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Efficient internalization into low-passage glioma cell lines using adenoviruses other than type 5: an approach for improvement of gene delivery to brain tumours

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INTRODUCTION

Recently, adenoviruses have been attracting much attention because of their use as gene-delivery vectors for gene therapy. Adenovirus is the second most common virus vector in clinical trials, and most work has been focused on the established adenovirus vectors based on serotype 5 (Ad5), which belongs to species C. Use of this vector has often met with problems caused primarily by two phenomena: the immunological vulnerability of Ad5 due to its high prevalence in all human populations; and difficulties in transducing various types of tissue. Not all kinds of cells are permissive for Ad5 infection.

There are at least 51 different human adenovirus serotypes, and these are grouped into six different species based on their genome size, DNA homology, oncogenicity in newborn hamsters and haemagglutination pattern (Wadell, 1984). Species B is further divided into two clusters, B:1 and B:2, based on their DNA restriction enzyme patterns. Generally speaking, the different adenovirus species have different tropisms. Adenoviruses can infect the respiratory tract (species B:1, C and E), the ocular tract (species D and E), the kidney and urinary tract (species B:2) and the gastrointestinal tract (species A and F) (Wadell, 1984). The icosahedral virus capsid is made up of seven different proteins. More than 75% of the capsid mass consists of the trimeric hexon protein. Proteins VI, VIII and IX are minor polypeptides associated with hexons as cementing proteins. The 12 vertices are each composed of a pentameric penton base, which is held in place by protein IIIa, and a protruding trimeric fibre (van Oostrum & Burnett, 1985).

The initial step in virus infection is attachment of the fibre to a cell receptor. Several adenoviruses, some of non-human origin, bind to a receptor called the coxsackie-adenovirus receptor (CAR) (Bergelson et al., 1997; Roelvink et al., 1998; Soudais et al., 2000). Members of human adenoviruses from species B, and also some members of species D, use receptors other than CAR (Arnberg et al., 2000a, 2000b; Gaggar et al., 2003; Roelvink et al., 1998; Segerman et al., 2003a, b). The second step of infection is internalization, which for some serotypes is mediated through binding of the RGD motif on the penton base to cellular αv integrins. Thus αvβ3 and αvβ5 integrins are involved in the
internalization process, but not the actual attachment of the virus (Wickham et al., 1993). The binding induces actin reorganization and internalization of the virion. Not all adenoviruses have the RGD motif, and these are internalized by an integrin-independent mechanism (Soudais et al., 2000).

Several attempts have been made to retarget the commonly used Ad5 vector by providing the Ad5 capsid with a non-CAR-binding fibre, or introducing a ligand at the fibre knob region (Magnusson et al., 2002; Nakamura et al., 2003; Nicklin et al., 2001; Schoggins et al., 2003). There have also been attempts to circumvent the high prevalence of pre-existing immunity by constructing a chimeric Ad5 vector using the hexon protein from other serotypes (Youil et al., 2002). However, hexons from the species B serotypes could not be rescued into Ad5 virus particles; Ad12 was the only serotype not in species C that could generate chimeric infectious Ad5 particles. Other methods of circumventing the pre-existing immunity to Ad5 have included transient immune suppression at the time of vector delivery (Smith et al., 1996), coating the adenovirus vector with polymers (Fisher et al., 2001), development of non-human adenovirus vectors (Kremer et al., 2000), removal of adenovirus-specific antibodies using plasmapheresis (Rahman et al., 2001) and induction of tolerance to adenovirus capsid proteins (Ilan et al., 1998). These methods have not yet achieved satisfactory improvements in gene therapy.

Almost all adults have antibodies directed against the endemic Ad5, although only about 50–70 % of adults display neutralizing antibodies. Approximately 65 % of these individuals have a cell-mediated immunity against Ad5 (Chirmule et al., 1999; Fox & Hall, 1980). Importantly, administration of high titres of an adenovirus vector also activates the complement cascade, and not only Ad5-neutralizing antibodies, but also non-neutralizing anti-Ad5 antibodies are capable of complement activation (Cichon et al., 2001).

