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Adenoviral Transduction of Enterocytes and M-Cells Using *in Vitro* Models Based on Caco-2 Cells: The Coxsackievirus and Adenovirus Receptor (CAR) Mediates Both Apical and Basolateral Transduction

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Abstract: Understanding virus−cell interaction is a key to the design of successful gene delivery vectors. In the present study we investigated Ad5 transduction of enterocytes and M-cells utilizing differentiated Caco-2 cells and cocultures of Caco-2 cells with lymphocytes. Transduction inhibition studies showed that CAR is the major receptor mediating apical and basolateral virus entry in differentiated Caco-2 cells. Integrins and heparan sulfate glycosaminoglycans do not appear to play a significant role. Immunofluorescence localized CAR to sites of cell−cell contact, with staining mostly observed on the cell perimeter. Staining was observed even in nonpermeabilized monolayers, suggesting apical accessibility of the receptor. Cocultures with mouse Peyer's patch lymphocytes or Raji B human lymphocytes were more susceptible to transduction than Caco-2 cells, and the effects were dose-dependent. Similar to Caco-2 cells, CAR and not integrins mediated apical transduction. In conclusion, contrary to other epithelial cell lines, both apical and basolateral transduction of absorptive enterocytes and M-cells is mediated by binding to CAR. The coculture system can be used to study the interactions between M-cells and gene delivery vectors.

Keywords: Adenovirus; vector; coxsackievirus and adenovirus receptor; M-cells; Caco-2 cells; enterocytes; cell culture; coculture

Introduction

Adenoviruses are commonly used as gene delivery vectors for manipulation of cellular genes/processes, for both research applications and gene therapy strategies. Successful delivery of the transgene to the cells or tissue of interest requires efficient cell internalization of the viral particle, a process often governed by specific interactions between viral capsid elements and cell surface receptors. Thus, understanding virus−cell interaction is a key to the design of successful gene delivery vectors. *In vitro* cell culture models are one of the first tools employed in studying these interactions.

In the case of intestinal tissue, Caco-2 cells, a human colorectal adenocarcinoma cell line that undergoes spontaneous enterocytic differentiation in culture,[1] are commonly used as an *in vitro* model of small intestinal epithelium. Adenoviral gene delivery to Caco-2 cells has attracted significant attention in the past both to alter their phenotype for basic research applications and to provide an *in vitro* model for

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gastrointestinal gene therapy. Some aspects of adenoviral transduction of differentiated Caco-2 cells are well characterized. Transduction efficiency is low,[2,3] and this has been partly attributed to the fact that α_v-integrins, known to facilitate internalization of the virus into cells, are not accessible on the apical cell membrane.[3,4] Little is known about the role of the major adenovirus receptor, coxsackievirus and adenovirus receptor (CAR),[5] in Caco-2 cells. In airway epithelial cell lines, the differential distribution of CAR in the apical and basolateral cell membrane has been shown to be a major determinant of the lack of susceptibility of those cells to adenoviral transduction.[6] Furthermore, other membrane receptors such as heparan sulfate glycosaminoglycans were recently identified as receptors for adenoviruses.[7,8]

Apart from columnar enterocytes, which constitute the majority of intestinal epithelium, another intestinal cell type appears to be of particular interest for gene delivery purposes. M-cells are specialized intestinal epithelial cells that appear to be responsible for luminal antigen sampling by the intestinal mucosal immune system.[9,10] Due to their unique characteristics and pivotal role in mucosal immune function, M-cells have attracted increasing attention as targets for vaccine delivery, but could also offer a target for the delivery of therapeutics in the case of inflammatory bowel disease. The occurrence of inflammation in the ileocecal region, a region rich in lymphoid follicles, points to a relationship between inflammatory bowel disease pathogenesis and the lymphoid structures in the gut.[11] Due to their unique characteristics in cell-pathogen interactions, M-cells might represent a better target for adenoviral vectors.

The goal of the studies reported here was to investigate factors that influence adenoviral transduction of Caco-2 cells, including the role of CAR and integrins in apical and basolateral transduction. Furthermore, utilizing an *in vitro* model of follicle-associated epithelium developed and described by Kernéis et al.[12] we studied differences in transduction of enterocytes and M-cells. These experiments were supplemented by additional work using the model of Gullberg et al.[13] These *in vitro* models, based on the coculture of Caco-2 cells and lymphocytes (the Kernéis model using mouse Peyer's patch lymphocytes and the Gullberg model using human Raji B cells), provide an opportunity to study the interactions of adenoviral vectors and human M-cells. The availability of these models is important because, due to the large species variability in M-cell phenotype, the *in vivo* and *in situ* animal models that have been used in the past might not be applicable to human M-cells.

Materials and Methods

Materials. Tissue culture reagents were obtained from Invitrogen Co. (Carlsbad, CA), and tissue culture materials were from Becton Dickinson (Franklin Lakes, NJ) and Corning Inc. Life Sciences (Acton, MA). The GRGDSP peptide was purchased from Bachem AG (King of Prussia, PA). The RmcB monoclonal antibody was produced by the Hybridoma Core, University of Michigan, from a commercially available clone[14] (ATCC #CRL-2379) or alternatively was purchased from Upstate Biotechnology (Waltham, MA). The MOPC 195 mouse myeloma immunoglobulin control and heparin (bovine, sodium salt) were from Sigma (St. Louis, MO). FluoSpheres, Alexa Fluor 488 anti-mouse antibody and Prolong mounting medium were from Molecular Probes (Eugene, OR). All other chemicals used were of analytical grade and purchased from Sigma or Fisher Scientific (Pittsburgh, PA).

Adenoviral Vector. An adenoviral type 5 (Ad5) vector containing the luciferase reporter gene in the E1A/E1B region

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of the viral genome under the control of a CMV promoter[15] [Ad5CMV-luc(loxP)] was obtained from the University of Michigan Vector Core Laboratory. The vector was purified in the Vector Core using previously published methods,[16] titered in 911 cells, and the particle concentration was determined by measuring optical density at 260 nm. A single lot, with particle:pfu ratio of 27, was used for all experiments. Aliquots were stored at −80 °C until immediately before use. MOIs were calculated based on the concentration of viral particles.

Animals. Specific pathogen-free, male 8−12 week old BALB/c mice (Charles River Laboratories) were used in accordance with a protocol approved by the University Committee on Use and Care of Animals (University of Michigan).

Caco-2 Cell Culture. Caco-2 cells (ATCC HTB37) of passage 33−44 were seeded at a density of 4×10^5 cells/well into multiwell plates, onto collagen-coated coverslips, or into Transwell COL culture inserts (0.4 μm pore size). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 0.1 mM nonessential amino acids (NEAA), 45 nM vitamin E, 100 units/mL penicillin, and 100 μg/mL streptomycin until confluent (typically 2−3 days). Medium was changed to DMEM with 5% FBS, 0.1 mM NEAA, 45 nM vitamin E, 0.1 μM selenium (Na_2SeO_3), 0.003 nM zinc ($ZnSO_4 \cdot 7H_2O$), 100 units/mL penicillin, and 100 μg/mL streptomycin for two weeks to obtain fully differentiated cultures. Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in room air.

