

## NOTES

# A Human Cell Line Selected for Resistance to Adenovirus Infection Has Reduced Levels of the Virus Receptor

PAUL FREIMUTH\*

*Biology Department, Brookhaven National Laboratory, Upton, New York 11973*

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**To investigate determinants of host cell susceptibility to infection, cells partially resistant to infection were selected from the rare cells which remained adherent after infection of a culture of A549 cells with Ad2RAE, a mutant of adenovirus type 2 whose vertex capsomers lack an Arg-Gly-Asp (RGD) sequence which mediates binding of wild-type virus to integrins. Integrins promote the internalization of attached virions, whereas adsorption itself results from binding of the viral fibers to an unidentified cellular receptor. Following three rounds of selection, a persistently infected culture was established in which virus replication was detected in approximately 5% of the cells. Uninfected cells were readily cloned from the culture, indicating that at any particular time the majority of cells in the culture were uninfected. The resistance of one clone of uninfected cells to infection was correlated with a 10-fold reduction in the concentration of fiber receptors on these cells compared with the parental A549 cell line, indicating that efficiency of virus adsorption depends on the receptor concentration. Surprisingly, the rate at which host cells internalized RGD-negative virus also was strongly dependent on the fiber receptor concentration. While internalization of wild-type virus is promoted by the binding of integrins to the penton base RGD sequence, these results suggest that virus also can enter cells by an alternate pathway which requires binding of virions to multiple fiber receptors.**

Adenoviruses are thought to enter the host cell cytoplasm largely intact (6, 7, 14, 22) following penetration of the endosomal membrane (12), although the mechanisms of virus endocytosis and membrane penetration are not well understood. Adsorption of virus occurs through binding of the viral fibers, elongated proteins which project radially from each of the 12 vertices of the nonenveloped icosahedral capsid, to specific receptors in the cell plasma membrane (24). Receptors have not been identified for any of the more than 40 known human adenovirus serotypes. Fibers are anchored to the capsid through an interaction with the viral penton base subunit (5), a pentamer of identical polypeptides which forms each capsid vertex (30). The penton base polypeptides of some, but not all, human adenovirus serotypes contain an Arg-Gly-Asp (RGD) sequence which can bind to integrins in the host cell membrane (3, 21). Recruitment of integrins into the initial virus-receptor complex promotes the endocytosis of virions (32); however, the mechanism by which integrins facilitate internalization of virus is not known.

Integrins on adherent cells localize to the basal surface, where they mediate cell adhesion by binding to RGD elements and other ligands in proteins which form the extracellular matrix (18, 26), whereas integrins on cells grown in suspension are likely to be distributed evenly on the cell surface. Mutants of adenovirus type 2 (Ad2) lacking an intact penton base RGD sequence have a prolonged eclipse period in adherent cells but replicate with normal kinetics in suspension cells (3). Loss of the penton base RGD ligand suggests that RGD-negative viruses infect cells through an integrin-independent pathway. However, the dependence of the duration of the virus eclipse

on the adhesion state of host cells suggests that integrins also have a role in the internalization of RGD-negative viruses. Consistent with this view, others have reported that infection of cells by wild-type Ad2 is inhibited by RGD-containing peptides or integrin antibodies (4, 32), reagents which could interfere with recruitment of integrins into the virus-receptor complex or with integrin function. Thus, if RGD-negative viruses use an integrin-independent pathway to infect cells, then it is not clear why this pathway is not used by wild-type Ad2 in the presence of integrin inhibitors.

The rapid cell-detaching activity associated with the wild-type penton base subunit (9, 23, 25, 31), which results from competition with matrix protein RGD sequences for integrin binding, was not detected in the penton base subunits of RGD-negative Ad2 mutants (3). We also observed that although monolayer cultures became rounded late after infection with RGD-negative viruses, individual cells remained attached to the culture plate at times when the cells in identical cultures infected with wild-type Ad2 were quantitatively detached. In this report, the loss of cell-detaching activity of the RGD-negative viruses was exploited to select for rare cells which are resistant to infection and remain viable and adherent after infection of a culture at high multiplicity with an RGD-negative virus. It was expected that resistance to infection might result from loss of cellular functions involved in receptor-mediated endocytosis of adenovirus and that characterization of such cells might reveal aspects of the mechanism of this process.