Repetitive administration of the gene-delivery vector will be hampered by the humoral immune response, and a pre-existing immunity may even impair the initial delivery. The humoral response against an adenovirus can be circumvented by the use of a different serotype (Mack et al., 1997). Although the efficiency of gene delivery will probably be increased by using a different vector, the duration of expression may not be increased to an equivalent degree. It has been shown that about one-third of cell-mediated immunity against adenoviruses is targeted against a hexon epitope that is well conserved between different serotypes (Olive et al., 2002). T-cell responses against this epitope will constrain the duration of expression of the transgene. The duration of expression may not be as important for anti-cancer gene therapy as for other purposes, where stable transgene expression is necessary. Long-term expression using adenovirus vectors appears to be possible only when so-called gutless vectors, which completely eliminate leaky expression of virus genes, are applied. Combining the use of gutless vectors and the use of different adenovirus serotypes could allow multiple administrations of the vector and long-term expression of the transgene (Morral et al., 1999).

The commonly used Ad5 vector binds to CAR, which is expressed on many types of cells, but not on all. The level of CAR expression in glioma tumours varies between patients, and in some studies the success of gene delivery using the Ad5p vector has been shown to correlate with CAR expression level, rather than the level of αv integrins (Asaoka et al., 2000; Fuxe et al., 2003; Miller et al., 1998).

In this study we have compared six different adenoviruses, three of which are CAR-binding serotypes and three non-CAR-binding serotypes, to ascertain whether adenovirus serotypes other than the CAR-binding Ad5p could be used to infect tumour cells from glioblastoma multiforme patients. Some members of species B, including Ad11p, Ad16p and Ad21p, have been shown to bind the complement regulatory protein CD46 (Gaggar et al., 2003; Segerman et al., 2003b). At least for Ad11p and Ad35p (also belonging to species B : 2), CD46 is not used exclusively as a receptor (Segerman et al., 2003a). Ad11p has previously been shown to be a very interesting vector candidate based on data on established cell lines from brain tumours (Skog et al., 2002). This extended study evaluates three non-CAR-binding serotypes from species B (Ad11p, Ad16p and Ad21p) and three CAR-binding serotypes from species C, D and E (Ad5p, Ad17p and Ad4p, respectively) regarding their capacity to be expressed in low-passage glioblastoma cell lines. Ad4p was selected because of its efficacy in some established brain tumour cell lines and superior CAR-binding capacity (Skog et al., 2002). Ad5p was chosen as the reference serotype, as most gene-therapy vectors have been based on this serotype. Ad17p was chosen because a recombinant Ad2 with an Ad17 fibre has been shown to infect primary rat brain cells effectively (Chillon et al., 1999). Ad16 and Ad21p were chosen as representatives of species B : 1. The Ad21p fibre knob has a resemblance to a newly developed vector based on Ad35 (Seshidhar Reddy et al., 2003). There was a pronounced discrepancy in the infection pattern of the different Ad serotypes in glioblastoma cells from different donors. The non-CAR-binding serotype 16p was very efficient and less affected than the CAR-binding serotypes by biological diversity among the tumours.