Caco-2/PP Lymphocyte Coculture. Caco-2 cells (ATCC HTB37) of passage 40−44 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.1 mM nonessential amino acids (NEAA), 100 units/mL penicillin, 10 μg/mL streptomycin, and 100 μg/mL fungizone.

Polycarbonate membrane Transwell inserts (Corning Inc., Acton, MA) with 3 μm pores were inverted into 15 cm culture dishes containing 10 mL of DMEM for humidification purposes. The bottom surface of each Transwell insert's membrane was seeded with 100 μL of Caco-2 medium containing 3×10^5 Caco-2 cells. The cells were allowed to attach overnight at 37 °C in a humidified atmosphere of 5% CO_2 in room air. The Transwells were transferred, in standard orientation, to the wells of 24-well culture plates containing 600 μL of medium/well. Medium (100 μL) was added to the inner Transwell chamber. Medium was changed every two days, for 14−21 days. Transepithelial electrical resistance (TEER) was measured with a Millicell-ERS voltohmmeter (Millipore, Bedford, MA) to confirm differentiation (TEER >300 $\Omega \cdot cm^2$).

BALB/c mice (8−12 week old) were euthanized by CO_2 inhalation. The small intestines were removed and flushed with PBS containing penicillin, streptomycin, and fungizone to remove intestinal contents. Peyer's patches along the small intestine were carefully dissected. Five to ten Peyer's patches were collected per animal in DMEM supplemented with penicillin, streptomycin, and fungizone. The Peyer's patches were then smashed with a 10 mL plastic syringe piston and subsequently triturated through a 100 μm nylon mesh (Becton Dickinson, Franklin Lakes, NJ). The cell suspension was transferred to a 50 mL centrifuge tube; cells were pelleted by centrifugation at 101 × g and resuspended in Caco-2 culture medium. Viable cells were counted based on trypan blue exclusion.

The concentration of the Peyer's patch lymphocytes was adjusted to 10^7 cells/mL. Cell suspension (100 μL) was placed in the inner chamber of each Transwell (basolateral to the Caco-2 cell monolayers). Cell-free Caco-2 culture medium (100 μL) was added to the control Transwells. Apical medium was replenished concurrently by placing 600 μL of Caco-2 culture medium into the wells of the 24-well plate. The cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in room air for 3 days.

Prior to embarking on the transduction studies, cells of this model were characterized with respect to activity of the brush border hydrolase alkaline phosphatase and transcytotic activity.

Alkaline Phosphatase Activity Assay. Alkaline phosphatase activity was measured as described by Calhau et al.[17] with minor modifications. After aspirating the medium, a 2.86 mM solution of *p*-nitrophenylphosphate was added to the apical side of the monolayers and incubated at 37 °C for 1.5 h. The reaction was then quenched with 3 N NaOH, and absorbance at 405 nm was measured.

Transcytotic Activity. Transcytotic activity was assessed using the transport of fluorescent microspheres. FITC-labeled, carboxylate-modified polystyrene microspheres (FluoSpheres, Molecular Probes, Eugene, OR) of 0.2 μm size were dispersed at a concentration of 4.85×10^{10} particles/mL in HBSS with 1 mM HEPES (pH 7.4). After a 30 min preincubation in HBSS−HEPES at 37 °C, prewarmed FluoSphere suspension (600 μL) was added to the wells of the multiwell plate (apical side of the monolayers) and 100 μL of HBSS−HEPES was added to the inserts (basolateral side). After 1 h incubation at 37 °C, the solution was collected from the inserts and kept protected from light at 4 °C until imaged using an Olympus BX-51 epi-fluorescence microscope.

Adenoviral Transduction Studies of Caco-2 cells (in Monoculture or Peyer's Patch Lymphocyte Coculture). Differentiated Caco-2 monolayers were infected with the

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Ad5CMV-luc(loxP) vector at the desired MOI, either apically or basolaterally. Basolateral transduction was carried out after aspirating the medium and inverting the culture inserts in a sterile vessel. Adenovirus transduction was allowed to proceed for 2 h at 37 °C. The medium was then aspirated, inverted Transwell inserts were returned to standard orientation in a 24-well plate, fresh medium was placed on both sides of the insert membrane, and the cultures were returned to the incubator. Luminescence activity was assayed after 48 h.

For transduction inhibition studies, cells were preincubated with blocking solutions before the vectors were applied, according to protocols in the literature.[4,5] The following blocking solutions were used: RmcB anti-CAR monoclonal antibody (1:50 dilution of ascites in culture medium), MOPC 195 mouse myeloma control immunoglobulin (1:50 dilution in culture medium), an RGD peptide (GRGDSP; 1 mM). In the case of heparan sulfate blocking studies, the vector was preincubated with a 10 μg/mL solution of sodium heparin in DMEM with 0.1% FBS for 1 h at 37 °C. The vector was subsequently applied to the Caco-2 cells at the desired MOI, and the cultures were placed at 4 °C for 1 h. At the end of the incubation, the medium was aspirated and replaced with fresh medium and the cultures were returned to the 37 °C incubator for 48 h before assessing transduction efficiency by assaying luminescence activity.

Caco-2/Raji B Cell Coculture and Adenoviral Transduction. For use in the second M-cell model coculture system, Raji B cells (ATCC CCL-86) were maintained in medium RPMI 1640 supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2 in room air. Caco-2 cells (6×10^5/cm^2) were seeded onto polycarbonate membrane Transwell inserts (24-well size; Corning Inc., Acton, MA) with 0.4 μm pores. After 15−17 days, TEER was measured to confirm confluence, and Raji B cells (5×10^5/well) in a 50:50 mixture of Raji B cell medium and Caco-2 cell medium were added to selected wells of the multiwell plate. Transduction with Ad5CMV-luc(loxP) vector was carried out 4 days later (after again confirming confluence of the monolayers using TEER) as described below. The vector was added in the desired concentrations in a volume of 50 μL to the apical aspect of the monolayers within the culture inserts. After incubating for 2 h at 37 °C, 500 μL of medium was added to each insert and incubation was continued for a total of 48 h. The inserts were then transferred to fresh multiwell plates containing 500 μL of medium per well so that assessment of transduction could be carried out in the absence of the Raji B cells. Bioluminescence assay was then carried out as described below.