**Selection of A549 cells that are resistant to infection.** It seemed possible that if any cells in a culture escaped infection by an RGD-negative virus, then they would remain adherent and could be expanded in culture. Reinfection of the expanded culture might enrich the population for cells that are resistant to infection. To test this hypothesis, a 10-cm diameter dish of confluent A549 cells, a human epithelial cell line that is per-

\* Phone: (516) 344-3350. Fax: (516) 344-3407. Electronic mail address: freimuth@bnlxl1.bnl.gov.

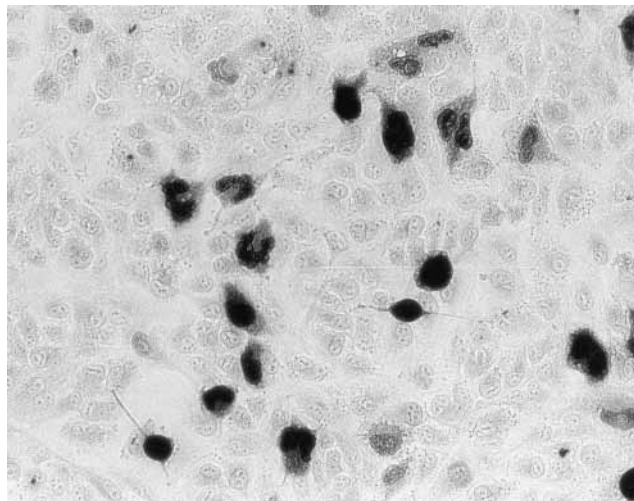


FIG. 1. Viral hexon antigen production in persistently infected cultures. A sample of the persistently infected culture at confluence was fixed with 90% methanol, stained with anti-hexon antibodies, and developed with horseradish peroxidase-conjugated anti-immunoglobulin G, followed by metal-enhanced diaminobenzidine (16), which deposits a dark precipitate on hexon-producing cells.

missive for adenovirus replication, was infected at 5 focus-forming units (FFU) per cell with Ad2RAE, an Ad2 mutant whose penton base RGD sequence was converted to RAE (Arg-Ala-Glu) by site-directed mutagenesis (3). At 24 h after infection, the cells were trypsinized and replated in complete medium supplemented with neutralizing antibodies specific for the viral hexon antigen. To decrease the virus burden in the culture, at 4-day intervals the plates were rapped sharply to detach round infected cells, and the residual adherent cells were fed with fresh complete medium containing neutralizing antibodies. An apparently healthy population of cells grew out in about 1 month. These cells were infected a second time with Ad2RAE at 20 FFU per cell and then processed exactly as described for the first round of infection. The cytopathic effect (CPE) was extensive following the second round of infection, and again it took about 1 month to recover an apparently healthy cell population. These cells were infected a third time with Ad2RAE at 40 FFU per cell. In striking contrast to the first two rounds of infection, only a minimal CPE was observed following the third round of infection. Neutralizing antibodies were then omitted from a portion of the culture, and the cells have since been in continuous culture in the presence or absence of neutralizing antibody for over 1 year. The cells are trypsinized and replated at a 1:5 dilution two or three times a week.

**The resistant cell cultures are persistently infected.** Although the cultures generally appear healthy, a small number of cells that display a characteristic adenovirus CPE are shed into the medium of both the antibody-treated and untreated cultures. To test for the presence of virus, samples of each culture were lysed by freezing and thawing and the lysates were applied to normal A549 cells. A CPE was evident in both cultures after 72 h, indicating that high concentrations of virus were present in both lysates. Monolayers of each resistant cell population were fixed and stained with anti-hexon antibodies to estimate the frequency of infected cells in each population. About 5% of cells in the neutralizing antibody-treated culture and 2% of cells in the untreated population contained detectable levels of hexon antigen. The frequency of hexon-positive cells in the cultures remained constant at these levels for over 1 year, and virus was detected in lysates of the cultures

throughout this period, indicating that the cultures are persistently infected. A representative field of cells from the persistently infected culture stained with hexon antibodies is shown in Fig. 1. In addition to the intensely stained, swollen nuclei typically seen in normal A549 cell cultures infected with either wild-type or RGD-negative Ad2, many cells in the persistently infected culture have nuclei that react less intensely with hexon antibodies and are irregularly shaped or multilobed, a morphology often associated with cells undergoing apoptosis.

