAM-L cells and HeLa B cells. Flow cytometric analysis. Cells (10^6) in 100-μl aliquots were incubated with MAb IH4 or an isotype-matched MAb diluted in PBS containing 1% bovine serum albumin on ice for 30 min, after which the cells were washed with 5.0 ml of PBS-bovine serum albumin. The cells were then pelleted at 1,000 × *g* for 5 min and resuspended in 100 μl of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (heavy plus light chains) (Silenus, Melbourne, Australia) diluted in PBS-bovine serum albumin. Following incubation on ice for 30 min, the cells were washed and pelleted as described above, resuspended in PBS-bovine serum albumin, and analyzed with a FACStar analyzer (Becton Dickenson, Sydney, Australia). The open histograms represent binding of the isotype-matched control antibody; the closed histograms represent binding of MAb IH4.

CAV21 binds ICAM-1. The level of ICAM-1 expression on the surface of stable mouse ICAM-1-transfected L cells (ICAM-L cells; supplied by Andrew Boyd, WEHI, Melbourne, Australia) and HeLa B cells was assessed by flow cytometry and immunoprecipitation using anti-ICAM-1 MAb IH4 (MAb IH4 binds to N-terminal domain 1 of ICAM-1 [1]). The fluorescence histograms in Fig. 1 indicate a lower level expression of ICAM-1 on the surface of ICAM-L cells compared to expression on CAV21-susceptible HeLa B cells. No detectable ICAM-1 was found on nontransfected mouse L cells. Immunoprecipitation of surface-biotinylated HeLa B and ICAM-L cells with MAb IH4 identified a polypeptide of approximately 95 kDa from both cell lysates (data not shown).

To determine whether CAV21 binds directly to ICAM-1 and if the process can be inhibited by anti-ICAM-1 MAbs IH4 and 15.2 (MAb 15.2 recognizes an epitope on the N-terminal domain of ICAM-1 [1]), confluent 24-well-plate monolayers of L cells and ICAM-L cells were pretreated with MAbs 15.2 and IH4 (25 μg/ml) for 1 h at room temperature and then incubated with approximately 3×10^4 cpm of ^{35}S-labeled CAV21 (Kuykendall strain). Radiolabeled preparations of purified 160S CAV21 were prepared by the method described previously (32). ICAM-L cells bound significant levels of CAV21, binding that could be reduced to background levels by pretreating the cells with anti-ICAM-1 MAb IH4 or 15.2 and not by a control MAb (MAb 204, directed against poliovirus type 1 [27]) (Fig. 2A).

To investigate whether sICAM-1 inhibited the binding of CAV21 to the surface of ICAM-L cells, ^{35}S-labeled CAV21 was preincubated with sICAM-1 (25 μg/ml) for 1 h at 22°C and then incubated on confluent 24-well plate monolayers of ICAM-L cells. The data in Fig. 2B indicate that sICAM-1 inhibited CAV21 attachment to ICAM-L cells by approximately 35%. This result is comparable to that reported for HRV14, where sICAM-1 (25 μg/ml) reduced the attachment of this virus to HeLa cells by approximately 40% (23).

ICAM-1 induces formation of altered CAV21 particles. A prerequisite for cell entry of most enteroviruses is a receptor-mediated conformational change in viral capsid architecture, resulting in the loss of VP4, an increased sensitivity to the action of proteases, and a reduced sedimentation coefficient in velocity gradient centrifugation. These altered particles are referred to as A-type particles (A-particles) (11). Detection of receptor-mediated conformational changes in virion structure needs a test virion population that is free of contaminating host cell proteins. In this study CAV21 160S particles were separated by velocity sucrose gradient centrifugation of ^{35}S-labeled CAV21 grown in HeLa B cells (32). Gradient-separated virus typically contained two distinct peaks, a larger peak at 160S and a smaller peak at 125S (Fig. 3A). Heated (56°C for 10 min) 160S particles migrated as a single peak at 80S (Fig. 3A). Protein profiles of both 160S and 80S particles were identical except that heating at 56°C resulted in the loss of VP4, while 125S particles were shown to consist of VP0, VP1, and VP3 (data not shown). To examine the relative binding abilities of the 160S, 125S, and 80S particles to the ICAM-L and HeLa B cells, confluent cell monolayers in 24-well culture plates were incubated with 10^5 cpm of each three CAV21 viral fractions. 160S particles attached to HeLa B and ICAM-L cells (Fig. 3B); the higher levels of binding to HeLa B cells are probably due to the higher level of ICAM-1 expression on these cells (Fig. 1A). Neither 125S or 80S particles showed significant binding (Fig. 3B).