Bioluminescence Imaging. At the end of the desired virus incubation time, medium was replaced with feeding medium containing 0.15 or 0.4 mg/mL luciferin. After incubating for 30 min at 37 °C, luciferase expression was quantified as bioluminescence photon counts as previously described[18] using a cryogenically cooled Xenogen IVIS Imaging system (Xenogen Corporation, Alameda, CA) coupled to a data acquisition computer at the Center for Molecular Imaging, University of Michigan. A digital grayscale image was acquired followed by acquisition and overlay of a pseudocolor image representing the spatial distribution of detected photon counts emerging from active luciferase within the wells. Signal intensity was quantified as the sum of all detected photons within the region of interest during a 1 or 3 min luminescence integration time. In some cases, results were expressed as fold photon counts compared to untransduced cells present in the plate.

Electron Microscopy. Three days after the addition of Peyer's patch lymphocytes to Caco-2 monolayers, Transwell membranes were placed in cold 2.5% glutaraldehyde in 0.1 M Sorensen's phosphate buffer, pH 7.4, and kept at 4 °C overnight. The samples were postfixed in osmium tetroxide, *en bloc* stained in uranyl acetate, and dehydrated in ethanol. They were then infiltrated in increasing concentrations of Epon and, finally, embedded in Epon and polymerized at 60 °C for 24 h. Ultrathin (70 nm) sections were prepared and poststained with uranyl acetate and lead citrate. Transmission electron microscopic images were obtained using a Philips CM100 electron microscope operated at 60 kV.

Immunofluorescent Detection of CAR. Caco-2 cells were cultured on Transwell inserts or collagen-coated coverslips. In the latter case, cell growth was confined to a 0.79 cm^2 circular area by a stainless steel ring fitted with an O-ring. Monolayers were washed with PBS and fixed with cold 4% paraformaldehyde in PBS. In the case of Transwells, the insert membrane was excised and placed on a glass coverslip for immunofluorescent staining.

Fixed cells were rinsed in PBS and blocked with a solution of 1% BSA or 10% goat serum in PBS for 20 min. In certain cases cells were permeabilized with 0.2% Triton X-100. Primary antibody (RmcB ascites 1:50) was added for 1 h at room temperature or alternatively overnight at 4 °C. Cells were washed twice with PBS and goat anti-mouse Alexa Fluor 488 conjugated secondary antibody in 1% BSA or 10% goat serum was added (1:100 dilution). Cells were incubated in the dark for 1 h at room temperature. Cells were rinsed twice with PBS; coverslips were mounted on glass slides with Prolong mounting medium. Cells were imaged using an Olympus BX-51 epi-fluorescence microscope equipped with a digital camera or a Bio-Rad MRC600 confocal microscope.

Data Analysis. Statistical significance was determined using the Student's *t* test or one-way ANOVA. *P* values less than 0.05 were considered statistically significant.

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Results

Adenoviral Transduction Inhibition Studies in Differentiated Caco-2 Cells. The main pathway through which adenoviruses gain entry into the cell is a two-step receptor-mediated endocytosis.[19,20] The first step in this process is the attachment of the fiber "knob" to the cell surface receptor, CAR. The second involves the binding of the pentose base to vitronectin receptors (integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$) on the cell surface.[21,22] In order to study the effect of those two factors on the transduction of intestinal epithelial cells, we carried out transduction inhibition studies using a monoclonal antibody against CAR (RmcB)[14] and an RGD peptide (GRGDSP) that blocks integrin binding.[4] The studies were carried out at a MOI of 200, which represents a submaximal MOI that provides for adequate transduction levels. The RGD peptide concentration of 1 mM was chosen based on previously published studies.[4]

When blocking solutions and vectors were applied to the apical cell surface, preincubation with the RmcB antibody resulted in a 32.7% decrease in transduction efficiency (20.19 $\pm$ 1.83 vs 13.59 $\pm$ 0.91, $p < 0.001$, mean of photon counts (fold untransduced control) $\pm$ SE (4 experiments, each with duplicate or triplicate cultures) (Figure 1A). Preincubation with the GRGDSP peptide resulted in only a 4.8% decrease in transduction levels which was not statistically significant (Figure 1A; $p = 0.37$). Similarly, blocking experiments were performed with basolateral application of blocking solution and vector. Again, preincubation with the RmcB antibody resulted in significant reduction in transduction efficiency (12.26 $\pm$ 1.45 vs 9.82 $\pm$ 2.14, $p < 0.05$, mean of photon counts (fold untransduced control) $\pm$ SE (6 experiments, each with duplicate or triplicate cultures) (Figure 1B). Reduction in transduction efficiency observed after GRGDSP preincubation was not statistically significant (Figure 1B; $p = 0.19$). It is worth mentioning that, consistent with previous studies in our laboratory, the basolateral transduction levels appear significantly lower compared to apical.

More recently, heparan sulfate glycosaminoglycans which are found on the cell membrane of several cell types, including Caco-2 cells, have been shown to be capable of acting as adenovirus receptors. To study the role of heparan sulfate binding in transduction of differentiated Caco-2 cells, we carried out transduction inhibition experiments by preincubating the viral vectors with heparin prior to application of the vectors to the monolayers. We did not observe any significant inhibition of adenoviral transduction by heparin pretreatment of Ad5 vector when the vector was applied either apically (Figure 2A; $p = 0.27$) or basolaterally (Figure 2B; $p = 0.31$).

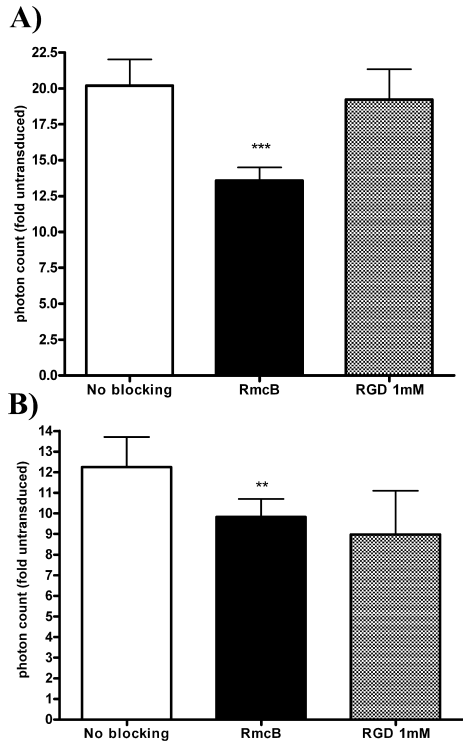

Figure 1. Transduction inhibition in fully differentiated Caco-2 monolayers: (A) after apical application of the vectors; (B) after basolateral application of the vectors. Caco-2 cell monolayers grown for 2 weeks on Transwell inserts were preincubated for 2 h at room temperature with the following: RmcB anti-CAR monoclonal antibody (1:50 ascites), an RGD peptide (GRGDSP, 1 mM) that blocks integrin binding, or culture medium (control). Subsequently cells were exposed to Ad5CMV-luc(loxP) (MOI 200) for 2 h at 37 °C. Basolateral application of solutions/vectors was carried out on inverted inserts. Luminescence activity was assayed after 48 h. *** = $p < 0.001$, ** = $p < 0.05$ compared to control. Values are mean $\pm$ SE of 4 duplicates/triplicates.

