Improved Gene Delivery to Intestinal Mucosa by Adenoviral Vectors Bearing Subgroup B and D Fibers

S. Lecollinet,¹ F. Gavard,¹ M. J. E. Havenga,² O. B. Spiller,³ A. Lemckert,²
J. Goudsmit,² M. Eloit,¹ and J. Richardson^{1*}

UMR01161 ENVA-INRA-AFSSA de Virologie, Ecole Nationale Vétérinaire d'Alfort, 94704 Maisons-Alfort, France¹;
CRUCCELL Holland BV, 2301 CA Leiden, The Netherlands²; and Virus Receptor and Immune Evasion Group,
Department of Child Health, Cardiff University, Wales School of Medicine, Fifth Floor Heath Hospital,
Health Park, Cardiff CF4 4XN, United Kingdom³

Received 13 October 2005/Accepted 15 December 2005

A major obstacle to successful oral vaccination is the lack of antigen delivery systems that are both safe and highly efficient. Conventional replication-incompetent adenoviral vectors, derived from human adenoviruses of subgroup C, are poorly efficient in delivering genetic material to differentiated intestinal epithelia. To date, 51 human adenovirus serotypes have been identified and shown to recognize different cellular receptors with different tissue distributions. This natural diversity was exploited in the present study to identify suitable adenoviral vectors for efficient gene delivery to the human intestinal epithelium. In particular, we compared the capacities of a library of adenovirus type 5-based vectors pseudotyped with fibers of several human serotypes for transduction, binding, and translocation toward the basolateral pole in human and murine tissue culture models of differentiated intestinal epithelia. In addition, antibody-based inhibition was used to gain insight into the molecular interactions needed for efficient attachment. We found that vectors differing merely in their fiber proteins displayed vastly different capacities for gene transfer to differentiated human intestinal epithelium. Notably, vectors bearing fibers derived from subgroup B and subgroup D serotypes transduced the apical pole of human epithelium with considerably greater efficiency than a subgroup C vector. Such efficiency was correlated with the capacity to use CD46 or sialic acid-containing glycoconjugates as opposed to CAR as attachment receptors. These results suggest that substantial gains could be made in gene transfer to digestive epithelium by exploiting the tropism of existing serotypes of human adenoviruses.

Improved strategies for oral vaccine delivery are highly desirable for optimizing vaccination against infectious diseases. Orally delivered vaccines are attractive not only for the ease with which they may be administered but also for their potential to deliver antigen to the vast expanse of gut-associated lymphoid tissue, permitting induction of immune responses in both mucosal and systemic compartments. At its strategic position between the external milieu and intestinal lymphoid tissue, the intestinal epithelium provides underlying immune cells with antigen, either by vesicular transport of intact luminal antigen from apical to basolateral poles, such as via specialized M cells, or after infection of epithelial cells and endogenous expression of antigen (41). For oral vaccination, antigen delivery to intestinal epithelium is currently limited by the safety or efficacy of currently available vaccine vectors. Thus far, the best compromise is provided by mammalian viruses that have been rendered incompetent for replication and engineered to encode antigens of interest.

Replication-incompetent vectors derived from adenoviruses (Ads) are particularly potent gene delivery vehicles, in that they allow the efficient transduction of a wide range of human and animal cells, including nonmitotic cells, and give rise to high levels of gene expression in vivo. As vaccine carriers they are capable of eliciting vigorous and long-lasting B- and CD8⁺

T-cell responses in experimental animals after parenteral administration, and the immune responses elicited against the transgene product have been shown to surpass those achieved with other types of gene vectors, such as vaccinia virus recombinants and genetic vaccines (10, 60, 66, 79).

Two prototypic viruses, Ad2 and Ad5, which belong to human Ads of subgroup C, have been the most extensively characterized biochemically and genetically and thus have been widely used in gene therapy and recombinant vaccine trials. The entry of subgroup C Ads in most of their target cells involves an initial high-affinity interaction between the fiber protein and its cellular receptor, the coxsackie B virus and Ad receptor (CAR), which mediates attachment (7, 68), and subsequent interaction between a second viral protein (the penton base), via its arginine-glycine-aspartic acid (RGD) motif, and $\alpha_v\beta$ integrins, which mediates rapid internalization (75) via receptor-mediated endocytosis (43). In studies pertaining to the respiratory mucosa, the low level of expression of both CAR and $\alpha_v\beta$ integrins at the apical pole appears to limit the efficiency of transduction of epithelial cells by Ad2 and Ad5 upon respiratory mucosal delivery (13, 47, 74, 83). The acidic glycocalyx could also serve as a repulsive barrier, preventing the fiber of Ad5 from reaching the CAR molecule (46).

Data concerning the efficiency of Ad2 and 5 in transferring genetic material into intestinal epithelial cells are quite limited but suggest that such transfer is also limited by physical and biochemical barriers. These vectors have been shown to ensure focal and short-lived (<14 days) transduction of enterocytes in vivo after intraluminal administration (29) or injection into the

* Corresponding author. Mailing address: ENVA, UMR01161 de Virologie, 7 Avenue du Général de Gaulle, 94704 Maisons-Alfort, France. Phone: 33 (0)1 43 96 73 71. Fax: 33 (0)1 43 96 73 96. E-mail: jrichardson@vet-alfort.fr.

superior mesenteric artery (56) and even highly efficient gene transfer to small and large intestinal epithelia subsequent to chemical or physical treatments aimed at enhancing accessibility of enterocytes by removal of the mucus layer, at least (11, 23). Moreover, in vivo and in vitro studies have suggested that as intestinal epithelial cells differentiate, they become much more refractory to transduction by this class of vectors (15, 72), possibly in relation to the sequestration of relevant receptors at the basolateral pole. Although harsh mucolytic treatments are unlikely to be clinically applicable, vector tropism may be amenable to optimization.

To date, 51 human Ad serotypes have been identified and classified into 6 subgroups, denoted A to F (19). They have been identified as causative agents of widely different diseases (35, 71), with clinical manifestations in gastrointestinal (species F primarily [70] and A), respiratory (subgroups B, C, and E) and ocular (species D and E) mucosa. The tissue specificity of the physiopathology of infection by different Ads is potentially related to the utilization of distinct cellular receptors for virus attachment and entry. Whereas the globular C-terminal knob of the fiber protein, by interacting with diverse membrane-bound receptors, confers the specificity of receptor recognition (26, 31, 65), the length and flexibility of the fiber shaft (1, 59, 77) modulates the efficacy of recognition. The primary structure of both the fiber knob and shaft domain diverges substantially among different subgroups, and even among serotypes within the same subgroup, providing ample scope for diversification in tropism.