**METHODS**

**Cell lines and culture conditions.** A549 cells (human oat cell lung carcinoma) are permissive for most adenovirus serotypes and were used for propagation of the different adenovirus strains. A549 cells were grown in Dulbecco’s modified Eagle’s medium containing 0.75 % NaHCO3 (w/v), 5 % fetal bovine serum (FBS), 20 mM HEPES (pH 7.4) and penicillin-streptomycin (10 IU ml⁻¹ and 10 μg ml⁻¹, respectively) (PEST) at 37 °C. Primary brain tumours diagnosed as glioblastoma multiforme, oligodendroglioma or giant cell astrocytoma were taken from seven different patients. The brain tumour biopsies were taken from the operation and placed immediately in
cold sterile Iscove’s modified Dulbecco’s medium (IMDM) without serum. Brain tumour biopsies were then transported to the tissue culture facility and cut into small pieces, about 1 mm in diameter. They were then incubated with collagenase at a concentration of 200 U ml\(^{-1}\) (Sigma) and DNase I at a concentration of 100 U ml\(^{-1}\) (Sigma) in IMDM medium without serum at 37 °C for 3 h. The cell suspension was left in a centrifuge tube for 5 min at room temperature to allow sedimentation of large pieces. Floating cells were transferred to a new tube and centrifuged for 8 min at 150 g and washed once in IMDM without serum. The cell pellet was suspended in IMDM supplemented with 15 % FBS and plated in Primaria tissue culture flasks (BD Biosciences Labware) at 10% CO\(_2\) 37 °C in a humidified incubator. Cells were karyotyped at various passage numbers. About 2–4 months after primary culture, all tumour cell cultures showed a dominating malignant karyotype. All tissue cultures were grown without antibiotics for at least 2 weeks and then analysed for mycoplasma. All cultures were mycoplasma-free.

Cells were plated and grown in IMDM with L-glutamine and 25 mM HEPES supplemented with 1 mM sodium pyruvate ( Gibco), 1 x non-essential amino acids ( Gibco), PEST and 15% FBS. The primary culture is a mixture of normal (endothelial) cells and glialoma cells, so cultures were allowed to proliferate until they were composed only of malignant cells (evaluated by karyotyping). Cells were kept below 20 passages after dissection of tumours. Lines 1141, 1151, 1175, 1199 and KNI53 were derived from glioblastoma multiforme, while 1206 and 1212 were from an oligodendroglioma and giant cell astrocytoma, respectively.

Line 1141 was derived from a 74-year-old male with the karyotype 108–121, XX, add(1)(q21), del(1)(q12), add(6) (q15), add(19)(p13), +der(1)(t;10)(q11). Line 1151 was derived from a 69-year-old male with the karyotype 73–81, XXY, +Y, +1, add(1)(p11)×2, +2, +3, –4, +7, +7, +7, +8, +9, add(9)(p11)×2, –10, +11, –14, –15, –17, –18, +19, +19, der(19)(t(17;19)(q12q13))×2, +20, +21, +22, +2mar. Line 1175 was derived from a 67-year-old male with the karyotype 60–61, XXY, +Y, der(1)(add(1)(p36))del(1) (q11q21)×2, –2, –4, +9, –10, –11, –13, –14, –16, –17, –12, –13, –16, –17, add(2)(q13), +mar. Line 1199 was derived from a 50-year-old male with the karyotype 85–92, XXY, –Y, –4, +5, –7, –9, –10, del(12)(q13q21), +5mar. KnI53 was derived from a 65-year-old female with the karyotype 74–91, XX, –X, –X, –1, –3, +7, +7, –8, –8, –9, add(9) (p11–12)×2, –10, –10, der(11)(add(1)(p14–15) del(12)(q13q23))×2, –13, –16, –17, –19, –22, –22. Line 1206 was derived from a 48-year-old male and this oligodendroglioma was never karyotyped. Line 1212 was derived from a 55-year-old female with the karyotype 41–44, XX, del(2)(p22–23), del(4)(p11), del(5)(p11), +7, –10, –11, –13, –16, –17, add(22)(q13), +mar.