Immunofluorescent Detection of CAR. In epithelial cell lines, CAR has been suggested to be localized at the tight/apical junction complex, playing a role in preserving integrity of the epithelial barrier. We carried out immunofluorescent studies with the RmcB anti-CAR antibody on fully differentiated Caco-2 monolayers. Consistent with the literature, CAR appeared to be localized at sites of cell−cell contact and staining was mostly observed on the cell perimeter (Figures 3, 4). Confocal microscopy studies (Figure 4) indicate a location near the apical region of the lateral membrane, possibly at the area of the tight junction. Similar staining, but with lesser intensity, was observed in nonpermeabilized cells, consistent with apical accessibility of CAR in the Caco-2 cell line (Figure 3E). In both cases, incubation with the MOPC 195 control immunoglobulin resulted only in diffuse cytoplasmic background staining (Figures 3B, 3F). Omission of the primary antibody resulted in little change

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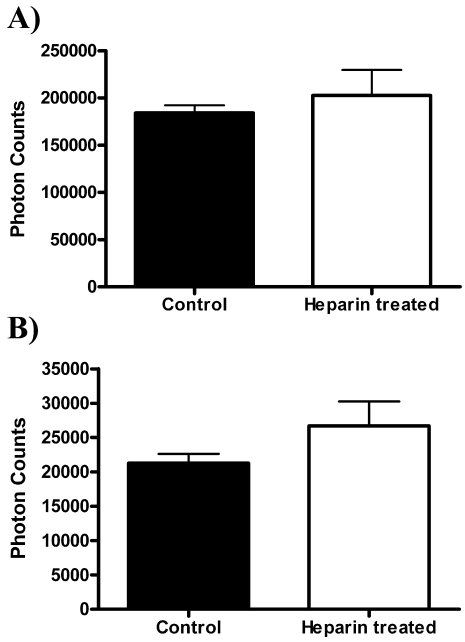

Figure 2. Effect of blocking heparan sulfate glycosaminoglycan binding on transduction of fully differentiated Caco-2 monolayers: (A) after apical application of the vectors; (B) after basolateral application of the vectors. Vectors were preincubated with a 10 μg/mL solution of sodium heparin in DMEM with 0.1% FBS for 1 h at 37 °C. The vector was subsequently applied to the cells which were incubated at 4 °C for 1 h. At the end of the incubation, medium was replenished and cells were returned to the 37 °C incubator for 48 h before assessing transduction efficiency ($n = 3-6$ wells).

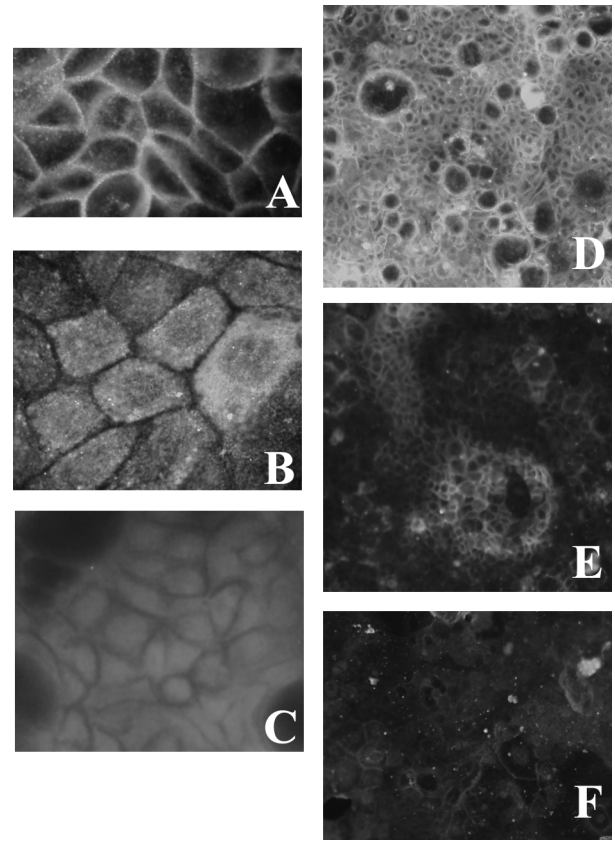

Figure 3. Immunofluorescence for CAR on fully differentiated Caco-2 monolayers. (A) anti-CAR RmcB mAb on permeabilized monolayer; (B) MOPC control immunoglobulin on permeabilized monolayer; (C) PBS (no primary mAb) on permeabilized monolayer) (D) anti-CAR RmcB mAb on permeabilized monolayer (low magnification); (E) anti-CAR RmcB mAb on nonpermeabilized monolayer (low magnification); (F) MOPC control immunoglobulin on nonpermeabilized monolayer (low magnification). Immunofluorescence was performed with standard techniques using an antibody to CAR (RmcB, 1:100 stock) or a control immunoglobulin (MOPC) followed by detection with an Alexa Fluor 488 labeled secondary anti-mouse immunoglobulin mAb. Cells were imaged using an Olympus BX-51 epi-fluorescence microscope equipped with a digital camera.

in the diffuse cytoplasmic background staining (Figure 3C) confirming its lack of specificity. No membrane staining was seen in either type of negative control.

Adenoviral Transduction Studies in Caco-2/PP Lymphocyte Cocultures. Prior to transduction studies, our application of the Caco-2/mouse Peyer's patch lymphocyte model was characterized. A transmission electron micrograph of a coculture (Figure 5A) shows a lymphocyte incorporated into the base of the Caco-2 cell monolayer. The overlying epithelial cells show evidence of transformation to an M-cell phenotype, with decreased density of the cytoplasm and a more sparse and more disorganized brush border than is seen in neighboring epithelial cells. TEER measured after 3 days of coculture was not different from that of concurrent Caco-2 monocultures (Figure 5B), confirming that tight junctions remained intact. One characteristic of M-cells is a decreased expression of brush border hydrolases, including alkaline phosphatase. The alkaline phosphatase activity of the cocultures was found to be approximately 25% lower than that of concurrent Caco-2 monocultures (Figure 5C; $p < 0.0001$), consistent with a transformation of at least some of the Caco-2 cells in the cocultures to an M-cell-like phenotype. Another property of M-cells is their ability to transport particles such as bacteria and viruses. Synthetic microbeads are commonly used to assess the transcytotic activity of M-cell models.[13,37,38] Figure 5D demonstrates a roughly 6-fold increase in apical to basolateral movement of 0.2 μm fluorescent microbeads after 1 h at 37 °C. This is comparable to an approximately 11-fold increase reported in the literature[37] for this model using flow cytometry for the analysis.