The rapid development of a CPE following infection of normal A549 cells with lysates of the resistant cell populations suggested that viruses with replication defects were not selected during establishment or maintenance of the persistent infection. To examine this in more detail, virus recovered from a lysate of cells at passage 30 after the final round of selection was amplified by one round of infection in normal A549 cells, and the multiplication kinetics of this virus, Ad2PI, was compared to that of the parent Ad2RAE and wild-type Ad2 (Fig. 2). The final yields of virus per cell were similar for all three viruses. In addition, Ad2PI and Ad2RAE were both delayed by about 3 h in entry into the log phase of virus multiplication, consistent with our earlier observation that the replication of Ad2RAE and other RGD-negative mutants in adherent cells is delayed by several hours (3). Retention of the mutant phenotype by Ad2PI indicates that revertants were not selected during the long period of continuous culture. Thus, the persistence of virus in the resistant cell population reflects an altered host cell phenotype rather than an altered virus phenotype.

**The persistently infected culture consists predominantly of uninfected cells.** The low frequency of hexon-positive cells in the persistently infected cultures suggested that, at any particular time, a large fraction of the cells might be uninfected. This hypothesis was tested by cloning cells from the culture by limiting dilution. Serial twofold dilutions of the cells were made in a 96-well plate, starting with approximately 100 cells in each well of the initial row (row A) and ending with approximately one cell in each well of the final row (row H). The cells were cultured in the presence of neutralizing antibodies. Cells grew in 8 wells in row H, in 10 wells in row G, and in all other wells. Four of the cultures from row H and one culture each from rows B, C, and D were expanded in the absence of

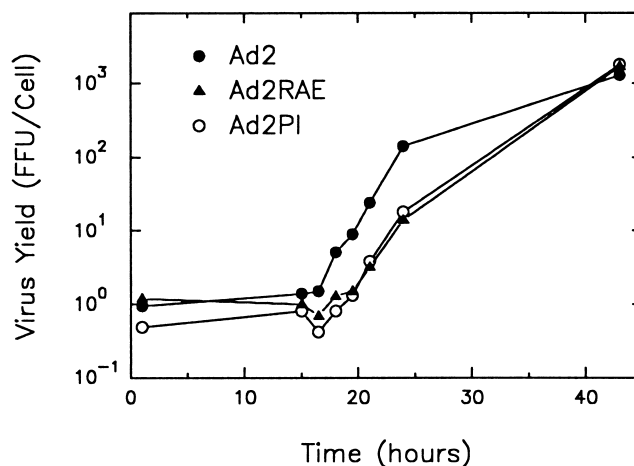


FIG. 2. Analysis of Ad2PI replication in A549 cells. Virus was recovered from the persistently infected culture at passage 30 and amplified by one round of infection in A549 cells. The replication kinetics of the resulting virus stock, Ad2PI, was determined in A549 cells and compared to the replication of parental Ad2RAE and wild-type Ad2. Samples of the infected cultures were taken at the indicated times, and the virus titers were determined on A549 cells.

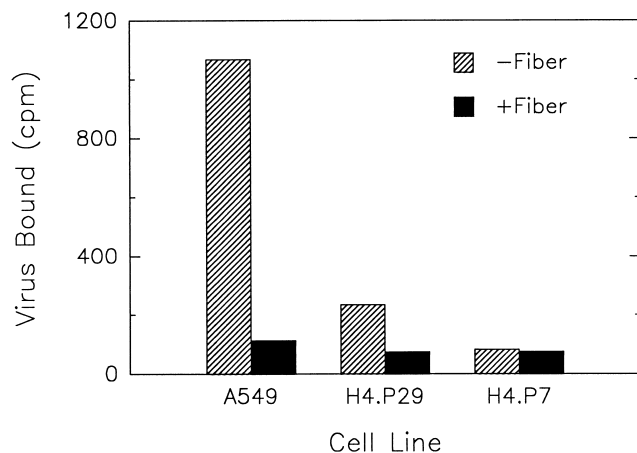


FIG. 3. Binding of Ad2RAE to A549 and H4 cells. Confluent monolayers of cells were incubated with buffer containing 10  $\mu$ g of Ad2 fiber protein per ml or with buffer alone prior to incubation with  $^3$ H-thymidine-labeled Ad2RAE virions. After washing to remove unbound virus, the cell-associated radioactivity was measured by liquid scintillation counting. H4 cells were compared at passages 7 and 29 (H4.P7 and H4.P29, respectively).