**Viruses propagation and purification.** The adenoviruses tested in this study were Ad4p (p = prototype; strain RJ-67), Ad5p (strain Ad75), Ad11p (strain Slobitski), Ad16p (strain Ch79), Ad17p (strain Ch22) and Ad21p (strain 1645). Viruses were propagated in A549 cells, which were then pelleted and resuspended in 20 mM Tris/HCl pH 7-4. Cells were freeze–thawed and the cell lysate purified on a discontinuous CsCl gradient at 25 000 r.p.m. (Beckman SW41 rotor) for 2-5 h (Mei et al., 1998). The virion band was collected and the density measured in a refractometer. Virions were purified from the CsCl by desalting on a NAP-10 column and eluted with PBS. Virion concentration was determined using a spectrophotometer: an OD of 1 unit (OD\(_{300-330}\)) corresponds to 280 μg virions or 10\(^7\) virus particles ml\(^{-1}\). Glycerol was added to a concentration of 10% and aliquots were stored at –80 °C. The different strains were stored for the same duration, to a maximum of 6 months after purification. Purity and protein levels of the virus preparations were assessed by SDS-PAGE analysis. Serotypes were all checked according to their DNA restriction enzyme patterns.

**Flow cytometry analysis of infected cells.** Cells were seeded in a 12-well plate and grown for 24 h. On the day of infection the medium was replaced with 500 μl IMDM containing 2% FBS and either 0-1, 1 or 5 μg virions per cell (corresponding to approx. 357, 3570 or 17 850 particles). After a 1 h adhesion step on a gyrating platform at 37 °C, fresh medium was added and cells were incubated for 48 h. Cells were harvested, washed in PBS and fixed for 15 min using 2% formaldehyde followed by permeabilization and blocking using PBS supplemented with 2% BSA and 0-1% saponin (BS buffer) for 30 min. The hexon produced was detected using MAB8052 (Chemicon) diluted 1:200 (5 μg ml\(^{-1}\)) in BS buffer for 60 min. After a wash, an FITC-conjugated goat anti-mouse Ab (Sigma) diluted in BS buffer was added for 60 min. The cells were then washed twice in PBS and analysed in a FACScan flow cytometer (Becton Dickinson) using the CELQuest software.

**Fluorescent in situ hybridization.** Two cell lines were chosen: 1199, which showed poor expression of hexon from the CAR-binding serotypes; and 1175, which was well infected by Ad5 and the species B adenoviruses. Fifty thousand cells were plated in a well of a microscope slide the day before infection. Virions (10 pg per cell) were added to a volume of 200 μl cold medium. The cells were incubated at 4 °C for 30 min. The medium was replaced by pre-warmed medium and incubated at 37 °C for 0, 30 or 240 min. Cells were washed with PBS and fixed as described previously (Greber et al., 1997). A DIG-labelled adenovirus probe from the respective serotype was constructed using the DIG–nick translation mix (Roche). Complete genomes were digested to 200–550 bp fragments and DIG labelled according to the manufacturer’s protocol. A probe cocktail (10 μl) containing 0-01 μg Ad probe, 6 μg herring sperm DNA, 50% deionized formamide, 2.5% dextran sulfate and 2× SSC was added to each well. The sample was denatured at 80 °C for 6 min and reannealed at 37 °C in a moist chamber. The samples were washed using 2× SSC for 10 min at room temperature, 50% formamide–2× SSC solution at 42 °C for 10 min, 2× SSC at room temperature for 10 min and then 0.1× SSC at 60 °C for 10 min. Endogenous peroxidase activity was quenched with 1% H\(_2\)O\(_2\) for 15 min. The samples were then stained using the TSA fluorescein system (Perkin-Elmer, NEN Life Sciences) according to the manufacturer’s protocol. Slides were washed and counterstained using 0-05% Evans blue solution for 2 min, washed with three rounds of 0.1× SSC and then mounted on a coverslip. Pictures were obtained using a confocal microscope (Leica DM IRBE, TCS SP2 confocal, 63× oil immersion objective) (see Fig. 2).