The primary exception to the CAR-binding paradigm is provided by the subgroup B Ads, which show no affinity for CAR (18, 26, 51, 65) and for whom CD46, a complement regulatory molecule, has recently been identified as a receptor (25, 55, 62). Certain members of subgroup B (Ad11 and Ad35) have been previously shown to infect epithelial cells more readily than Ad5 (42). Other Ads that warrant investigation are the enteric subgroup F viruses (Ad40 and 41), whose natural and narrow tropism for the intestinal tract (20) and physicochemical properties (e.g., resistance to extreme pH conditions) (21) suggest that they may be inherently apt for intestinal gene delivery. These serotypes are unique among human Ads in encoding two fibers (37, 81): one long (20.5 to 21.5 β -repeats), known to interact with CAR, and one short (11.5 β -repeats), which does not (51), and whose function is still poorly understood (45, 54). Finally, not all subgroup D serotypes use CAR as an attachment receptor. The EKC (epidemic keratoconjunctivitis)-causing serotypes use sialic acid (3, 4), as well as an additional protein recently identified by Wu et al. as CD46 (78).

Fiber-chimeric vectors have been elaborated to take advantage of the natural diversity in receptor selection by different Ad serotypes (30, 38, 64, 82). A similar strategy has been applied successfully for redirecting AAV vectors to specific target tissues such as the lung, retina, or central nervous system (27). We have used a library of Ad5-based vectors, bearing fibers derived from representative serotypes of five of the six subgroups of human Ads, to identify suitable adenoviral vectors for efficient gene delivery to the human intestinal epithelium. In the present study, differentiated intestinal epithelial monolayers were reconstituted in vitro, and the capacity of different fiber-pseudotyped Ads for transduction, binding, and translocation toward the basolateral pole was compared. In

addition, antibody-based inhibition and RNA interference technology were used to gain insight into the molecular interactions needed for efficient attachment. The results obtained suggest that substantial gain could be made in the efficiency of intestinal gene delivery by exploiting the tropism of existing serotypes of human Ads.

MATERIALS AND METHODS

Cell lines and culture. The HEK-293 cell line (28) (ATCC number CRL-1573) was used for multiplication and titration of E1-deleted recombinant Ads derived from serotype 5 (Ad5). Two intestinal epithelial cell lines, the human col carcinoma Caco-2 (Te7 clone) (22) cell line and the mICl2 (6) cell line derived from the small intestine of transgenic C57BL/6 mice harboring the simian virus 40 large T gene under the control of the L-PK promoter, were kindly provided by M. Rousset and A. Vandewalle, respectively. CHO cells were purchased from the European Collection of Cell Cultures (Health Protection Agency, Salisbury, United Kingdom) and were stably transfected with either the pCDNA 3.1 eukaryotic expression vector (Invitrogen) or the pDR2 Δ EF1 α expression vector containing the full human CAR cDNA as previously described (63).

HEK-293 cells were grown in Dulbecco modified Eagle medium containing 10% fetal calf serum (FCS; Invitrogen), Caco-2 cells in Dulbecco modified Eagle medium plus 20% FCS (Eurobio) and 1% nonessential amino acids, and CHO cells in RPMI 1640 with 10% FCS (Invitrogen). The medium used for mICl2 has been previously described (6). Culture media were supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (50 IU/ml), and streptomycin (50 μ g/ml; Invitrogen). Cells were grown at 37°C in a 5 or 8% (Caco-2 cells only) CO₂ incubator.

Intestinal epithelial cells were cultured on permeable supports (Transwell polycarbonate filters of 6.5-mm diameter, 0.3-cm² area, and 0.3- μ m pore size; Corning). Cells (10^5 in 100 μ l of medium) were deposited onto the internal or external surface of the filter so as to provide simple access to the apical or basolateral pole of the polarized epithelium. Medium was replaced every 2 days until terminal differentiation of the cells, which required 14 days for Caco-2 and 9 days for mICl2 cells. Transepithelial resistance (TER) was regularly measured (Ohmmeter; Millipore) in order to monitor formation and maintenance of tight junctions and hence assess monolayer integrity. TER values equal to 150 ± 15 or $29 \pm 1.5 \Omega \cdot \text{cm}^2$ for Caco-2 or mICl2 cells, respectively, were considered indicative of full polarization and optimal monolayer integrity. Furthermore, TER was monitored during the course of the experiments and remained constant whichever treatment was applied to the cells (see below).

Pseudotyped viruses. Ad5-based vectors encoding the luciferase (Luc) reporter gene and expressing fibers from adenoviral serotypes of subgroups A (serotype 12), B (serotypes 7, 7B, 11, 16, 35, and 50), C (serotype 5), D (serotypes 8, 9, 10, 13, 17, 24, 27, 30, 32, 33, 38, 45, 47, and 49), and the long and short fibers of the subgroup F serotype 40 were used to study the interaction of Ads with epithelial intestinal cells in vitro. Their construction, including insertion of the Luc transgene in the E1 region and replacement of fiber genes, as well as their production in PER.C6 cells, has been previously described (30, 48). Viruses were amplified in HEK-293 cells and purified by two rounds of CsCl density gradient centrifugation. Viruses were dialyzed against phosphate-buffered saline (PBS), divided into aliquots, and stored at -80°C . Titers were determined by using three different methods. The concentration of viral physical particles was determined by measurement of absorbance at 260 nm, after dilution of the viral solution in sodium dodecyl sulfate (1% final concentration) and incubation at 100°C (67). Viral infectious titers expressed as TCID₅₀ were determined by endpoint dilution in HEK-293 cells, following the Reed and Muench method (49). Quantification of viral genomes by quantitative real-time PCR is described below. A good correlation between the amount of physical particles and the concentration in genomes was observed, their ratio being ca. 2.5 (range, 2 to 3.9). Absence of cross-contamination between viral stocks was verified by subgroup-specific PCR in the fiber gene.

Virus-cell contact. For transduction and translocation experiments, duplicate Transwell units were infected with 10^9 viral genomes inside the insert in 100 μ l of medium for 2 h at 37°C. The inoculum was then replaced with fresh medium. Transduction was assessed 48 h after application to apical or basolateral poles by means of bioluminescence measurement as described below. To assess translocation, aliquots of the medium containing translocated viral particles were removed from the basolateral chamber at different times after the onset of infection, and viral genomes were enumerated by quantitative real-time PCR as described below. Virus-binding studies were initiated in the same manner as for

transduction and translocation experiments, but at 4°C, and evaluated by quantitative real-time PCR.

Diminution of surface accessibility to sialic acid, CAR, and CD46. To assess the role of sialoglycoconjugates in attachment to polarized intestinal epithelial cells, cells were treated, prior to binding experiments, with 12.5 mU of neuraminidase from *Vibrio cholerae* (Sigma) for 2 h at 37°C. To analyze involvement of CAR and CD46 proteins in viral attachment, two different strategies were used. First, specific antibodies, i.e., rabbit polyclonal anti-CAR antibody (63) and rabbit polyclonal anti-CD46 antibody (sc-9098; Santa Cruz), were used as competitive inhibitors. Cells were incubated with 5% anti-CAR serum or 4 µg of anti-CD46 antibody/well for 1 h at 37°C, washed, and chilled to 4°C before virus-cell contact. Second, small interfering RNAs (siRNAs) were used to down-regulate human CD46. A 381-bp CD46 mRNA segment was amplified by reverse transcription-PCR on total RNA extracted from Hep-2 cells using F-ATG GCT ACC TGT CTC AGA TGA CGC and R-GTC ACC ACA ATA AAT CGT GC (9), containing T7 promoter extensions. After in vitro T7 polymerase-driven synthesis of long double-stranded RNA, siRNA molecules were prepared by digestion with RNase III, according to the protocol supplied with the siRNA cocktail kit (Ambion). Polarized human intestinal epithelium was transfected with 250 nM CD46-specific or irrelevant (Block-It Fluorescent Oligo; Invitrogen) siRNAs after basolateral contact by using Lipofectamine 2000 (Invitrogen). Cells were used for binding experiments or assayed for CD46 expression by Western blotting at 72 h posttransfection.