To demonstrate that the formation of CAV21 A-particles could be induced by surface-expressed ICAM-1, 160S particles were incubated with ICAM-L cells for 60 min at 22°C. Unbound particles were removed by washing, and cell monolayers were then incubated at 37°C for 60 min. The cells were pelleted, and the supernatant was centrifuged on a 5 to 30% sucrose gradient. The unbound particles sedimented at 160S, while eluted particles had a reduced sedimentation coefficient (approximately 135S) indicative of A-particle formation (Fig. 3C). The small peak in the middle of both gradients probably represents 125S particles contaminating the original 160S pooled fraction.

CAV21 can replicate in ICAM-1-expressing mouse cells. As surface-expressed ICAM-1 bound CAV21 and induced the formation of A-particles, the next step was to determine whether ICAM-L cells were permissive for cell entry and subsequent replication of CAV21. Confluent monolayers of L cells, ICAM-L cells, and HeLa B cells were each prepared in six-well tissue culture plates and infected with 10^5 50% tissue

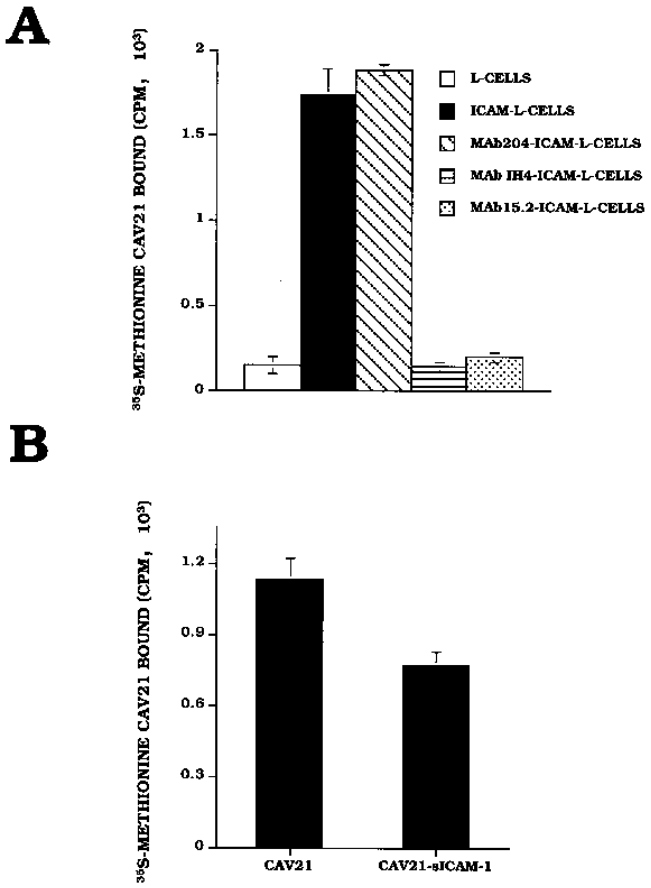

FIG. 2. Inhibition of CAV21 binding to ICAM-1 by using anti-ICAM-1 MAb blockade and sICAM-1. (A) Inhibition of ^{35}S-labeled CAV21 binding to ICAM-L cells by MAbs IH4 and 15.2. Mouse L-cell or ICAM-L-cell monolayers in 24-well tissue culture plates were first incubated for 1 h at 22°C with MAbs 204, IH4, and 15.2 (25 μg/ml) in DMEM containing 1% bovine serum albumin and then washed and incubated with ^{35}S-labeled CAV21 (3×10^4 cpm) for the same period. Following four washes with PBS, the cell monolayers were dissolved in 200 μl of 0.2 M NaOH–1% sodium dodecyl sulfate, and the amount of labeled virus that was bound was measured by liquid scintillation counting. Results are expressed as the means from triplicate wells plus 2 standard deviations. (B) Ability of sICAM-1 to inhibit CAV21 binding. Labeled CAV21 (2×10^4 cpm) was incubated in the presence and absence of sICAM-1 (25 μg/ml) diluted in DMEM-bovine serum albumin at 22°C for 1 h and then incubated with confluent monolayers of ICAM-L cells in 24-well tissue culture plates as described above. Results are expressed as the means from triplicate wells plus 2 standard deviations.