**Detection of CAR, α/β\(_3\) and α/β\(_5\) levels.** Cells were harvested using PBS/0.05% EDTA. They were washed in medium and then PBS, and blocked using 2% FBS/PBS supplemented with 0-01% NaN\(_3\) (FBS/PBS/NaN\(_3\)). Five hundred thousand cells were stained using 100 μl Ab diluted in FBS/PBS/NaN\(_3\) [anti-CAR Ab Rmcb at 1:200 dilution, anti-α/β\(_3\) Ab MAB1976 (Chemicon) at 1:100 dilution, or anti-α/β\(_5\) Ab MAB1961 (Chemicon) at 1:500 dilution] for 1 h at room temperature. Cells were washed and visualized using an FITC-labelled goat anti-mouse Fab (Sigma) diluted 1:100 in 2% FBS/PBS/NaN\(_3\). Cells were washed and analysed by FACScan flow cytometer as outlined above. The geometric mean level of fluorescence was measured and the value for the positive control cell line A549 was set to 100. The result is presented as the mean ± SEM from at least three independent experiments.

**RESULTS**

We chose to assay some parameters of relevance to adenovirus expression in low-passage brain tumour cells: expression of the CAR receptor and expression of α/β\(_3\) and α/β\(_5\) integrins mediating internalization, at least for the
Seven different low-passage (<20) glioma tumour cell lines were infected with three CAR-binding serotypes [Ad4p (■), Ad5p (▲), Ad17p (◆)] and three non-CAR-binding serotypes [Ad11p (▼), Ad16p (□), Ad21p (△)]. The proportion of infected cells was measured by staining cells for hexon production 48 h p.i., followed by flow cytometry analysis. Three different virus concentrations were used (0-1, 1 and 5 pg virions per cell). The results are presented as the mean±SEM of at least three independent experiments.
well-studied Ad5. Two methods were used to ascertain
two parameters of the adenovirus infection of these cells: in situ hybridization, monitoring the localization and extent of DNA delivery, and FACS analysis of infected cells, monitoring the number of cells expressing adenovirus hexons, the major constituent of the virus capsid, as an indication of efficient intranuclear expression of viral DNA.

**Infectivity assay**

To evaluate the efficiency of each adenovirus serotype to become internalized and start gene expression, the number of cells expressing the virus hexon protein 48 h post-infection (p.i.) was counted. There were significant differences between CAR-binding serotypes in their ability to infect cells (Fig. 1). Of these viruses, Ad5p appeared to be more efficient than both Ad4p and Ad17p. Ad17p showed more variation between cell lines than did Ad5p. The 1199 cell line was relatively refractory to infection by all the CAR-binding adenoviruses.

Overall, the non-CAR-binding serotype Ad16p was the most efficient serotype in this study. It showed the least variation of infection capacity of different cells and also gave the highest overall percentage of infected cells. Interestingly, Ad11p and Ad21p also manifested a high capacity of infection in the different gliomas. The species B serotypes were capable of efficiently infecting the cells that were shown to be more refractory to the CAR-binding serotypes (line 1199).