neutralizing antibodies and tested for hexon antigen by antibody staining. Hexon antigen was not detected in any of the four row H cultures or in the row C and D cultures, but 0.5% of cells in the row B culture were hexon positive. All of the hexon-negative cultures remained negative in three subsequent assays. Normal A549 cells failed to develop a CPE after cocultivation for eight passages with a freeze-thaw lysate of one of the row H cultures (H4). These results support the conclusion that a large fraction of cells in the persistently infected population are uninfected.

**Cells cloned from the persistently infected culture have decreased levels of the virus receptor.** One of the row H clones, H4, was chosen for further study. Virus titers on A549 cells were 40 to 100 times greater than on H4 cells, indicating that H4 cells are less susceptible to infection than are A549 cells. However, virus yields in A549 cells were only two to four times greater than those in H4 cells 48 h after infection with 5 FFU of Ad2 or Ad2RAE per cell. Therefore, the large reduction in virus infectivity on H4 cells does not result from a high frequency of abortive infection in these cells. The slight depression of virus yields on H4 cells is consistent with the observation that some infected cells appeared to undergo apoptosis (Fig. 1), a physiological state that may limit virus production.

To investigate whether the resistance of H4 cells to infection results from impaired virus adsorption, the abilities of H4 and A549 cells to bind  $^3$ H-thymidine-labeled Ad2RAE virions were compared. A549 cells bound approximately 10-fold more radiolabeled Ad2RAE virus than did H4 cells, and incubation of both cell lines with 10  $\mu$ g of purified Ad2 fiber protein per ml inhibited subsequent virus binding (Fig. 3). These results indicate that the fiber receptor on H4 cells either is present at a reduced concentration on the cell membrane or has decreased affinity for the viral fiber protein. To distinguish between these two possibilities, equilibrium binding of  $^{125}$ I-labeled Ad2 fiber protein to H4 and A549 cells was compared (Fig. 4). The results indicate that the concentration of fiber receptors on H4 cells was about 10-fold lower than on A549 cells (920 versus 9,600 sites per cell), whereas the dissociation constants were nearly identical (0.32 versus 0.37 nM). Therefore, the resistance of H4 cells to infection results predominantly from a reduction in the concentration of virus receptors on these cells.

The wild-type Ad2 penton base subunit binds to integrins on cultured human cells with a  $K_d$  of 55 nM (32) or about 100-fold lower affinity than the interaction of fiber with its receptor reported here. In view of the fact that Ad2 adsorption is mediated predominantly by the viral fiber (24) and not to an appreciable extent by the interaction of the penton base with integrins (2, 32), these results indicate that the efficiency of virus adsorption depends on both the concentration of receptors on host cells and a minimum threshold of receptor affinity for ligands on the capsid.

**Endocytosis of RGD-negative virus requires interaction of virions with multiple fiber receptors.** To determine whether H4 cells support other early events of infection subsequent to virus adsorption leading to the internalization of virions by endocytosis, the rates of endocytosis of virus adsorbed to H4 and A549 cells were compared. Wild-type Ad2 or Ad2RAE virus was adsorbed to cells (multiplicity of infection, 0.01) at 4°C to block endocytosis. The cultures were then warmed to 37°C to permit endocytosis and were challenged with neutralizing anti-hexon antibodies at different times after warming. Two days later, the number of infected cells was determined by