Analysis of transduction. Luciferase gene transfer efficiency was assessed 48 h after infection by quantification of luciferase activity using commercially available reagents (Luciferase Reporter Gene Assay; Roche Diagnostics) on a FluoroskanAscentFL luminometer (ThermoLabsystems). Filters were excised so as to exclude less differentiated cells at the periphery. Cells were lysed in 100 µl of buffer, and then 20 µl of lysate was analyzed after addition of 50 µl of substrate. Transduction results were normalized against total protein concentration of the lysate determined by using commercially available reagents (microBC Assay; Uptima, Interchim). Cell transduction efficiency of viruses bearing the LacZ transgene was evaluated at 2 days postinfection. After fixation with 0.2% glutaraldehyde and 1% paraformaldehyde, cells were analyzed for β -galactosidase activity by X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) histochemistry and photographed under a light microscope at a magnification of $\times 10$ or $\times 20$. Visible blue nuclei and total cells were counted in order to determine the percentage of transduced cells.

Quantification of viral and cellular genomes by real-time PCR. Viral genomes were quantified by amplification of a portion of the luciferase gene using the LightCycler instrument (Roche Diagnostics). The PCR was performed with 700 nM concentrations of the primers LucF (CAT GCC AGA GAT CCT ATT TTT GG) and LucR (AAG ACG ACT CGA AAT CCA CAT ATC) and the Quantitect SybrGreen PCR kit (QIAGEN), with MgCl₂ (Roche) added to a final concentration of 4 mM.

For normalization of binding experiments, the β -globin gene was quantified by PCR. The PCR was performed as described above, except that the primer concentration was 500 nM for β -globinF (CAT GGT GCA CCT GAC T) and β -globinR (CTC ACC ACC AAC TTC ATC) and MgCl₂ was not added.

Amplification reactions were carried out with 2 µl of 1/2 dilution of basolateral chamber medium, 2 µl of pure cellular DNA extracted using QIAamp DNA blood kit (QIAGEN), or 2 µl of serially diluted standard plasmid pSP bearing the Luc gene, or genomic DNA derived from C57BL/6 cells). After *Taq* activation at 94°C for 15 min, each cycle consisted of denaturation at 94°C for 15 s, hybridization at 61°C for 15 s (Luc PCR) or 52°C for 20 s (β -globin PCR), and elongation at 72°C for 10 s (for 47 or 40 cycles for Luc or β -globin PCR, respectively). PCR specificity was checked systematically at the end of the experiment by melting-curve analysis.

Attachment experiments on undifferentiated intestinal cells and fluorescence-activated cell sorting analysis. A total of 2.5×10^4 freshly trypsin-treated cells were placed on ice in contact with 2,500 viral genomes per cell for 2 h in duplicate wells of a 96-well plate. After three washes with PBS, cells were incubated with a 1/2 dilution of tissue culture supernatant of the hybridoma 1D2, containing monoclonal antibody directed against the Ad penton base protein (33), for 1 h on ice, and then with a 1/20 dilution of goat anti-mouse immunoglobulin coupled to phycoerythrin (Dako) for 15 min. After four washes in PBS, cells were fixed in 1% paraformaldehyde and viral binding was analyzed by flow cytometry in the FL2 channel on a FACScan flow cytometer using the CellQuest Pro Software (Becton Dickinson).

Expression and localization of CAR and CD46 by Western blotting and confocal microscopy. CAR and CD46 expression were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cell lysates after sample heating and the addition of β -mercaptoethanol on 12 or 10% polyacrylamide gels, re-

spectively. Proteins were transferred to nitrocellulose membrane and probed overnight at 4°C with the anti-CAR (1/100) or anti-CD46 (1/500) antibodies described earlier. Primary antibody reactivity was visualized after incubation of membranes with a 1/1,000 dilution of anti-rabbit immunoglobulin coupled to horseradish peroxidase (Pierce) for 1 h at room temperature and development with the West Pico or Femto substrate (Pierce).

CAR localization was studied in our cell lines by confocal microscopy. Transwell units were fixed in 4% paraformaldehyde for 20 min and then treated with 50 mM NH₄Cl for 15 min. Permeabilization and saturation of unspecific sites were performed with PBS–1% bovine serum albumin plus 0.05% saponin for 1 h. Cells were incubated with a 1/25 dilution of polyclonal anti-CAR antibody for 1 h in the continuous presence of saponin and then with 1/40 anti-mouse immunoglobulin conjugated to fluorescein isothiocyanate or TRITC (tetramethyl rhodamine isothiocyanate) (Dako). Cell labeling was analyzed at a $\times 40$ magnification with a Zeiss confocal microscope.

Data analysis. Ordinary one-way analysis of variance and Dunnett's post-tests were performed using GraphPad Prism 4.0 Software (GraphPad Software). Differences were considered statistically significant at $P \leq 0.05$.

RESULTS

Attachment of fiber-pseudotyped Ads to undifferentiated human intestinal epithelium. Attachment of viral vectors to targeted cell types often represents a limiting factor in gene transfer strategies. In an effort to identify suitable adenoviral vectors for gene delivery to the human intestinal epithelium, we compared the capacity of a library of fiber-pseudotyped Ads to attach to undifferentiated Caco-2 cells. In particular, attachment of fluorescently labeled viral capsids was evaluated by flow cytometry. Different pseudotyped vectors exhibited substantially different capacities for attachment, with mean fluorescence intensity varying from 7.7 to 219.2 U for vectors bearing the short fiber of serotype 40 (F40-S) and the fiber of serotype 16 (F16), respectively (Fig. 1A).

Several vectors, bearing fibers from serotype 12 of subgroup A, serotypes 11, 16, 35, or 50 of subgroup B, serotype 32 of subgroup D, or the long fiber of serotype 40 (F40-L), bound more efficiently than Ad5F5. Such vectors included those bearing F12 and F40-L, which are presumed to bind, like Ad5F5, to CAR, either on the basis of capacity to bind to recombinant CAR protein (F12) (51), or the high degree of similarity with the CAR-binding F41-L (F40-L). Despite their shared capacity to use CAR, these vectors displayed wide-ranging binding efficiencies (42.3, 81.4, and 174.8 U, for Ad5F5, Ad5F12, and Ad5F40-L, respectively).