Adenoviral transduction studies were carried out in 3-day cocultures for comparison with concurrent cultures of differentiated Caco-2 cells alone. As seen in Figure 6, similarly to the Caco-2 cell monocultures, the cocultures exhibited a dose-dependent increase in transduction efficiency. When cells were infected with 100 viral particles/cell, transduction levels were similar for cocultures and Caco-2 cells ($p = 0.79$). However, when the adenovirus dose was increased to MOI 1000, cocultures exhibited a significantly higher trans-

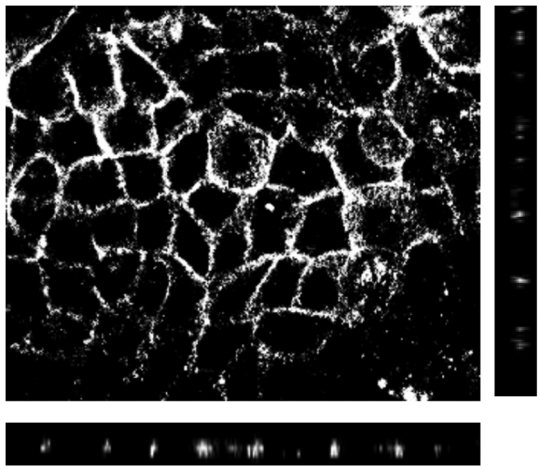

Figure 4. Confocal microscope image of a permeabilized Caco-2 monolayer stained for CAR with the RmcB mAb. Immunofluorescence was performed with standard techniques, using an antibody to CAR (RmcB, 1:100 stock) followed by detection with an Alexa Fluor 488 labeled secondary anti-mouse immunoglobulin mAb. Cells were imaged using a Bio-Rad MRC600 confocal microscope. Slice reconstruction was done in ImageJ imaging software.

duction level compared to Caco-2 monocultures (1.55-fold increase; $3.4 \times 10^5 \pm 4.2 \times 10^4$ vs $2.2 \times 10^5 \pm 2.2 \times 10^4$, mean of photon counts ± SE, $p < 0.001$). For both Caco-2 cell monocultures and cocultures, the bioluminescence at MOI 1000 was significantly greater than that at MOI 100 ($p < 0.001$).

Blocking studies were performed to study possible interactions between the vectors and the two major adenovirus receptors, CAR and integrins. All blocking studies were performed at two virus concentrations, MOI 200 and MOI 1000. When CAR binding was blocked after preincubation of the cells with an anti-CAR mAb (RmcB),[14] there was a significant reduction in transduction compared to cultures blocked with a control antibody. At the MOI of 200, cocultures exhibited a 17.7% reduction in bioluminescence activity ($p < 0.05$; Figure 7). Similarly, in Caco-2 cells (MOI 200) bioluminescence activity decreased by 24.9% ($p < 0.05$). At the higher concentration (MOI 1000), the observed reduction was 39.6% for the cocultures ($p < 0.001$) and 29.6% for the Caco-2 cells ($p < 0.05$; Figure 7). In contrast to the results obtained after RmcB preincubation, preincubating the cells with an integrin-binding RGD peptide (GRGDSP) prior to vector application did not affect transduction levels in either monocultures ($p = 0.10$ and 0.33 for MOI 200 and 1000, respectively) or cocultures of Caco-2 cells ($p = 0.28$ and 0.30 for MOI 200 and 1000, respectively; Figure 8).

Adenoviral Transduction Studies in Caco-2/Raji B Cell Cocultures. The effect of adenoviral transduction was studied in a second *in vitro* M-cell model. This model employs the human Raji B lymphocyte cell line rather than mouse Peyer's patch lymphocytes to alter the phenotype of Caco-2 cells to resemble M-cells. A feature of this model is

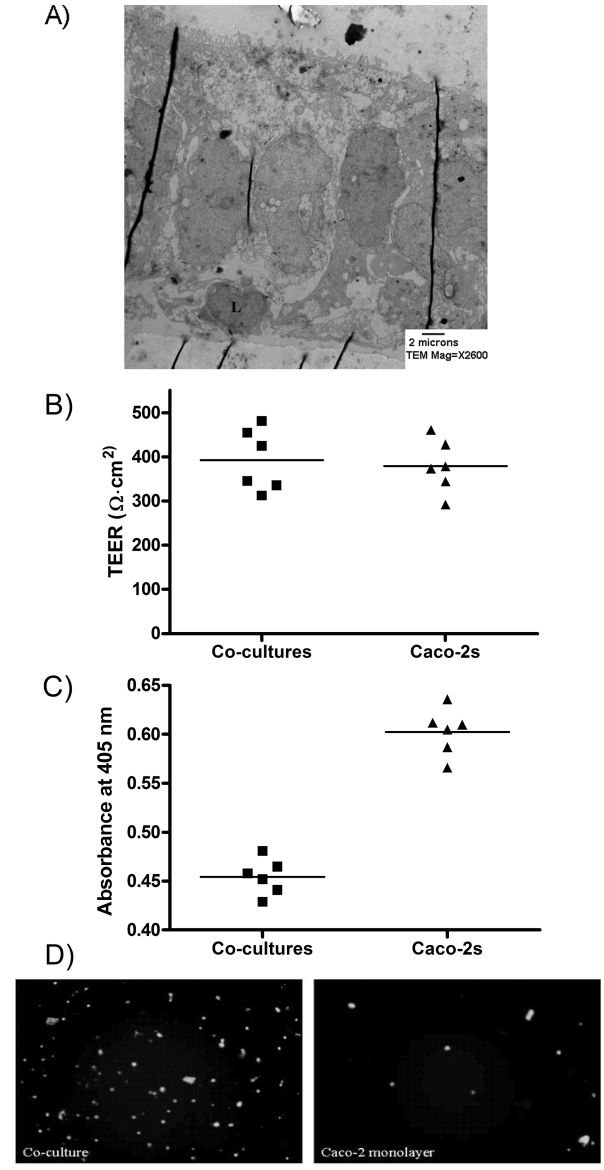

Figure 5. Characterization of Caco-2 cell/mouse Peyer's patch lymphocyte cocultures. (A) Transmission electron micrograph of a Caco-2 cell monolayer cocultured for three days with mouse Peyer's patch lymphocytes. A lymphocyte (L) can be seen within the Caco-2 monolayer. The epithelial cells above the lymphocyte exhibit a more sparse and less organized brush border as well as less dense cytoplasm compared to neighboring epithelial cells not in contact with lymphocytes. (B) TEER is unchanged in the cocultures, confirming that tight junctions remain intact. Results of two experiments are shown, each with triplicate wells. (C) Alkaline phosphatase activity assayed using *p*-nitrophenylphosphate (2.86 mM) as substrate. Absorbances at 405 nm after a 1.5 h incubation at 37 °C were 0.454 ± 0.018 and 0.603 ± 0.024 (mean ± SD) for cocultures and Caco-2 monocultures, respectively ($n = 6$; $p < 0.0001$). (D) Transcytosis of fluorescent 0.2 μm microbeads from apical to basolateral medium, assessed by fluorescence microscopy. Images representative of two experiments, $n = 3-6$.