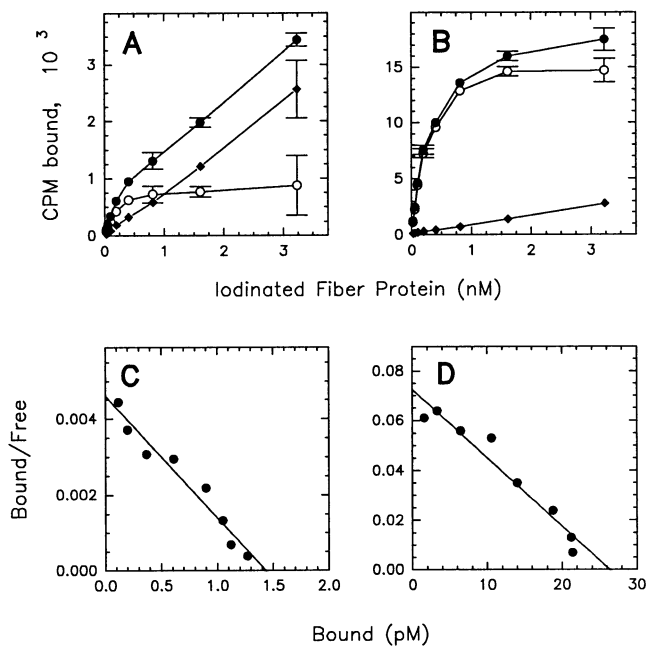


FIG. 4. Isotherms of  $^{125}$ I-labeled Ad2 fiber binding to adherent H4 and A549 cells. A 10- $\mu$ l volume of a 2.7-mg/ml solution of Ad2 fiber protein in 10 mM phosphate buffer (pH 8.2) was incubated on ice for 3 h with 1 mCi of  $^{125}$ I-labeled Bolton-Hunter reagent (Dupont NEN) and then desalted on a column (0.7 by 7 cm) of BioGel P6DG (Bio-Rad) conditioned in phosphate-buffered saline containing 1% gelatin. (A and B) Confluent H4 and A549 cultures in 24-well plates (containing  $1.9 \times 10^5$  and  $3.3 \times 10^5$  cells per well, respectively) were incubated in 0.2 ml of Dulbecco's modified Eagle medium containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2), 0.4% bovine serum albumin, and increasing concentrations of  $^{125}$ I-labeled fiber protein ( $1.9 \times 10^4$  cpm/ng) in the presence ( $\blacklozenge$ ) or absence ( $\bullet$ ) of a 100-fold molar excess of unlabeled fiber protein for 2.5 h at 25°C. Wells were then washed five times with 0.4 ml of phosphate-buffered saline, and cells were lysed in 0.2 ml of 0.3 N NaOH and transferred to scintillation vials for determination of radioactivity. Specific binding ( $\circ$ ) was calculated as the difference between binding in the absence and presence of unlabeled ligand. Each point represents the mean  $\pm$  the standard deviation of triplicate determinations. (C and D) Scatchard analysis of the data for H4 and A549 cells, respectively. The data were fitted to straight lines with correlation coefficients of 0.97 (C) and 0.95 (D). The apparent  $K_{d,s}$ , determined from the slopes, were 0.32 and 0.37 nM, and the numbers of sites per cell, determined from the x intercepts, were 920 and 9,600 for H4 and A549 cells, respectively.

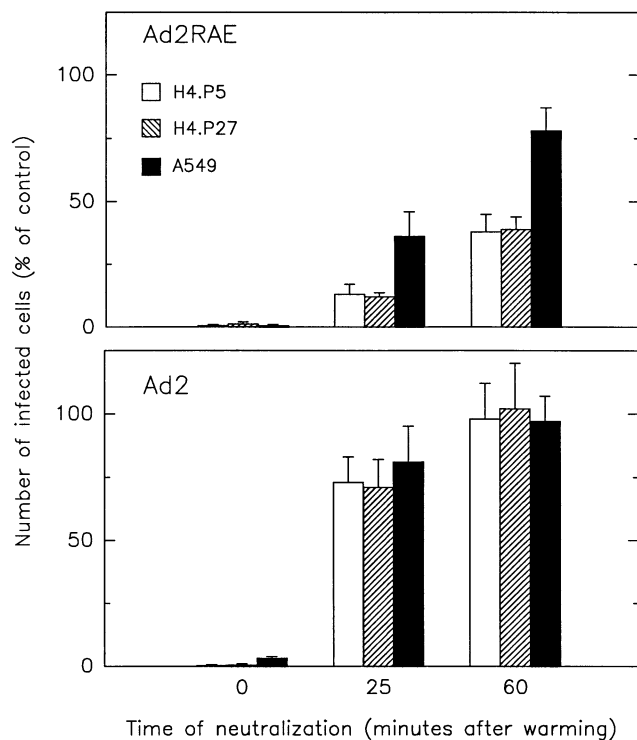


FIG. 5. Kinetics of virus endocytosis into H4 and A549 cells. Cells were infected (multiplicity of infection, 0.01) at 4°C to block endocytosis and then washed and warmed to 37°C. Neutralizing anti-hexon antibodies were then added to the wells either immediately or after 25 or 60 min of incubation at 37°C. Two days later, the infected cells were counted; the data are expressed as a percentage of the number of infected cells in control cultures that were not treated with neutralizing antibodies. H4 cells were compared at passage 5 and 27 (H4.P5 and H4.P27, respectively).