Attachment of fiber-pseudotyped Ads to differentiated human intestinal epithelium. Studies have suggested that, as intestinal epithelial cells differentiate, the apical pole becomes more refractory to attachment and subsequent internalization (15, 72) of subgroup C derived vectors, thereby limiting gene transfer efficiency. We therefore compared the capacity of vectors to attach to a polarized monolayer of Caco-2 cells. After a 2-h incubation of vector with apical or basolateral poles of fully differentiated cells, maintained at 4°C so as to preclude vector internalization, attachment was assessed by quantitative real-time PCR. This technique allowed quantification of the luciferase transgene and hence cell-associated viral genomes in a reproducible manner over a range of at least 8 logs of magnitude. Under these conditions, pseudotyped vectors exhibited substantially different capacities for attachment to differentiated intestinal epithelium and, notably, after application to the apical pole, the surface targeted by oral delivery (Fig. 1B). In particular, the level of attachment after apical administration

FIG. 1. Interaction of fiber-pseudotyped Ads with the human intestinal epithelial cell line, Caco-2. (A) Binding to undifferentiated Caco-2 cells was measured after a 2-h incubation with 2,500 viral genomes/cell at 4°C and staining of bound viral capsids by flow cytometry. (B) Attachment to a polarized Caco-2 monolayer was performed at 4°C for 2 h with 10⁹ viral genomes per filter and quantified by real-time PCR on viral and cellular DNA. The results are expressed as the ratio of viral and cellular genomes. (C) Transduction of a polarized Caco-2 monolayer was assessed by luminometric measurement of the luciferase transgene activity 2 days after viral infection with 10⁹ viral genomes per filter. The luciferase activity is expressed after normalization for protein concentration for the different fiber-pseudotyped Ads. The figures depict representative results of at least two independent experiments performed in duplicate. Means $\pm$ the standard error of the mean (SEM) are represented. Statistically significant differences with Ad5F5 are indicated: *, $P < 0.05$; **, $P < 0.01$.

ranged from 6.2 to 225.8 viral genomes/cell for vectors bearing the F9 and F32 fibers, respectively. The highest level of attachment was observed for the Ad5F32 vector, with little difference observed between apical and basolateral application (225.8

and 318.3 viral genomes/cell, respectively). Regarding the reference Ad5F5 vector, its level of attachment after apical contact was among the lowest (14.0 viral genomes/cell). The two other vectors capable of binding to CAR in appropriate circumstances, Ad5F12 and Ad5F40-L, bound with greater efficiency (173.5 and 291.5 viral genomes/cell, respectively) than Ad5F5 (99.1 viral genomes/cell) from the basolateral pole, closely paralleling their relative efficiency for attachment to undifferentiated cells. These three vectors—Ad5F5, Ad5F12, and Ad5F40-L—exhibited marked differences between apical and basolateral binding capacities. Vectors bearing fibers from subgroup B Ads also displayed markedly different capacities for apical and basolateral attachment (19.5 and 501.9 viral genomes/cell, respectively, in the case of Ad5F7B).

Altogether, the results of binding studies on differentiated and undifferentiated cells suggested that the capacities of certain fiber-pseudotyped vectors to attach to human intestinal epithelia are greatly reduced at the apical pole during cell polarization. This diminution is likely to be related to the relocalization of their cognate receptors toward the basolateral pole of Caco-2 cells.

Transduction of differentiated human intestinal epithelium by fiber-pseudotyped Ads. The different fiber-pseudotyped Ads were then compared with regard to their capacity to transduce a polarized monolayer of Caco-2 cells grown on permeable filters. Expression of the luciferase transgene was assessed 48 h after application to apical or basolateral poles by means of bioluminescence measurement.

Under these conditions, pseudotyped vectors exhibited vastly different capacities for gene transfer to differentiated intestinal epithelium and, notably, after application to the apical pole (Fig. 1C). In particular, luciferase activity after apical contact ranged from 2.5 to 190.4 relative light units (RLU)/mg of protein for vectors bearing the F40-S and F35 fibers, respectively. With regard to the vector bearing the fiber F5 of the highly characterized serotype 5 (subgroup C), apical application gave rise to a very low level of transgene expression (9.8 RLU/mg of protein). Such inefficiency did not reflect any general defect in fiber function, since a markedly higher level of transgene expression (92.4 RLU/mg of protein) was observed after basolateral application. This pattern, inefficient transgene expression after apical but not basolateral application, was exhibited by many other fiber-pseudotyped vectors, and in particular those presumed to interact with CAR in appropriate contexts, such as vectors bearing fibers F12 or F40-L. This pattern also held true for several other vectors bearing fibers derived from subgroup D Ads for whom recognition of CAR has not been evaluated and paralleled the apparent differences between apical and basolateral binding capacities of these vectors. It is worthy of note that, among vectors bearing fibers described to bind to CAR, those bearing F12 or F40-L, derived from Ads responsible for symptomatic infections of the gastrointestinal tract, were much more efficient than Ad5F5 in transducing differentiated epithelium from the basolateral pole (221.3, 397.7, and 92.4 RLU/mg of protein for Ad5F12, Ad5F40-L, and Ad5F5, respectively). Nevertheless, transduction by the vector bearing the short fiber (F40-S) derived from serotype 40 (subgroup F), despite the intestinal tropism of the parental virus, was very inefficient whichever pole considered. While Croyle et al. (15) observed that Ad41, a subgroup F Ad,

was superior to Ad5 with regard to the binding and uptake at the apical pole of differentiated Caco-2 cells, such increased efficiency was not evidenced in our study for Ad5-based vectors bearing a single subgroup F fiber. Perhaps other structural proteins such as the RGD-independent penton base, or the combinatorial use of short and long fibers displayed by the same viral particles, may be required for efficient interaction of subgroup F Ads.

By contrast, some pseudotyped vectors transduced intestinal epithelium from the apical pole considerably more efficiently (>40 RLU/mg of protein) than Ad5F5 and exhibited a reduced difference between the efficiency of gene transfer from apical and basolateral poles. This held true for all vectors bearing fibers derived from subgroup B Ads, with levels of transgene expression ranging from 42.7 to 205.1 RLU/mg of protein for Ad5F7 and Ad5F35, respectively, and for some but not all vectors bearing fibers derived from subgroup D Ads, and in particular Ad5F32. Thus, for vectors bearing subgroup B fibers, relatively high levels of apical transduction were associated with rather low levels of apical attachment, suggesting that these vectors gained access to more efficient pathways leading to gene expression when applied to the apical rather than basolateral surface.

These data implied that the efficiency with which differentiated intestinal epithelium was transduced by adenoviral vectors was influenced by the efficiency of not only attachment but also subsequent steps, depending on the fiber taken into consideration.

Interaction of fiber-pseudotyped Ads with the murine intestinal epithelium. In an effort to assess species-specificity of fiber-mediated interactions, we investigated the capacity of fiber-pseudotyped vectors for transduction of murine intestinal epithelium. The mICcl2 cell line, which has been shown to assume the characteristics of differentiated intestinal epithelium upon 10-day culture (6), was selected for the study. Transduction experiments were conducted under the same conditions as for Caco-2 cells.