culture infective doses ($TCID_{50}$) of CAV21. The CAV21 inoculum was removed by four washes with phosphate-buffered saline (PBS), 2 ml of Dulbecco minimal essential medium (DMEM) was then added, and the plates were incubated at 37°C. Both monolayers and culture supernatants were collected at 0, 24, and 48 h postinoculation (p.i.) and pooled; CAV21 yields were determined by a cell lysis assay on HeLa B-cell monolayers in 96-well plates. A complete cytopathic effect was observed in HeLa B cells at 24 h p.i.; therefore, no further samples were collected. ICAM-L cells showed a slight change in cell morphology at 24 h p.i. which did not intensify following incubation for a further 24-h period. CAV21 yields at 0, 24, and 48 h are shown in Fig. 4A. The ICAM-L cells were permissive for CAV21 replication, as shown by an increase in titer from $10^{2.46}$ (0 h p.i.) to $10^{4.25}$ (24 h p.i.) $TCID_{50}$/ml, although no further increase in CAV21 titer was observed 48 h p.i. ($10^{4.08}$ $TCID_{50}$/ml). HeLa B cells supported significantly

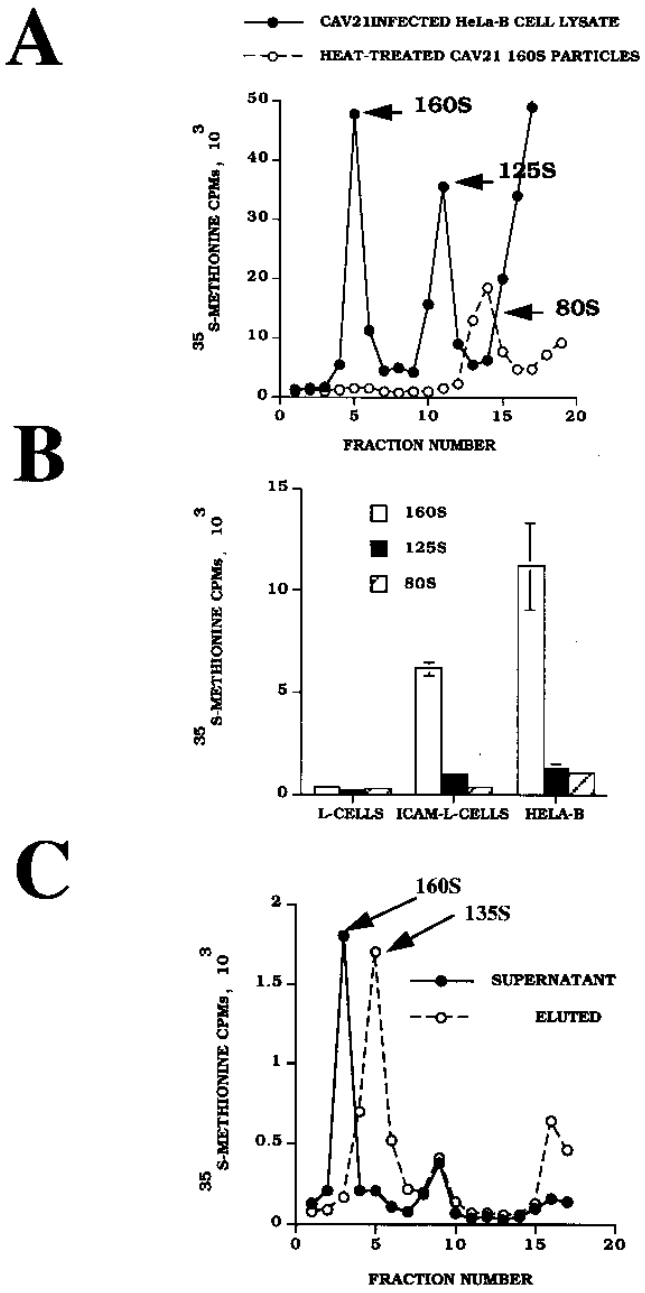

FIG. 3. ICAM-1-induced alteration of CAV21 virions. (A) Analysis of the sedimentation of 160S (virion), 125S (provirion), and 80S (heat-treated 160S virion) CAV21 particles. One milliliter of [^{35}S]methionine-labeled CAV21-infected HeLa B-cell lysate was layered on a 12-ml 5 to 30% linear sucrose gradient and centrifuged for 90 min at 4°C in an SW 41Ti rotor at 36,000 rpm (32). The sedimentation of CAV21 80S particles (prepared by heating 160S particles for 10 min at 56°C in the presence of 0.05% Nonidet P-40) in a parallel gradient is also represented. Fractions (~700 μl) were collected from the bottom of the gradient, and radioactivity was determined by liquid scintillation counting. (B) Cell binding capacity of CAV21 160S, 125S, and 80S particles. Approximately 8×10^4 cpm of CAV21 160S, 125S, and 80S