antibody staining. The ratios of the number of infected cells in the presence and absence of neutralizing antibodies are displayed in Fig. 5. Addition of anti-hexon serum at the time of warming reduced the number of infected cells by approximately 100-fold relative to untreated samples, demonstrating that anti-hexon antibodies can efficiently neutralize virions that are adsorbed to the host cell membrane. The rates of internalization of wild-type Ad2 were equivalent on both cell types, and Ad2 internalization was nearly complete after 25 min at 37°C, while the uptake of Ad2RAE by both cell lines occurred at a substantially slower rate. Furthermore, the rates of internalization of Ad2RAE were not equivalent in both cell lines, and Ad2RAE internalization was markedly slower in H4 cells.

The data in Fig. 5 suggest a correlation between the rate of endocytosis of RGD-negative virus and the concentration of Ad2 receptors on the cell membrane. To investigate this relationship further, the concentration of unoccupied receptors available for interaction with adsorbed virions was varied on both A549 and H4 cells by incubation of the cells in the presence or absence of 10 µg of fiber protein per ml following virus adsorption (multiplicity of infection, 0.01) at 4°C. Warm medium was then added to the wells, and the cultures were incubated at 37°C to permit endocytosis. After 20 min for A549 cells or 60 min for H4 cells, anti-hexon antibodies were added to the wells to neutralize extracellular virus. Two days later, the number of infected cells was determined. Titration of receptors on both A549 and H4 cells with fiber protein reduced the fraction of Ad2RAE virus resistant to neutralizing antibodies (internalized virus) by 30 and 45%, respectively, compared

with parallel cultures that did not receive fiber protein (Fig. 6). In contrast, the fraction of wild-type Ad2 resistant to neutralizing antibodies on fiber-treated cells was reduced by 8% on A549 cells and 15% on H4 cells compared with the untreated controls. These data are consistent with the model in which internalization of virus can be promoted either by recruitment of integrins into the initial virus-receptor complex or by recruitment of one or more additional fiber receptors into the complex. The greater inhibition of Ad2RAE uptake seen in the experiment shown in Fig. 6 might reflect that whereas wild-type Ad2 can enter cells by either pathway, RGD-negative viruses are restricted to using multiple fiber receptors.

Persistent infections could be reestablished in H4 cells and all other uninfected cell clones derived from the persistently infected culture by infecting them at a low multiplicity (0.01 FFU per cell) with Ad2RAE; however, most clones infected with a low multiplicity of wild-type Ad2 developed a complete CPE after only two or three serial passages. The basis for the greater transmission efficiency of wild-type Ad2 in these cultures has not been determined but could be related to the accelerated rate of wild-type Ad2 internalization by host cells. In addition, previous experiments suggested that direct interaction of wild-type Ad2 with integrins may be sufficient for infection, albeit at a low efficiency (2).

The present study demonstrates that the level of virus receptors on cultured A549 cells can be modulated and that persistent infection can be established *in vitro* in cells that express low levels of the virus receptor. In another study, selection of HeLa cells resistant to infection by poliovirus also led to the establishment of a persistent infection from which cells lacking expression of the poliovirus receptor were isolated (19). Loss of receptor expression was correlated with down regulation of poliovirus receptor RNA in the variant cells, perhaps resulting from methylation of the gene (20). About 20 cell clones were derived at different times from the persistent infection described in the present study, all of which exhibited some residual susceptibility to infection by RGD-negative Ad2 (data not shown). These results imply that unlike the poliovirus