Ad5F5 and certain fiber-pseudotyped Ads (Ad5F12, Ad5F40-L, and Ad5F32) transduced differentiated monolayers of mICcl2 cells much more efficiently than Caco-2 cells (Fig. 2). Three such vectors bore CAR-binding fibers F12, F5, or F40-L, although vectors bearing CAR-binding fibers derived from subgroup D serotypes (F9) did not display such high transduction levels. The fourth vector that transduced mICcl2 cells efficiently, Ad5F32, bore the fiber of a subgroup D serotype that does not recognize CAR. Ad5F32 was the sole representative among 14 subgroup D derived vectors to transduce mICcl2 cells efficiently (data not shown), underscoring the high degree of heterogeneity of Ads belonging to this subgroup.

The decreased transduction of murine intestinal epithelium by all vectors pseudotyped with subgroup B fibers was particularly striking, suggesting they recognize one or more receptors poorly conserved between humans and mice, with regard to either molecular structure or tissue localization. The capacity of the vector bearing the short fiber of serotype 40 (F40-S) for transduction of polarized intestinal epithelium appeared to be as low in mICcl2 as in Caco-2 cells. Certain differences between the mICcl2 and the Caco-2 cell lines became apparent in the course of our experiments. First, the difference in the efficiency of transduction at apical and basolateral poles was

FIG. 2. Transduction of a polarized murine intestinal epithelium composed of mICcl2 cells by fiber-pseudotyped Ads. Transduction was measured by luminometric measurement of the luciferase transgene activity 2 days after viral infection with 10^9 viral genomes per filter. Luciferase activity is expressed after normalization for protein concentration for the different fiber-pseudotyped Ads and depicts representative results of at least two independent experiments performed in duplicate. Means $\pm$ the SEM are shown. Statistically significant differences with Ad5F5 are indicated: *, $P < 0.05$; **, $P < 0.01$.

much reduced in mICcl2 cells (see Ad5F5 or Ad5F40-L, for example). Moreover, and quite surprisingly, although the level of transduction was higher in mICcl2 cells than in Caco-2 cells for Ad5F5, Ad5F12, Ad5F40-L, and Ad5F32, attachment was not (data not shown), suggesting that pathways leading to gene expression after attachment were more efficient in mICcl2 than in Caco-2 cells.

Translocation of viral particles across differentiated human or murine intestinal epithelia. It is not clear to what extent gene delivery to the intestinal epithelium by viral vectors will depend upon transduction of intestinal epithelial cells via the apical pole or, subsequent to transcytosis of vectors, via the basolateral pole. Indeed, transduction of underlying cell types, such as antigen-presenting cells, may be critical for oral vaccination. It is plausible, however, that vectors using different receptors might differ in their capacity to gain access to transcytotic membrane-bound vesicular traffic. We therefore compared the translocation of fiber-pseudotyped vectors from the apical to the basolateral pole, analogous to the penetration of epithelium from the intestinal lumen to the lamina propria. Medium in the basolateral reservoir was sampled at different time points, and viral genomes were quantified by real-time quantitative PCR.

With the exception of Ad5F40-L, translocation of viral particles across the Caco-2 monolayer was not detected 2 h after virus application (Fig. 3A). In contrast, translocation was observed 2 days after apical application of viruses pseudotyped with subgroup D fibers, some subgroup B fibers (F7, F16, and F35), and the fibers (F12 and F40-L) derived from Ads displaying a natural intestinal tropism.

The mICcl2 monolayer was crossed with greater efficiency, since every virus could be recovered in the basolateral medium 2 days after cell infection (Fig. 3B). Moreover, passage across the murine epithelium was also observed at earlier time points. At 2 h postcontact, for example, viral particles were detected for vectors bearing fibers capable of binding to CAR (F5, F12, and F40-L) and some subgroup B fibers (F7B, F16, and F35).

FIG. 3. Translocation of viral particles from the apical toward the basolateral pole of the human (A) and murine (B) intestinal epithelium. Medium from the basolateral chamber was sampled for quantification of viral genomes by real-time PCR 2 h and 2 days postinfection with 10^9 viral genomes per filter. The figures depict representative results of two independent experiments performed in duplicate. Means $\pm$ the SEM are shown. Statistically significant differences with Ad5F5 are indicated: *, $P < 0.05$; **, $P < 0.01$.

These viruses were recovered at higher levels in later samples, showing that translocation continued over time.

Thus differences in penetration of Caco-2 and mICcl2 monolayers mainly concerned the rate of translocation and not the nature of the viruses which were efficiently transported, with vectors bearing subgroup B fibers (F7, F7B, F16, and F35) and some fibers presumed capable of binding to CAR (F12, F40-L) appearing to penetrate both Caco-2 and mICcl2 monolayers more efficiently than Ad5F5. Translocation from the basolat-

eral to the apical pole was also evaluated and appeared to be much faster and less selective in the two cell lines (data not shown).

Molecular basis of binding to polarized intestinal epithelial cells. Three major adenoviral receptors have been recently described in several cellular contexts (Table 1); namely, the coxsackie B virus and Ad receptor (CAR), CD46 and sialoglycoconjugates. In order to address the role of these three receptors in the context of differentiated intestinal epithelium, we investigated

TABLE 1. Diversity in physiopathology and attachment receptor usage of human Ads whose fibers were used to construct fiber-pseudotyped Ads^a

Subgroup	Serotype	Pathogeny	Primary receptor	Cellular context (reference)
A	12	Cryptic enteric infections	CAR	12, 31: A549, Ramos, CHO (51)
B	7, 7b, 11, 16, 35, 50	Epidemic respiratory infections, persistent renal infections	CD46	35: CHO, HeLa, A549, K562, primary bladder epithelial cells, primary human CD34 ⁺ PBMC (25)
				11: CHO (55)
				3: BHK, HeLa, K562, THP-1 (62)
				3: CHO, HeLa, human DC (61)
C	5	Endemic respiratory infections	CD80-CD86	2, 5: A549, Ramos, CHO (51)
			CAR	5: CHO (7)
				5: NIH 3T3 (68)
				2, 5: A549, CHO (17)
				5: MM-39 (24)
			Sialoglycoconjugates	5: CF-KM4 (24)
			VCAM1	5: NIH 3T3 (12)
			α_2 domain of MHC1	5: HeLa, Daudi (34)
			β_2 integrins	2: macrophages, THP-1, CHO (36)
D	8	Keratoconjunctivitis, ocular infections	Sialoglycoconjugates	37: CHO, A549, HCE, Daudi (3)
				8, 19a, 37: A549 (4)
			CD46	37: HeLa, Chang, CHO (78)
	9, 17		CAR	9, 15: A549, Ramos, CHO (51)
			α_v integrins	9: A549 (4)
	10, 13, 24, 27, 32, 33, 38, 45, 47, 49		?	
	30		ND	
F	40	Infantile gastroenteritis	40-S: ND	41-L: A549, Ramos, CHO (51)
			40-L: CAR	

^a Cellular contexts in which receptor usage was examined are given in the form "serotype(s): cell line(s)." A549, human lung carcinoma; Ramos, human B lymphoblasts; CHO, hamster ovary carcinoma; HeLa, human cervical carcinoma; K562, human erythroid leukemia; PBMC, peripheral blood mononuclear cells; BHK, hamster kidney fibroblasts; THP-1, human monocytic leukemia; DC, human dendritic cells; NIH 3T3, murine embryonic fibroblasts; MM39 and CF-KM4, human tracheal glandular cells (healthy and cystic fibrosis transmembrane conductance regulator deficient, respectively); Daudi, human B lymphoblasts; HCE, human corneal epithelium; Chang, human conjunctiva. HS, heparan sulfate; ?, unknown receptor but different from CAR; ND, not determined.