particles was incubated with confluent 24-well monolayers of L cells, ICAM-L cells, and HeLa B cells and treated as described in the legend to Fig. 2A. (C) ICAM-1-mediated alteration of 160S CAV21 virions. Labeled CAV21 160S particles (3×10^5 cpm) were incubated with ICAM-L cells (10^7) for 60 min at 22°C in DMEM. The cells were washed, and the unbound virions were saved. The CAV21–ICAM-L-cell complexes were incubated at 37°C for 45 min, cells were then removed by centrifugation, and the eluted and unbound particles were analyzed in parallel 5 to 30% linear sucrose gradients as described for panel A.

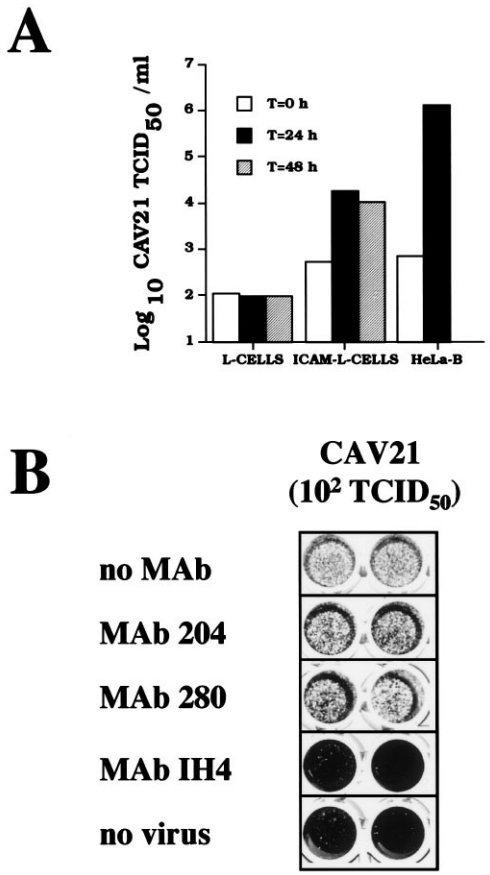

FIG. 4. Replication of CAV21 in ICAM-1-expressing mouse and HeLa B cells. (A) Confluent monolayers of L cells, ICAM-L cells, and HeLa B cells in six-well tissue culture plates were inoculated with 10^5 $TCID_{50}$ of CAV21. The virus was allowed to adsorb for 1 h at 37°C, and the monolayers were then washed four times with PBS. Two milliliters of DMEM containing 1% fetal calf serum was added to each well and incubated at 37°C for the times indicated. The pooled cells and supernatants were subjected to three freeze-thaw cycles, and the yield of infectious virus was determined by a cell lysis assay in 96-well monolayers of HeLa B cells. (B) Inhibition of CAV21-induced cell lysis. Monolayers of HeLa B cells in 96-well plates were incubated with MAbs 204, 280, and IH4 (25 μg/ml) diluted in DMEM containing 1% fetal calf serum for 1 h at 22°C. CAV21 (10^2 $TCID_{50}$) was added to each well, and the plate was incubated for 48 h at 37°C. The cell monolayers were then stained with a crystal violet-methanol stain and examined for evidence of CAV21-induced cell lysis.

higher levels of CAV21 replication, reaching a titer of $10^{6.16}$ $TCID_{50}$/ml at 24 h p.i. No increase in CAV21 yield was observed in nontransfected L cells at the assayed times.