**In situ hybridization experiment**

To analyse further the efficiency of DNA delivery to the nucleus, we conducted a fluorescent in situ hybridization experiment (Fig. 2). A higher virus concentration was used than in the flow cytometry experiment (10 pg per cell compared with a maximum of 5 pg per cell in flow cytometry), but the results reflected well the results of the flow cytometry experiment. Two different cell lines were chosen based on the flow cytometry data: 1199 cells, which
Fig. 2. In situ hybridization. The glioma cell lines 1175 (a) and 1199 (b), permissive and non-permissive, respectively, for infection by CAR-binding serotypes, were infected with 10 pg virions per cell. Thirty and 240 min p.i., cells were fixed and hybridized using a DIG-labelled adenovirus-genomic probe. The probe was visualized using FITC-Tyramide signal amplification and cells were counterstained using Evans blue. NC, Mock-infected negative controls. Pictures were taken using a Leica DM IRBE, TCS SP2 confocal microscope with a 63× oil-immersion objective.
were relatively inefficiently infected by the CAR-binding serotypes, and 1175 cells, which were refractory to infection by Ad17p and Ad4p, but were reasonably well infected by Ad5p and the species B viruses. A restriction to the infection is passing the nuclear membrane, as a proportion of the particles accumulate on the outside of the nuclear membrane. The majority of particles from species B that crossed the cell membrane had not been able to cross the nuclear membrane by 4 h p.i., although accumulation around the nucleus could be seen. An exception to this observation was the infection of 1175 by Ad11p, where the majority of particles had crossed the nuclear membrane by 4 h p.i. The species B serotypes all manifested the most efficient internalization of virions. The number of particles inside every cell was higher than for the CAR-binding serotypes. It is possible that some of the intranuclear genomes have replicated. As indicated by the flow cytometry assay, the internalization capacity of Ad16p was superior to all other serotypes tested, followed by Ad21p and Ad11p. Ad5 had fewer adenovirus genomes internalized per cell. The transport of Ad5 to the nucleus was quick in the permissive 1175 line, but less efficient in line 1199. The internalization of Ad4p was surprisingly inefficient, especially in cell line 1199.

CAR expression

CAR expression on low-passage brain tumour cells was significantly lower than in the permissive A549 cell line, which was used as a positive control for CAR expression (Fig. 3). A Chinese hamster ovary (CHO) cell line was used as a negative control for human CAR expression. Four of the brain tumour cells had CAR expression corresponding to about one-tenth of that in A549 cells, and three showed a moderate degree of expression which was between 40 and 50 % of the CAR expression level in A549 cells. Interestingly, there was no clear correlation between the level of CAR expression and permissiveness to infection in the glioma cells (Fig. 4).

Integrin expression

The A549 cells contained very small amounts of $a_v b_3$ integrins and showed relatively high expression of $a_v b_5$ integrins (Fig. 5). Both 1151 and 1206 cells manifested very high expression of $a_v b_3$ integrins, about 30 times more than the permissive A549 cell line. All the brain tumour cells contained more $a_v b_3$ integrin and less $a_v b_5$ integrin than the A549 cells. The $a_v b_5$ content in the brain tumour cells was 20–45 % of the level in A549 cells, with the exception of line 1212, which had only about 5 % of the level of A549 cells. A high level of $a_v b_3$ was not associated with a high level of $a_v b_5$. There was no clear correlation between the level of integrin expression and efficiency of infection; the deviation of the slope from zero was not statistically significant for any of the serotypes (Fig. 6).
This study investigates adenovirus serotypes that are potential alternatives to Ad5 as vectors for gene transfer to cells of neural tumour origin. This study accentuates the diversity among gliomas from different patients. It would be of great interest to have alternative vectors to Ad5 to compensate for this variability.

A trait of vectors based on serotype 5 that reduces their effectiveness is the high prevalence of this virus in all populations. The endemic nature of this serotype gives ample opportunity for reinfection and boosting of the antibody titre throughout life, resulting in higher titres than the less prevalent serotypes (Seshidhar Reddy et al., 2003). A study analysing neutralizing antibodies against different adenovirus serotypes showed the species B members to be of low prevalence. Ad11p, Ad16p and Ad21p only had about 2, 12 and 15% seroprevalence, respectively, and the corresponding numbers for Ad4p, Ad5p and Ad17p were around 45, 78 and 32% (Vogels et al., 2003).

Although CAR is conserved in many mammalian species, its normal physiological function remains unknown (Carson, 2001). CAR is a component of the tight junction and is therefore not exposed on the apical side of polarized cells, making the receptor inaccessible to the virus (Cohen et al., 2001). Furthermore, CAR has recently been shown to be a tumour suppressor in malignant glioma cells, both in vitro and in vivo (Kim et al., 2003). Downregulation of CAR is often seen in malignant glioma cells, and the level of CAR mRNA expression in other cancers such as bladder cancer specimens correlates inversely with the aggressiveness of the tumour (Fuxe et al., 2003; Miller et al., 1998; Okegawa et al., 2001). To circumvent this problem, a vector based on a serotype with a more efficient internalization capacity for glioma cells could be used, preferably one that does not use CAR as a primary receptor.