FIG. 4. Role of sialoglycoconjugates in attachment of fiber-pseudotyped Ads at the surface of a polarized human (A) and murine (B) intestinal epithelium. Binding was quantified by real-time PCR after a 2-h pretreatment of cells with *Vibrio cholerae* neuraminidase. The results are expressed as the ratio of attachment to treated and untreated cells. The figures depict representative results of at least two independent experiments performed in duplicate. Means $\pm$ the SEM are shown. Statistically significant differences between attachment to treated and untreated cells are indicated: *, $P < 0.05$; **, $P < 0.01$.

their involvement in binding of fiber-pseudotyped vectors to monolayers of Caco-2 and mICcl2 cells.

The role of sialoglycoconjugates was first evaluated by treating the in vitro reconstituted intestinal epithelium with neuraminidase from *Vibrio cholerae*, which hydrolyzes sialic acid residues of oligosaccharides and glycoproteins with broad substrate specificity. Upon comparison of virus binding with or without prior neuraminidase treatment (see Fig. 4A and B), attachment to Caco-2 and mICcl2 cells was inhibited at both apical and basolateral poles for two vectors (Ad5F8 and Ad5F32) bearing subgroup D-derived fibers. The level of inhibition was similar in the two cell lines, though slightly higher in the mICcl2 cells, and was similar whichever pole was considered. For certain vectors, Ad5F5, Ad5F12, Ad5F40-L, and Ad5F40-S in Caco-2 cells, and Ad5F5, Ad5F12, Ad5F35, and Ad5F40-S in mICcl2 cells, neuraminidase treatment increased attachment at the apical pole. Several teams have in fact reported enhanced interaction of Ad5 with the cell surface after sialic acid removal (2, 3), probably due to alleviation of electrostatic repulsion between the acidic exposed loops of Ad5 hexon (14) and sialic acids. In our case, enhanced binding did not appear to be mediated by the fiber, since it was not abolished in the presence of purified adenoviral fibers or antibodies blocking established adenoviral receptors (data not shown).

We next sought to assess the importance of CAR and CD46, by pretreating the cells with polyclonal rabbit antibodies raised against each of these proteins. Three vectors appeared to require the CAR molecule to a large extent for attachment to both Caco-2 (Fig. 5A and B) and mICcl2 (data not shown) cells. These included Ad5F5, Ad5F12, and Ad5F40-L, bearing fibers that are known or supposed to interact with CAR in other cell systems. We observed a 50 to 60% inhibition in apical binding, and greater inhibition, of ca. 80%, in binding to the basolateral pole. The attachment of two other viruses bearing subgroup D fibers, Ad5F8 and F9, seemed to depend more weakly on the CAR molecule, with a 40% inhibition after application of the virus at either pole.

Regarding CD46, attachment of two vectors (Ad5F35 and Ad5F8) at both poles of Caco-2 cells appeared to require the CD46 molecule (Fig. 5C). Binding was inhibited by 70 to 90% for Ad5F35, but by only 30 to 40% for Ad5F8, which we have shown to interact with other molecules at the surface of Caco-2 cells (sialic acids and CAR). More unexpectedly, CD46 also seemed to play a significant role in the attachment of Ad5F12 and Ad5F40-L to the basolateral pole of Caco-2 cells, since anti-CD46 antibody inhibited attachment by 30%. In order to confirm the critical role of CD46 in Ad5F35 binding to Caco-2 cells, the RNA interference approach was used. Upon efficient transfection of cells with CD46-specific small interfering RNAs (siRNAs), total CD46 expression could be reduced by ca. 40%, leading to a 30% decrease in Ad5F35 binding to the basolateral pole of Caco-2 cells, while under the same conditions Ad5F5 binding was moderately enhanced (Fig. 5D).

With the exception of Ad5F40-S, all tested vectors, bearing fibers derived from at least one representative of each subgroup and from all vectors that displayed efficient transduction, were capable of interacting with at least one of the three molecules, i.e., CAR, CD46, or sialoglycoconjugates. Only Ad5F40-S did not display a measurable level of attachment to any receptor. The attachment of several vectors appeared to depend in a significant manner upon multiple receptors: sialic acid, CAR, and CD46 for Ad5F8; and CAR and possibly CD46 (basolateral pole only) for Ad5F12 and Ad5F40-L.

Observed discrepancies in transduction of polarized Caco-2 and mICcl2 cells correlate with differences in expression or localization of CAR and CD46 receptors. To determine whether the discrepancies observed in the transduction of human Caco-2 and murine mICcl2 intestinal epithelium were attributable to species- or cell type-specific differences in receptor expression, we assessed expression and localization of CAR and CD46 in polarized Caco-2 and mICcl2 monolayers.

CAR expression, as evaluated by Western blotting, was detected in both Caco-2 and mICcl2 lysates by the presence of a specific protein doublet at 46 and 48 kDa, resulting from post-

FIG. 5. Role of CAR (A and B) and CD46 (C and D) in attachment of fiber-pseudotyped Ads at the surface of a polarized human intestinal epithelium. Binding was quantified by real-time PCR either after a 1-h pretreatment of cells with polyclonal anti-CAR (A and B) or anti-CD46 (C) antibodies or 72 h posttransfection with 250 nM CD46-specific siRNAs (D). Transfection efficacy was checked by using fluorescently labeled siRNAs (Block-It Fluorescent Oligo) and specific down regulation of CD46 expression by Western blotting. The results are expressed as the ratio of attachment in the presence of specific (antisera or siRNA) and nonspecific (irrelevant polyclonal antibodies or siRNA) reagents. The figures depict representative results of two independent experiments performed in duplicate. Means $\pm$ the SEM are shown. Statistically significant differences between attachment in the presence of specific inhibitory molecules and their respective controls are indicated: *, $P < 0.05$; **, $P < 0.01$.

transcriptional N glycosylation modifications of CAR in the murine (80) and human contexts (68) (Fig. 6A). Confocal microscopy revealed substantial differences in the distribution of CAR at the surface of polarized monolayers of murine and human intestinal epithelial cell lines. CAR was rather uniformly distributed at apical and basolateral poles of mICcl2 cells, while in Caco-2 cells CAR seemed to be expressed preferentially at the basolateral pole, appearing to lie beneath tight junctions (indicated by ZO-1 staining). This distribution provided a plausible explanation for the observation that mICcl2 cells were transduced with similar efficiency at either pole by CAR-dependent Ads (for example, Ad5F40-L), while Caco-2 cells were transduced with much greater efficiency via the basolateral rather than the apical pole.