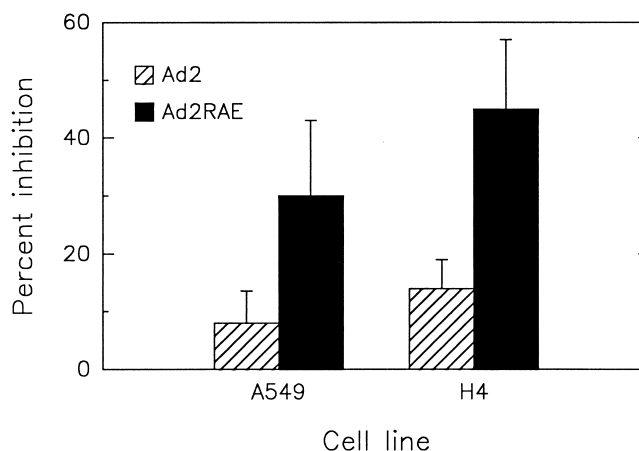


FIG. 6. Dependence of virus internalization rate on the concentration of unoccupied fiber receptors. Virus was adsorbed to cell monolayers (multiplicity of infection, 0.01) at 4°C for 30 min. The cultures were then washed and incubated for an additional 30 min on ice in the presence or absence of 10 µg of Ad2 fiber protein per ml. After washing, the cultures were warmed to 37°C. Neutralizing antibodies were then added to A549 and H4 cultures after 20 or 60 min, respectively, of incubation at 37°C. Two days later, the infected cells in each well were counted. The number of infected cells in fiber-treated wells is expressed as the percent reduction of the number of infected cells in control wells that did not receive fiber treatment.

receptor, which is nonessential for cell viability (20), the Ad2 receptor may be essential, in which case complete loss of its expression would be lethal. Identification of the fiber receptor will make possible an investigation of the mechanism of its regulation in H4 and other resistant cell clones. It would also be interesting to determine if down regulation of the Ad2 receptor is a specific response to infection and whether persistent adenovirus infections in vivo (28) result from inefficient virus transmission as occurs in the in vitro model described here.

Chronic or persistent adenovirus infections of human lymphoid and monocyte cell lines also has been observed (1, 8, 10, 13, 27) and may also result from low levels of either virus receptors or integrin coreceptors. Adsorption of Ad2 to Raji and MOLT-3 lymphocyte cell lines is nearly as efficient as adsorption to HeLa cells, indicating that these cells have comparable fiber receptor levels (27). However, the majority of virus adsorbed to the lymphocyte cell lines remains on the cell membrane, and at any given time, only about 5% of cells in the culture become infected (27). A recent study indicates that human peripheral blood monocytes and T lymphocytes have low levels of  $\alpha$ v integrins and that induction of integrin expression increases the susceptibility of these cells to infection by Ad5 (17). The membranes of lymphoid cells could serve as a reservoir of virus that might eventually be shed from the membrane and infect permissive cells. In vivo, however, it is likely that surface-bound virus would be inactivated by neutralizing antibodies.

The correlation between fiber receptor concentration and the rate of endocytosis of Ad2RAE suggests that internalization of virus can be promoted by recruitment of additional fiber receptors into the initial virus-receptor complex. Simultaneous interaction of virus with multiple receptors could facilitate virus uptake by at least two possible mechanisms. In one case, interaction of virions with multiple receptors could facilitate "zippering" or circumferential spreading of the cell plasma membrane around the virus particle, a mechanism proposed for the uptake of bacteria by animal cells (15, 29). Whereas wild-type Ad2 might employ both integrins and fiber receptors to enter cells along this pathway, RGD-negative viruses would be restricted to multiple interactions with the fiber receptor. A greater abundance of integrins on the plasma membrane relative to fiber receptors, which was measured for one cell line (32), could explain the accelerated uptake of wild-type virus. Close apposition of the cell membrane and the viral capsid at multiple points also might facilitate subsequent penetration of the membrane by the virion. In an alternative mechanism, interaction of virus with multiple receptors might generate a signal enabling endocytosis of the receptor. Recently it was reported that dimerization of integrins with antibodies enhances endocytosis of the integrin-antibody complexes (11). The integrin-mediated uptake of wild-type Ad2 could occur by a similar mechanism, considering that each capsid vertex contains five RGD elements. Further characterization of the fiber receptor is required to determine whether multimerization of this molecule by interaction with antibodies or with multiple fibers on the adenovirus capsid can generate signals that promote its endocytosis.

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