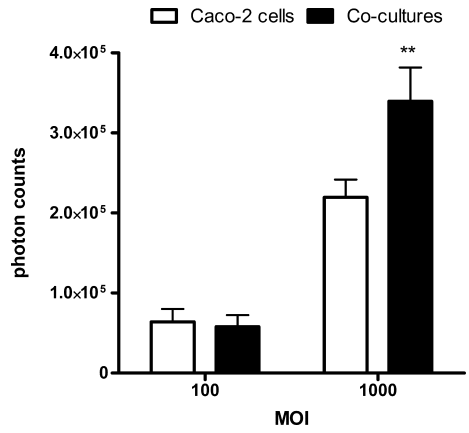

Figure 6. Differences in transduction efficiency of Caco-2 cells and Caco-2/PP lymphocyte cocultures. Cell monolayers were exposed to Ad5CMV-luc(loxP) for 2 h at 37 °C. Additional maintenance medium was added to the cells, and infection was allowed to occur for 24 or 48 h. Data are mean photon counts ± SE, n = 8−10 cultures, ** = $p < 0.01$ for the comparison with Caco-2 monocultures at the same MOI.

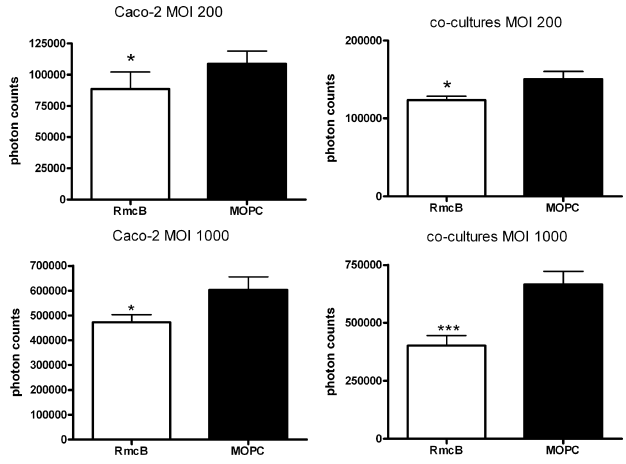

Figure 7. Transduction inhibition experiments with an anti-CAR mAb (RmcB) on Caco-2 cells and Caco-2/PP lymphocyte cocultures. Cell monolayers were preincubated for 2 h with 1:50 dilution of RmcB mAb or MOPC control immunoglobulin. Subsequently cells were transduced with Ad5CMV-luc(loxP) for 2 h at 37 °C. Luminescence activity was assayed after 48 h. *** = $p < 0.001$, * = $p < 0.05$ compared to control. Values are mean ± SD of 8−10 cultures.

that the Raji B cells are not incorporated into the Caco-2 cell monolayer. To help ensure that this was the case, 0.4 μm pore size culture inserts were employed in our performance of this model. In addition, the culture inserts containing the vector-exposed Caco-2 cells or M-like cells were transferred to fresh multiwell plates, leaving the Raji B cells behind, prior to the addition of luciferin and bioluminescence imaging.

TEER measurements prior to application of the adenoviral vector were not different from those of concurrent Caco-2 monocultures (Figure 9A), indicating that tight junctions remained intact. As in the Caco-2 monocultures, the level

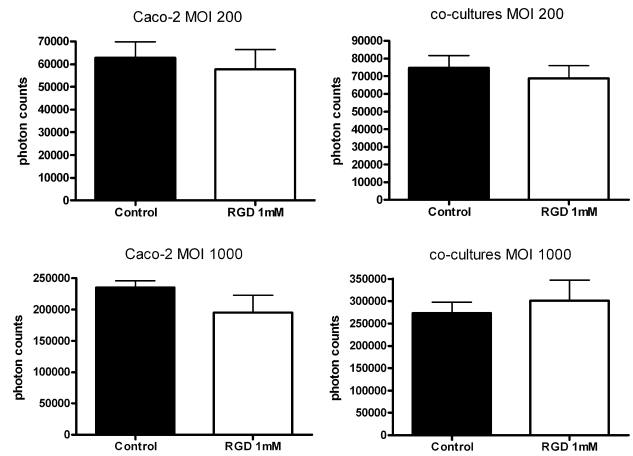

Figure 8. Transduction inhibition experiments with an integrin-binding peptide on Caco-2 cells and Caco-2/PP lymphocyte cocultures. Cell monolayers were preincubated with the RGD peptide GRGDSP (1 mM in culture medium). Subsequently cells were exposed to Ad5CMV-luc(loxP) for 2 h. Luminescence activity was assayed after 48 h. Values are mean ± SD of 6−7 cultures.

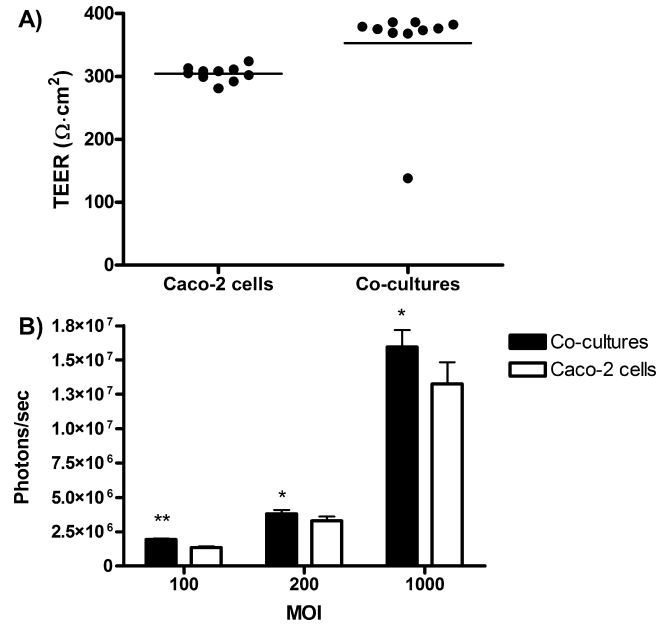

Figure 9. Caco-2/Raji B cell cocultures: (A) Assessment of tight junction integrity by TEER. Results of one representative experiment are shown, with ten replicate wells. (B) Differences in transduction efficiency of Caco-2 cells and Caco-2/Raji B cell cocultures. Cell monolayers were exposed to Ad5CMV-luc(loxP) for 2 h at 37 °C. Additional maintenance medium was added to the cells and infection was allowed to occur for 48 h. Data are mean photon counts ± SE; n = 4 experiments, each with 2−3 replicate cultures; ** = $p < 0.005$, * = $p < 0.05$ for the comparison with Caco-2 monocultures at the same MOI.

of bioluminescence was dose-dependent in the Raji B cell cocultures ($1.97 \times 10^6 \pm 6.81 \times 10^4$, $3.84 \times 10^6 \pm 1.64 \times 10^5$, and $16.0 \times 10^6 \pm 7.77 \times 10^5$ photons/s for MOI 100,

200, and 1000, respectively; mean of photon counts ± SE for individual wells of 5 experiments, each with duplicate or triplicate wells; Figure 9B). Consistent with the findings with mouse Peyer's patch lymphocyte cocultures, an increase in bioluminescence as compared to parallel Caco-2 monocultures was seen in the Raji B cell cocultures (mean 1.33, 1.15, and 1.21 fold at MOI 100, 200, and 1000, respectively; $p \leq 0.007$).