Regarding CD46 expression, two major CD46 isoforms could be visualized at 55 and 65 kDa only in the Caco-2 lysate (see Fig. 6B), whereas no expression could be detected in mICcl2 cells, by Western blotting with antibodies recognizing both human and murine CD46 (the murine CD46 molecule appeared as a faint 22-kDa band in the lysates of murine testis, to which murine CD46 expression has previously been reported to be restricted [69]). Thus, inefficient transduction of the murine intestinal cell line by CD46-dependent viruses may have been attributable to the absence or insufficient levels of CD46, although it is unlikely that murine CD46 would have been recognized, even had a substantial level been expressed, by vectors bearing human CD46-dependent fibers, since such vectors did not appear to recognize murine CD46 in other cellular contexts (30).

FIG. 6. Differential expression or localization of CAR (A) and CD46 (B) receptors in Caco-2 and mICcl2 cells. (A) CAR expression was assessed by Western blotting with polyclonal anti-CAR antibodies on lysates of Caco-2 and mICcl2 cells in comparison to lysates of the CAR-negative parental (CHO-pcDNA) and human CAR-positive (CHO-hCAR) CHO cell lines. MW, molecular weight marker. Actin expression levels are also displayed. CAR localization on polarized Caco-2 and mICcl2 cells was observed by confocal microscopy along the xz plane perpendicular to the filter, after staining for CAR (with the fluorescein isothiocyanate and TRITC fluorochromes for Caco-2 and mICcl2 cells, respectively) and the tight junction marker ZO-1 (with the TRITC fluorochrome for Caco-2 cells) ($\times 40$ magnification). CAR localization is shown in relation to transduction efficiency for apical and basolateral poles of a CAR-dependent Ad (F40-L) bearing the *lacZ* gene, as revealed by X-Gal immunohistochemistry and light microscopy ($\times 10$ magnification). The proportion of infected cells is indicated in the lower right corner. (B) CD46 expression was assessed by Western blotting with polyclonal anti-CD46 antibodies on lysates of Caco-2 and mICcl2 cells, in comparison to the positive murine testis lysate. (Inset) Reduced exposure of the Caco-2 lane.

DISCUSSION

Diverse serotypes of human Ads cause tissue-specific pathologies, presumably in relation to the recognition of different cellular receptors with different tissue distributions. This natural diversity was exploited in the present study to identify suitable adenoviral vectors for efficient gene delivery to the human intestinal epithelium. In particular, we investigated the interactions of a library of fiber-pseudotyped human Ads with differentiated intestinal epithelia in human and murine tissue culture models; namely, polarized monolayers of Caco-2 and mICcl2 cell lines cultivated on permeable filters.

Ad vectors bearing fibers derived from different serotypes exhibited strikingly different capacities for attachment to and transduction of human and murine intestinal epithelia, suggesting that receptor selection by fiber determines the efficiency of gene transfer to differentiated intestinal epithelium. For vectors derived from subgroup C serotypes, such as Ad5, the inaccessibility of their high-affinity receptor (CAR) at the apical pole of differentiated epithelium has been identified as a limiting factor in respiratory tissue (74). We obtained similar results for Ad5F5 in differentiated intestinal epithelium, that is, less efficient attachment and transduction via the apical

pole, and confirmed the use of CAR as an attachment receptor in this context. This difference was correlated with a sequestration of CAR at the basolateral pole of differentiated intestinal epithelium, as has been described for differentiated respiratory epithelium (47, 83).

More unexpectedly, vectors bearing CAR-binding fibers derived from serotypes of subgroups A and F behaved unlike Ad5F5. In particular, vectors pseudotyped with F12 or F40-L, derived from Ads with natural tropism for the gastrointestinal tract, were much more efficient than Ad5F5 in transducing differentiated epithelium from the basolateral pole. This difference cannot apparently be attributed solely to their capacity to use CD46 at this pole (discussed below), since Ad5F12 and Ad5F40-L were also more efficient than Ad5F5 in murine intestinal epithelium, in which CD46 does not appear to be expressed. Vectors bearing F12 and F40-L are perhaps capable of making better use of CAR, for reasons which are as yet unclear. It is tempting to speculate, however, that the pathophysiology of infections with the parental viruses is related to a greater capacity for local dissemination, by virtue of enhanced CAR-dependent infection via the basolateral pole once the epithelial barrier has been breached, such as after transcytosis

of virus by M cells or after initial low-level infection at the apical pole (73).

Certain members of the highly heterogeneous subgroup D have been shown to be capable of binding CAR. In particular, the attachment of the vector bearing F9 was inhibited by anti-CAR antibodies. This vector was approximately equivalent to Ad5F5 in transducing differentiated human intestinal epithelium but much less so in murine intestinal epithelium, suggesting that F9 and F5 interact with CAR in a different manner. Although subgroup D serotypes causing epidemic keratoconjunctivitis (EKC), including Ad8, have been reported to interact very weakly with the CAR protein in CHO-CAR cells (55), in our study the binding of the F8-pseudotyped vector was inhibited by anti-CAR antibodies to the same extent as Ad5F9. Although ocular tropism has been associated with the use of sialoglycoconjugates and CD46 as receptors, the capacity to use these receptors may not exclude the use of CAR. Indeed, the amino acid sequences of F8 and the CAR-binding F9 are highly similar (95%), allowing localization of a CAR-binding motif within the F8 knob (52). Moreover, the EKC-causing Ad37 has previously been shown to bind to CAR in overlay blot assays (75).

In inhibition of binding experiments, we showed that CD46 was involved in the binding of vectors bearing subgroup B (F35) and certain subgroup D fibers (F8) to polarized human intestinal epithelium. CD46 seemed to be used by F8 in a different manner than F35, since we observed moderate levels of transduction and binding on Caco-2 cells compared to F35-bearing vectors. Wu et al. (76) and Segerman et al. (55) have previously demonstrated that F8 and F11 (the latter belonging to subgroup B, like F35) use different binding domains on CD46, with F8 attachment to the SCR1 domain being dependent on bivalent cations while F11 binding to SCR3/4 domains was not. Our result thus provided support for the idea that members of the adenoviridae family may exploit the same receptor by different types of interactions. CD46-dependent vectors bound more efficiently to the basolateral rather than apical pole of Caco-2 cells, perhaps reflecting a predominantly basolateral distribution of CD46, as has been observed in polarized simian Vero, human rectal HRT-18, or canine MDCK cells (40). Curiously, in antibody inhibition experiments, F12 and F40-L, derived from Ads with intestinal tropism, appeared to bind to CD46 solely at the basolateral pole of human epithelium. Possible explanations include the recognition of splicing or glycosylation isoforms of CD46 preferentially expressed at the basolateral pole or the use of a basolaterally expressed receptor that is tightly associated with CD46. In this regard, $\alpha_{1/3/5/6}\beta_1$ integrins, which have been described to be associated with CD46 in HeLa cells (39) and expressed preferentially at the basolateral pole of differentiated epithelial cells (5), would merit investigation.