To analyze whether the lytic infection of HeLa B cells by CAV21 could be inhibited by pretreatment with anti-ICAM-1 MAb IH4, duplicate wells of a 96-well plate containing monolayers of HeLa B cells were preincubated for 1 h with MAb IH4 (25 μg/ml) or, as a control, with antipoliovirus MAb 204 or anti-poliovirus receptor MAb 280 (27) and inoculated with 10^2 $TCID_{50}$ of virus. The cells were incubated at 37°C for 48 h, then stained with a crystal violet-methanol solution, and examined for CAV21-mediated cell lysis. As shown Fig. 4B, a substantial cytopathic effect was observed in wells lacking MAb or pretreated with MAb 204 or 280, whereas MAb IH4 completely inhibited CAV21 lytic infection.

In this report we have established that CAV21 employs ICAM-1 as a receptor for attachment and cell entry. This finding confirms and extends previous reports that CAV21 uses the same cellular receptor as major-group rhinoviruses. The inhibition of CAV21 binding to ICAM-1 cells with anti-ICAM-1 MAbs (Fig. 2A), the reduced binding with sICAM-1 (Fig. 2B), and the inhibition of lytic infection of HeLa B cells with an anti-ICAM-1 (domain 1) MAb (Fig. 4B) together suggest specific blockade of the CAV21 binding epitope on ICAM-1 and not steric hindrance of an alternate receptor. Since ICAM-1 domain 1 MAbs inhibit CAV21 binding, it is highly likely that the CAV21 binding region is located in this domain, as is also the case for HRV14 (25).

Studies investigating the attachment of CAV21 to cells expressing ICAM-1 domain deletion mutants are required to validate this finding. Expression of ICAM-1 alone on the surface of mouse cells was sufficient to establish replication of CAV21 (Fig. 4A), a situation analogous to the replication of polioviruses in poliovirus receptor-transfected mouse cells (26). However, the yield of infectious CAV21 in ICAM-L cells was approximately 100-fold less than that obtained in HeLa B cells (Fig. 4B), which may be a direct consequence of HeLa B cells binding nearly twice the amount of CAV21 (Fig. 3B), because this cell type has a higher level of ICAM-1 expression (Fig. 1). These data could also be interpreted to indicate that HeLa-B cells may possess other receptor molecules that facilitate increased cell entry and/or more-efficient viral capsid uncoating of CAV21. However, the possibility that cell type differences between HeLa B and L cells influence CAV21 yields by affecting one or many stages of the viral replicative process cannot be disregarded. Although cell entry mechanisms by CAV21 are not the subject of this report, it is noteworthy that a recent study of HRV14 cell entry detected major differences in which virus was internalized in either HeLa cells or ICAM-1-expressing BHK cells (16); HRV14 uptake was similar in both cell types, but HeLa cell entry was by clathrin-coated pits and vesicles while entry into ICAM-1-expressing BHK cells occurred through an unknown channel-like system. However, HRV14 did not replicate in ICAM-1-expressing cells, possibly because of a block in virus uncoating or viral translation (16). In our experience, HRV14 does not replicate in ICAM-1-expressing mouse cells (13). As CAV21 can enter and replicate in ICAM-1-expressing murine cells, it would be of interest to determine whether CAV21 would use the same modes of entry into HeLa and ICAM-1-expressing BHK cells as employed by HRV14.

The production of A-particles as a result of specific binding to their cellular receptors is a common characteristic of many picornaviruses, and in this study ICAM-1-expressing L cells were shown to induce capsid alteration of CAV21 160S virions producing A-particles sedimenting at 135S (Fig. 3C). Elution of CAV21 A-particles from ICAM-L cells is consistent with previous reports on the elution of A-particles from susceptible cells for polioviruses (17), rhinoviruses (21), and coxsackievirus B3 (9). This CAV21 A-particle formation supports the concept that receptor-mediated capsid conformational change is a critical prerequisite for the cell entry of many picornaviruses. However, further studies of the interaction of CAV21 and sICAM-1 are needed to establish whether sICAM-1-mediated alteration of CAV21 produces intermediate viral structures that are more like those observed following interaction of HRV14 and sICAM-1 (i.e., particles sedimenting at 150S to 140S through to 120S and 80S [5, 15, 18, 24]) or those from poliovirus and soluble poliovirus receptor (160S to 135S [12, 20]).

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