The infection study was made in the ‘classical’ way for adenoviruses. It is our experience that a good yield of Ad2 and Ad5 can be obtained using subconfluent A549 cells on the day of infection. Thus, subconfluent cells were used throughout the study, but it should be kept in mind that the result could be different using cells grown to confluence. The fact that CAR localizes in the tight junctions to neighbouring cells, rendering the receptor inaccessible in confluent cells, is a great disadvantage to vectors that bind CAR. It is likely that the situation in vivo mimics the confluent situation, constraining the virus to use alternative receptors. For Ad2 and Ad5 these could be, for example, the α2 domain of major histocompatibility complex class 1 and heparan sulfate glycosaminoglycans (Dechecchi et al., 2001; Hong et al., 1997).

Fig. 5. Expression level of αvβ3 integrins (a) and αvβ5 integrins (b) on different glioma cells relative to permissive A549 cells. Cells were analysed by flow cytometry. The geometric mean value of the fluorescence was measured and the value for the A549 cells was set to 100. The result is presented as the mean±SEM of at least three independent experiments.

Ad16p was the most effective serotype regarding infection, as evaluated both by the capacity to induce hexon expression and by the low variability between cells from different donors. Ad21p was also very efficient on most of the donor cells. The CAR-binding serotypes performed poorly in 1199 cells, whereas both Ad16p and Ad21p achieved a high level of transduction. The third non-CAR-binding serotype, Ad11p, was more effective than both Ad4p and Ad17p, but was less efficient than expected from earlier studies on established brain tumour cell lines, where Ad11p was superior (Skog et al., 2002). Ad4p and Ad17p were not successful in transducing most of the cells compared with the other viruses. The CAR-interacting domains of the fibres of the CAR-binding serotypes are not completely conserved, which could account for this difference (Skog et al., 2002). Also, the rigidity of the fibre shaft may vary somewhat. It has recently been shown that utilization of CAR requires a flexible shaft, and species D serotypes have a short and relatively rigid shaft (Wu et al., 2003).

In this context, it is fair to mention that the binding characteristics of Ad17p are not well studied and, despite the fact that Ad17p has the CAR-interacting motif in the knob, alternative receptors may be of greater importance than CAR. Also, some of the variation among CAR-binding serotypes may be attributable to differences other than binding characteristics. Post-binding events such as...
translocation to the nucleus and initiation of gene expression involve complex events, and it is possible that some serotypes have optimized their tropism by mechanisms over and above just binding to the cell membrane. Ad5p performed well in most glioblastoma cells (being on a par with Ad11p), although not as well as the best vector candidate, Ad16p.

It has been shown previously that the species B:1 serotypes Ad3p and Ad7p bind to a receptor common to both species B:1 and B:2 and that the species B:2 serotypes Ad11p and Ad35 also recognize an additional receptor not utilized by Ad3p and Ad7p (Segerman et al., 2003a). Recently, it has been shown that Ad11p binds to CD46, and this was postulated to be the species B:2 receptor (Segerman et al., 2003b). CD46 is widely expressed in humans and acts as a protective barrier against inappropriate complement activation. Strong immunostaining against CD46 is present in the blood vessel endothelia and epithelial cells in a wide range of tissues, including lung alveoli, kidney tubules, skin, lymphoid tissues and intestine. Strong CD46 staining can also be found in cerebral endothelial cells throughout the brain and in the ependymal cells lining the ventricles (McQuaid & Cosby, 2002). The neurons and glial cells of the normal brain show comparatively more sporadic and weaker staining (McQuaid & Cosby, 2002). Also, CD46 expression in the brain appears to be restricted mostly to the isoform with cytoplasmic tail 2 (Buchholz et al., 1996). The fact that tumour cells often upregulate CD46 (Fishelson et al., 2003) might favour the use of CD46-binding adenoviruses in cancer therapy.