Discussion

Understanding virus−cell interactions is a key to the design of new gene delivery vectors. In the gastrointestinal tract, columnar enterocytes and M-cells represent two possible targets for gene delivery. Columnar enterocytes are extensively modeled *in vitro* with Caco-2 cell cultures. Recently two coculture systems of Caco-2 cells and lymphocytes have been developed to mimic follicle-associated epithelium *in vitro*. In this report we utilized these two *in vitro* models to study the interactions of Ad5 vectors with the intestinal epithelium. Previous reports on Caco-2 cells concentrated only on integrins and their effect in adenoviral transduction, while no studies have been performed on CAR, the main adenoviral receptor. Here we report that, contrary to other epithelial cell lines, in differentiated Caco-2 cells CAR appears to mediate both apical and basolateral entry of adenovirus. Furthermore, our data suggest that M-cells are more susceptible to adenoviral transduction than enterocytes. Our data also suggest that, similar to Caco-2 cells, CAR and not integrins appears to mediate apical transduction of M-cells.

Adenoviruses enter the cells mainly through receptor-mediated endocytosis. The major entry pathway involves the attachment of the fiber "knob" of the virus to CAR, followed by internalization of the virus through clathrin-mediated endocytosis that appears to be mediated by binding to integrins on the cell surface.[19,20] The accessibility of those two receptors appears to dictate adenoviral transduction efficiency.[19] Mature, differentiated enterocytes appear to be resistant to transduction by commonly used adenoviral vectors.[2,3] The same low levels of transduction have been reported for other epithelial cell types such as airway epithelial cells.[6,23−25] Studies utilizing Caco-2 cells suggest that the decrease in integrin expression as cells differentiate is responsible for the low transduction efficiency.[3,4] For airway epithelia, the low transducibility has been attributed to the absence of CAR on the apical cell membrane.[6,25] Furthermore, it was recently demonstrated that CAR acts as an adhesion molecule mediating homotypic cell−cell contacts, being localized in the apical junction complex helping maintain epithelial integrity.[26,27] To our knowledge CAR expression and its role in adenoviral transduction has not been studied in intestinal epithelial cells.

In earlier studies, we noticed that in Caco-2 cells, contrary to airway epithelia, apical transduction appears to be favored over basolateral.[18] As mentioned above, integrins have a minimal effect on the apical transduction of differentiated Caco-2 cells as their expression decreases as cells differentiate. Our data confirm these results as we saw no significant inhibition of apical transduction with the GRGDSP integrin-binding peptide (Figure 1A). It has been reported that during Caco-2 cell differentiation integrin expression is downregulated and integrin molecules redistribute to the basolateral surfaces.[28,29] It is interesting that we did not see a significant effect of integrins on basolateral transduction (Figure 1B), suggesting that integrins are not accessible to the virus during basolateral infection. However, the lower level of basolateral transduction coupled with smaller and less consistent effects of blocking agents limit the conclusions that can be drawn.

Contrary to the integrins, our data suggest that CAR is the receptor responsible for adenoviral transduction of enterocytes. We noticed significant inhibition in transduction efficiency for both apical (Figure 1A) and basolateral (Figure 1B) virus application following preincubation with the RmcB anti-CAR monoclonal antibody. While involvement of CAR in basolateral transduction is expected based on the available literature with other epithelial cell lines, the fact that we observed transduction inhibition with the anti-CAR antibody after apical virus application is in striking contrast to what has been reported for airway epithelia. Our data suggest that, contrary to other epithelial cell types, in Caco-2 cells CAR is accessible from the apical surface and mediates adenoviral cell entry.

Recently, heparan sulfate glycosaminoglycans have been suggested as an alternative receptor for adenoviruses.[7,8] Glycosaminoglycans are long polyionic carbohydrate chains consisting of repeating disaccharide units containing sulfate residues. They are widely expressed on cell surfaces and have a wide functional heterogeneity. In Caco-2 cells, it has been shown that heparan sulfate undergoes differentiation-de-

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pendent redistribution to the basolateral cell surface.[30,31] As a result, in fully differentiated Caco-2 monolayers the majority of heparan sulfate is found in the basolateral surfaces. Caco-2 differentiation also results in changes in apical and basal heparan sulfate structure and function.[32] Preincubation of the virus with heparin has been shown to result in decreased infection of undifferentiated Caco-2 cells.[33] We did not observe any inhibition in transduction efficiency for the Ad5 vectors after either apical or basolateral application of the virus (Figure 2). Our results suggest that binding to heparan sulfate does not play a significant role in transduction of fully differentiated Caco-2 cells.

We also performed immunofluorescence experiments to study the localization of CAR in the Caco-2 cell line. Consistent with the tight junction localization that has been suggested in other epithelial cell lines in the literature, we observed staining around the perimeter of the cells (Figures 3, 4). However, our transduction data point to a localization close to the apical surface where it is still accessible by the adenovirus. This is supported by the confocal microscopy images that reveal a CAR location toward the apical cell surface (Figure 4) and by the fact that staining was observed even in nonpermeabilized Caco-2 monolayers (Figure 3E).

While columnar enterocytes, modeled by Caco-2 cell cultures, represent the majority of intestinal epithelial cells, M-cells have attracted significant attention as targets for delivery of vaccines and therapeutics, due to their important role in mucosal immunity and inflammatory response. In the case of gastrointestinal gene delivery M-cells might provide a better target for adenoviral vectors compared to enterocytes that are known to exhibit low transduction.[3,4] There are a couple of reports in the literature supporting this hypothesis. Croyle et al. reported increased transduction of rat ileum compared to jejunum, after intraluminal administration of Ad5 vectors, and attributed the difference to the presence of Peyer's patches in the ileum.[4] Foreman et al. similarly reported that intraluminal administration of Ad5 vectors in rabbit ileal segments containing a Peyer's patch resulted in higher transduction of the epithelium compared to areas where lymphoid follicles were not present.[34] Furthermore, they demonstrated that the cells responsible for the increased transduction were epithelial cells of the lymphoid follicles, potentially M-cells.