To assess the role of sialoglycoconjugates in adenoviral attachment, monolayers were treated with a broad spectrum neuraminidase prior to application of pseudotyped vectors. We found that two subgroup D fibers, F8 and F32, were able to bind to sialoglycoconjugates. A requirement for sialic acid has been previously described for attachment of EKC-causing serotypes, and in particular for Ad8 (4). We have identified another fiber, F32, which required sialoglycoconjugates for efficient binding at the apical and basolateral pole of intestinal

epithelium. Ad32 has not been associated with ocular disease or tropism but rather with infections of the central nervous system or colon in immunocompromised hosts (32). Thus, the ability to interact with sialoglycoconjugates among subgroup D serotypes does not seem to be restricted to EKC-causing serotypes. Nevertheless, the manner in which F32 and F8 interact with sialoglycoconjugates may be quite different. Although neuraminidase treatment inhibited binding of Ad5F8 and Ad5F32 to similar extents, Ad5F32 bound and transduced Caco-2 and mICl2 cells much more efficiently than Ad5F8. This difference could be attributable to the use of the same sialoglycoconjugates with different affinities or to the use of different sialoglycoconjugates. Since neuraminidase treatment inhibited binding of Ad5F32 by only 50%, other attachment receptors for F32 may well have been involved. In any case, vectors bearing the F32 receptor would appear to be good candidates for oral delivery *in vivo* and, in light of their efficacy in both human and murine cell lines, could be amenable to evaluation in preclinical murine models.

Several serotypes of Ads, including Ad8, Ad12, and Ad40, have been described to make use of multiple receptors. The combinatorial use of diverse receptors by Ads could represent an evolutionary strategy to augment adenoviral fitness. This strategy has been evidenced for Ad5, which is able to use VCAM-1 (12), β_2 integrins (36), major histocompatibility complex class I (34), heparan sulfates (17), or sialoglycoconjugates (24) *in vitro* on cell types lacking the primary CAR receptor (Table 1). The use of alternative receptors might be expected to broaden host cell range. Indeed, since survival of Ads requires penetration of mucosal epithelium, local or systemic dissemination, and shedding from mucosa for transmission to new hosts, the ability to infect different cell types may be critically important. We may thus speculate that flexibility in receptor selection, even at the expense of reduced affinity for individual cognate receptors, might maximize overall efficiency.

According to our results, receptor selection appears to have consequences on postbinding steps as well. Whereas for most vectors, low levels of apical attachment were associated with low levels of apical transduction, for vectors bearing fibers derived from subgroup B, rather low levels of apical attachment gave rise to relatively high levels of transduction. These vectors thus appeared to gain access to more efficient pathways leading to gene expression when applied to the apical rather than basolateral surface. Receptor selection may influence the internalization pathway, either directly, if the attachment receptor is used for internalization, or indirectly, by influencing the efficiency with which the virion may interact with an internalization receptor. Replacement of a subgroup C fiber with a subgroup B fiber has been shown to alter intracellular trafficking, presumably in relation to the use of CD46 rather than CAR as an attachment receptor (18, 44, 58). Although an Ad5-based vector bearing a chimeric fiber composed of the Ad5 shaft and the Ad35 knob domain required integrins for internalization, it is not certain whether this holds true for vectors bearing entire subgroup B fibers (57).

Fiber-pseudotyped Ads displayed varied behavior in translocation across human and murine intestinal epithelium. In particular, vectors bearing fibers from subgroup B and some subgroup D members were observed to translocate more efficiently across both murine and human polarized epithelia. The

binding of these vectors required CD46 and was much reduced in the murine cell line, in which CD46 was not detected. Thus, translocation was almost certainly independent of CD46 in mICcl2 cells and perhaps as well in Caco-2 cells. If, however, translocation is receptor independent, it will be of interest to understand the basis of its selectivity for vectors bearing certain fibers, which may involve physicochemical properties of the fiber protein (shaft length and charge, for instance).

Most fiber-pseudotyped vectors behaved very differently in mICcl2 and Caco-2 cells, and in particular with regard to transduction. The discrepancies observed between the cell lines probably reflected both species- and cell-type differences, related to the expression pattern of CD46 and CAR receptors. Expression of CD46 was detected in Caco-2 but not mICcl2 cells, presumably in relation to species-specific tissue distribution, i.e., ubiquitous in humans versus restricted to the testis in mice (69). CAR, which is highly conserved between human and murine species, displayed differential localization at the surface of polarized Caco-2 and mICcl2 cells, i.e., predominantly basolateral or uniform, respectively, probably in relation to their respective differentiation states. Caco-2 cells, originating from a colon carcinoma, exhibit features of differentiated small intestine enterocytes, whereas mICcl2 cells retain crypt-like characteristics. Many *in vitro* studies have shown that intestinal crypt cells are easily transduced by various adenoviral vectors, and in particular by Ad5 vectors (11, 16), but that the efficiency of transduction diminishes as cells assume an increasingly differentiated state. This diminution has been shown to be correlated with reduction in expression of CAR and integrins at the apical pole during the polarization process (13, 47, 83). Unfortunately, crypts are not suitable targets for gene delivery after oral administration of Ads, since they are not readily accessible from the intestinal lumen, unless harsh pharmaceutical treatments are used to make them so (53). In light of our results, and to the extent that cell lines represent authentic intestinal epithelium, the mouse appears to be suitable pre-clinical model for the study of intestinal gene delivery for vectors derived from a limited number of adenoviral serotypes, and particularly for CAR-dependent Ads and Ad32. Even then, the tissue distribution of CAR may be dissimilar in humans and mice, with more pronounced mRNA levels in certain murine tissues such as liver or lung (8), thus complicating the extrapolation of results of trials performed in mice to humans. Vectors bearing most subgroup B or D fibers, some of which are attractive candidates for intestinal gene delivery, will need to be assessed in other species more related to humans, such as primates, or in transgenic mice expressing human receptors such as CD46 in relevant tissues.

Gene transfer to the intestinal epithelium represents an important challenge, demanding the design of better-adapted vectors for oral delivery. Our study suggests that substantial gain would be made in gene delivery to digestive epithelium by exploiting the tropism of existing serotypes of human Ads. Nevertheless, for certain applications and for oral vaccination in particular, transduction of not only intestinal epithelial cells but also other intestinal mucosal cells might be advantageous. Indeed, besides direct transduction of intestinal epithelium, which may behave as nonprofessional antigen-presenting cells, transcytosis through enterocytes or specialized M cells of the follicle-associated epithelium, as well as direct capture of viral

particles by dendritic cells from the intestinal lumen (50), could allow transduction of professional antigen-presenting cells. The relative contribution of these different antigen-presenting pathways to the induction of protective immunity after oral administration of Ad vectors will require *in vivo* assessment. The present study provides the rational basis for evaluation of selected vectors in appropriate animal models.

ACKNOWLEDGMENTS

We thank A. Vandewalle (INSERM, U478) for kindly providing the mICcl2 cell line. We are grateful to M. Bomsel (INSERM, U567) for fruitful advice and discussions concerning polarized epithelial cells. We thank M. Monteil, S. Mercier, K. Kacem, B. Klonjowski, and V. Massip for their support. We also thank P. Domanges (IFR 65) for technical support on confocal microscopy.

S.L. was supported by a fellowship from the AFSSA institution.

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