The classification of species B viruses as B:1 and B:2 is based on genomic differences. It does not necessarily differentiate their receptor usage. Both Ad16p and Ad21p belong to species B:1, but the sequence identity in the fibre knob between Ad16p and the other species B:1 serotypes, Ad3p and Ad7p, is only 61-7 and 46-4 %, respectively, making divergence in the receptor-binding region a distinct possibility. The corresponding figure for Ad21p is even lower. The Ad21p fibre knob is very similar (91-6 %) to the Ad35p fibre knob of species B:2 (Mei, 1996). This is in agreement with published data that Ad11p and Ad35p (species B:2), as well as Ad16p and Ad21p (species B:1), have affinity for CD46 (Gaggar et al., 2003).

Our data agree with earlier studies showing that there is no correlation between the level of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins
and transduction efficiency of Ad5 (or the other tested serotypes) (Fig. 6). There are conflicting results in the literature regarding the level of CAR expression and transduction efficiency (Bruning et al., 2001; Fuxe et al., 2003). The low-passage glioma cells showed no correlation of CAR level and transduction efficiency, suggesting that other features are also involved in this mechanism. The degree of CAR expression in gliomas is variable, but none of these glioma cells was completely negative for CAR. The cell with the lowest level of CAR (1212) still showed reasonably good transduction efficiency. This cell also expresses small quantities of $\alpha_v\beta_3$ integrin, but expresses larger amounts of $\alpha_v\beta_5$ integrin. Both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ promote internalization of adenoviruses from species C, but $\alpha_v\beta_5$ selectively facilitates membrane permeabilization and endosomal rupture to release the particle (Wang et al., 2000). Additional factors are probably involved in the internalization pathway.

Except for the well-studied Ad2 and Ad5, it is not known whether integrins are necessary for internalization. The penton base RGD (Arg–Gly–Asp) motif responsible for the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin interaction is conserved in the species E virus represented by the simian Ad25 virus (GenBank accession no. NP_478405), Ad5 (XZADH5), Ad11p (AAN62510) and Ad17p (CAC82545). The penton base of these four adenoviruses also contains the LDV (Leu–Glu–Val) motif involved in $\alpha_v\beta_1$ integrin binding. The corresponding sequences from Ad16p and Ad21p are not available in the database. However, the RGD motif appears to be conserved in most human adenovirus serotypes, except the enteric adenoviruses Ad40 and Ad41 (Albinsson & Kidd, 1999). This indicates that most serotypes can probably make use of the integrin pathway, but the presence of the integrin-binding motif does not necessarily mean that this represents the only mode of internalization. A chimeric Ad5 with an Ad35 fibre from species B:2 can internalize in an $\alpha_v$ integrin-independent mechanism (Shayakhmetov et al., 2000).

In conclusion, pronounced inter-individual differences occur between the gliomas concerning their susceptibility to transduction by different adenoviruses. However, Ad16p from species B:1 was most efficient and displayed the least variability in transduction efficiency between the different cells. This serotype is a very interesting alternative to Ad5 because of its low seroprevalence and higher capacity to internalize into glioma cells. We could not see any significant correlation between the level of CAR or integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ expression and the level of transduction in these seven low-passage glioma cells. The commonly used Ad5p is expressed relatively well in these cells, although not as efficiently as Ad16p. Nevertheless, Ad5 also has the disadvantage of being endemic in all human populations, with concomitantly high antibody titres that will prematurely terminate Ad5 vector-induced gene expression, even in the immunoprivileged region of the brain (Thomas et al., 2000). According to the in situ hybridization results, Ad16p and Ad21p internalized very efficiently in these cells.

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**REFERENCES**


Adenovirus infection of low-passage brain tumour cells