In the present study, an *in vitro* model of follicle-associated epithelium based on the conversion of Caco-2 cells was used to study interactions between adenoviral vectors and M-cells. The *in vitro* model initially developed by Kernéis et al.[12,35] offers the possibility of studying interactions between adenoviral vectors and human M-cells. The human origin of the M-cells of this model offers a significant advantage over the animal models used in the past due to the significant species differences observed for the M-cell phenotype. This, or Gullberg's similar model based on the coculture of Caco-2 cells and Raji B lymphocytes,[13] is attracting increased attention recently to study pathogen−M-cell interactions,[12,35,36] to study the transport of microparticles or macromolecules,[37,38] or to identify specific receptors on the M-cell surface that could be used for oral targeting.[39]

Although only a fraction of the Caco-2 cells in the Peyer's patch lymphocyte coculture model convert to M-cells, these cocultures exhibit several characteristics of follicle-associated epithelium. Figure 5A shows a lymphocyte incorporated into a Caco-2 monolayer, a feature of this model. Functional changes that are consistent with conversion of cells to an M-cell phenotype included decreased alkaline phosphatase activity (Figure 5C) and increased transcytotic activity (Figure 5D).

Similar to what has been reported for other cell lines, as well as the Caco-2 cell line, cocultures of both types exhibited a dose-dependent increase in transduction. It should be noted that significant transduction of the lymphocytes within the monolayer is not expected as lymphocytes are

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known to be resistant to transduction.[40] While we have not directly tested this, the species difference in Kernéis' mouse Peyer's patch lymphocyte coculture model may be an advantage for our experiments in that mouse cells may be somewhat less likely to be transduced by the human serotype 5 adenoviral vector than are human Raji B cells. In our experiments using the Kernéis model, any mouse Peyer's patch cells that had not migrated into the Caco-2 cell monolayer (or at least into the membrane of the culture insert) were aspirated from the insert prior to application of the adenoviral vector. When applied apically to the M-like cells, the vector would need to be transported through or between the cells of the monolayer to contact and transduce the lymphocytes. TEER measurements in the cocultures did not differ from those of the Caco-2 monocultures, indicating that tight junctions remained intact. Due to the transcytotic capabilities of M-cells, transport of the vector through the M-cells may be a theoretical possibility.

We therefore sought to confirm an increased transducibility of M-cells using Gullberg's Raji B cell coculture model.[13] Unlike the mouse Peyer's patch lymphocyte model in which the Caco-2 cells are on the outer surface of the culture insert membrane and the lymphocytes are added to the interior of the insert, in the Raji B cell model the Caco-2 cell monolayer is contained within the insert and the lymphocytes are added to the well of the multiwell plate in which the insert is suspended. Spontaneous settling of the Raji B cells to the bottom of the wells of the multiwell plates minimizes the likelihood that Raji B cells can come in contact with the insert membrane and migrate across it. To further minimize this possibility, we chose to use inserts with a pore size of 0.4 μm in performing the Raji B cell model, rather than 3 μm as used in the Peyer's patch lymphocyte model. The use of small pore size inserts is expected to preclude migration of Raji B cells through the membrane and subsequent incorporation into the Caco-2 cell monolayer. Overall, TEER measurements prior to application of the adenoviral vector were not different from those of concurrent Caco-2 monocultures (Figure 9A), indicating that tight junctions remained intact. As a final precaution, the culture inserts containing the vector-exposed Caco-2 cells or M-like cells were transferred to fresh multiwell plates, leaving the Raji B cells behind, prior to the addition of luciferin and bioluminescence imaging. For these reasons, it is unlikely that transduction of Raji B cells occurred and it is unlikely that this contributed to the bioluminescence measurements. Results obtained using the Raji B cell coculture model (Figure 9) therefore support the hypothesis that increased transduction in the coculture models as compared to Caco-2 monocultures is due to increased transduction of M-like cells (or other Caco-2 derived cells) and is not due to transduction of lymphocytes or other Peyer's patch cells.

The species difference inherent in the mouse Peyer's patch coculture model may be viewed as a limitation of the model but, as noted above, may instead be a theoretical advantage for experiments using viral vectors based on human viral serotypes. The fact that multiple cell types are present in the cell suspension prepared from the mouse Peyer's patches may be disadvantageous in experiments aimed at understanding the mechanisms of induction of the M-cell phenotype but are not likely to be problematic when the aim is to compare M-cell and enterocyte properties. The fact that the M-cells induced in this model are of human origin is key to the model's usefulness in that there are wide species differences in M-cell structure.

The analytical techniques used in the present studies do not demonstrate that it is specifically or only M-like cells within the Caco-2 cell monolayers that exhibit increased transduction. It is possible that increased adenoviral transducibility is a more generalized effect of lymphocytes on Caco-2 cells and that all of the epithelial cells are affected. More targeted methods will be needed to answer this question.

We further studied the involvement of the two major adenovirus receptors, CAR (coxsackievirus and adenovirus receptor) and integrins, in the transduction process by means of transduction inhibition experiments. Blocking binding to CAR with the anti-CAR monoclonal antibody (RmcB) resulted in significant reduction in transduction at the two MOIs tested in both Caco-2 cell monocultures and mouse Peyer's patch cocultures (Figure 7). The most pronounced effect (40% decrease) was observed with the coculture at the MOI of 1000. Although this could point to a higher involvement of CAR in transduction of M-cells, it is difficult to draw any conclusions as generally the inhibition levels were similar. We did not observe any effect of integrin blocking with the GRGDSP peptide on transduction efficiency, for either the Caco-2 cell monocultures or the mouse Peyer's patch cocultures (Figure 8).

In summary, using *in vitro* cell culture models based on Caco-2 cells we studied transduction of enterocytes and M-cells. Contrary to other epithelial cell lines, our data suggest that both apical and basolateral transduction of Caco-2 cells is mediated by binding to CAR. Binding to other receptors, such as integrins and heparan sulfate glycosaminoglycans, does not play a significant role. Our data also suggest that M-cells are more susceptible to adenoviral transduction than enterocytes. Similar to Caco-2 cells, CAR and not integrins appears to mediate apical transduction. These coculture systems can be used to study the interactions between M-cells and gene delivery vectors.

Acknowledgment. Adenoviral vectors were produced by the University of Michigan Vector Core. We thank the Center for Molecular Imaging of the University of Michigan Medical School, especially Dan E. Hall, for help with the bioluminescence imaging studies. We thank members of the Microscopy and Image Analysis Laboratory, University of Michigan, for the transmission electron microscopy and for their help with the fluorescence microscopy. This work was supported by grants from National Institutes of Health (RO1DK56750 and 1RO1DK073992-01A2 to E.M.Z.), National Cancer Institute (R24CA83099 to the MSAIR of the Center for Molecular Imaging) and Michigan Gastrointestinal Peptide Research Center (P30DK34933 to Vector Core Laboratory).

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