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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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There is a need for improvement of the commonly used adenovirus vectors based on serotype 5. This study was performed on three adenovirus serotypes with a CAR-binding motif (Ad4p, Ad5p and Ad17p) and three non-CAR-binding serotypes (Ad11p, Ad16p and Ad21p). The capacity of these alternative adenovirus vector candidates to deliver DNA into low-passage glioma cell lines from seven different donors was evaluated. The non-CAR-binding serotype Ad16p was the most efficient serotype with regard to import of its DNA, as well as initiation of hexon protein expression. Ad16p established hexon expression in 60–80 % of the cell population in gliomas from all donors tested. The other non-CAR-binding serotypes, Ad11p and Ad21p, showed hexon expression in 25–60 and 40–80 % of cells, respectively. The corresponding figure for the best CAR-binding serotype, Ad5p, was only 25–65 %, indicating greater variability between cells from different donors than serotype Ad16p had. The other CAR-binding serotypes, Ad4p and Ad17p, were refractory to some of the gliomas, giving a maximum of only 45 and 40 % hexon expression, respectively, in the most permissive cells. Interestingly, the transduction capacity of the CAR-binding serotypes was not correlated to the level of CAR expression on the cells.

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, b; 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 α_v integrins. Thus $\alpha_v\beta_3$ and $\alpha_v\beta_5$ 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 adeno-virus 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 circum-vented 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 sero-types 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 α_v 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 com-plement 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 exclu-sively 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 representa-tives of species B : 1. The Ad21p fibre knob has a resem-blance 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 % NaCO$_3$ (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 astrocy-toma 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 × non-essential amino acids (Gibco), PEST and 15 % FBS. The primary culture is a mixture of normal (endothelial) cells and glioma 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 KNf35 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) (q?15), add(19)(p13), +?der(?)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)(q21;q13)×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, −22, 1dmin. 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), +3mar. Knf35 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(11)(p14–15) del(11)(q23)×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.

Virus propagation and purification. The adenoviruses tested in this study were Ad4p (p=prototype; strain RI-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$_{260–330}$) corresponds to 280 µg virions or 10^{12} 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 pg 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 CELLQUEST 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, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ 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-$\alpha_v\beta_3$ Ab MAB1976 (Chemicon) at 1:100 dilution, or anti-$\alpha_v\beta_5$ 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 $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins mediating internalization, at least for the

CAR-binding serotypes
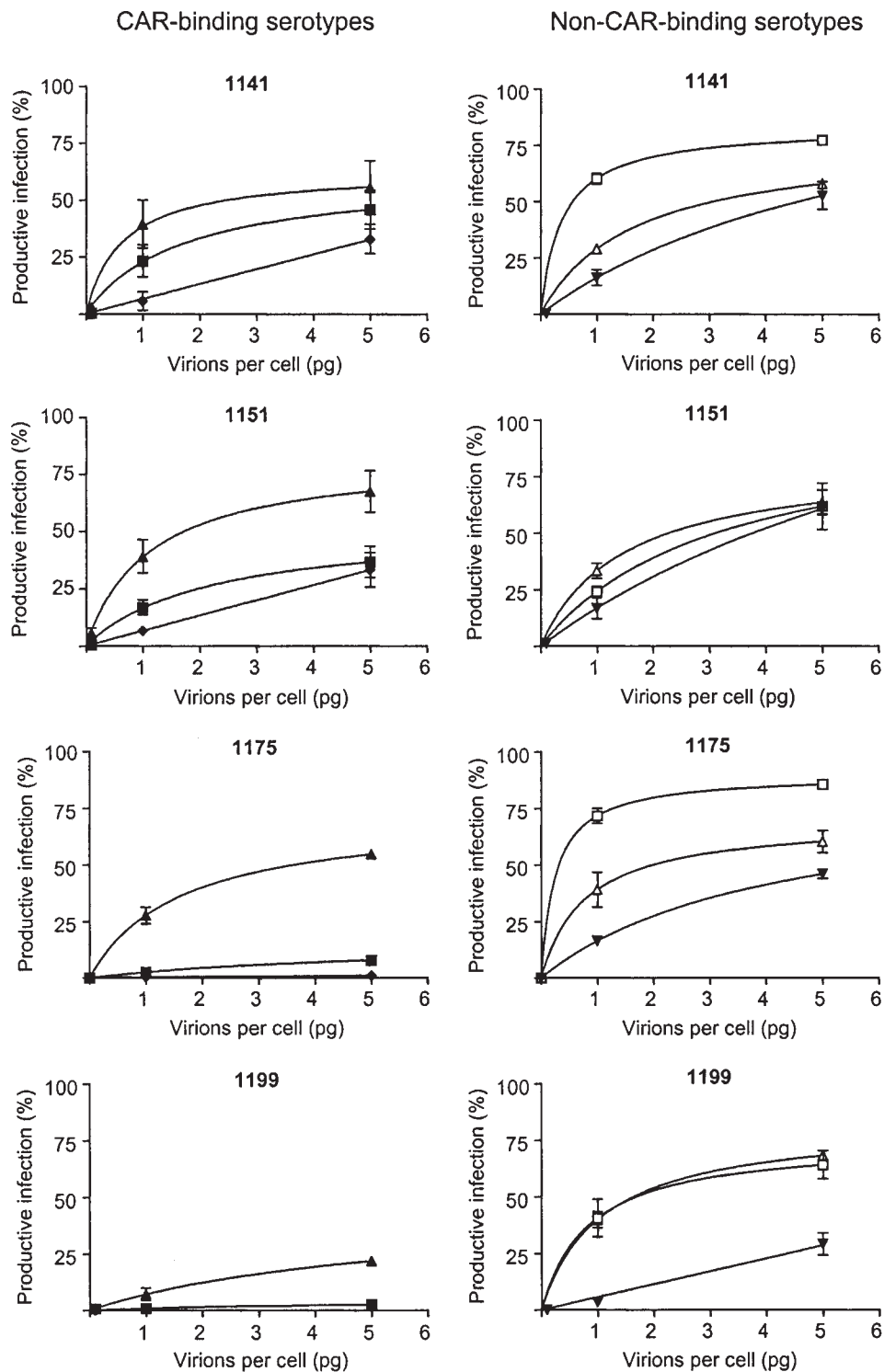
Non-CAR-binding serotypes

Fig. 1. 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
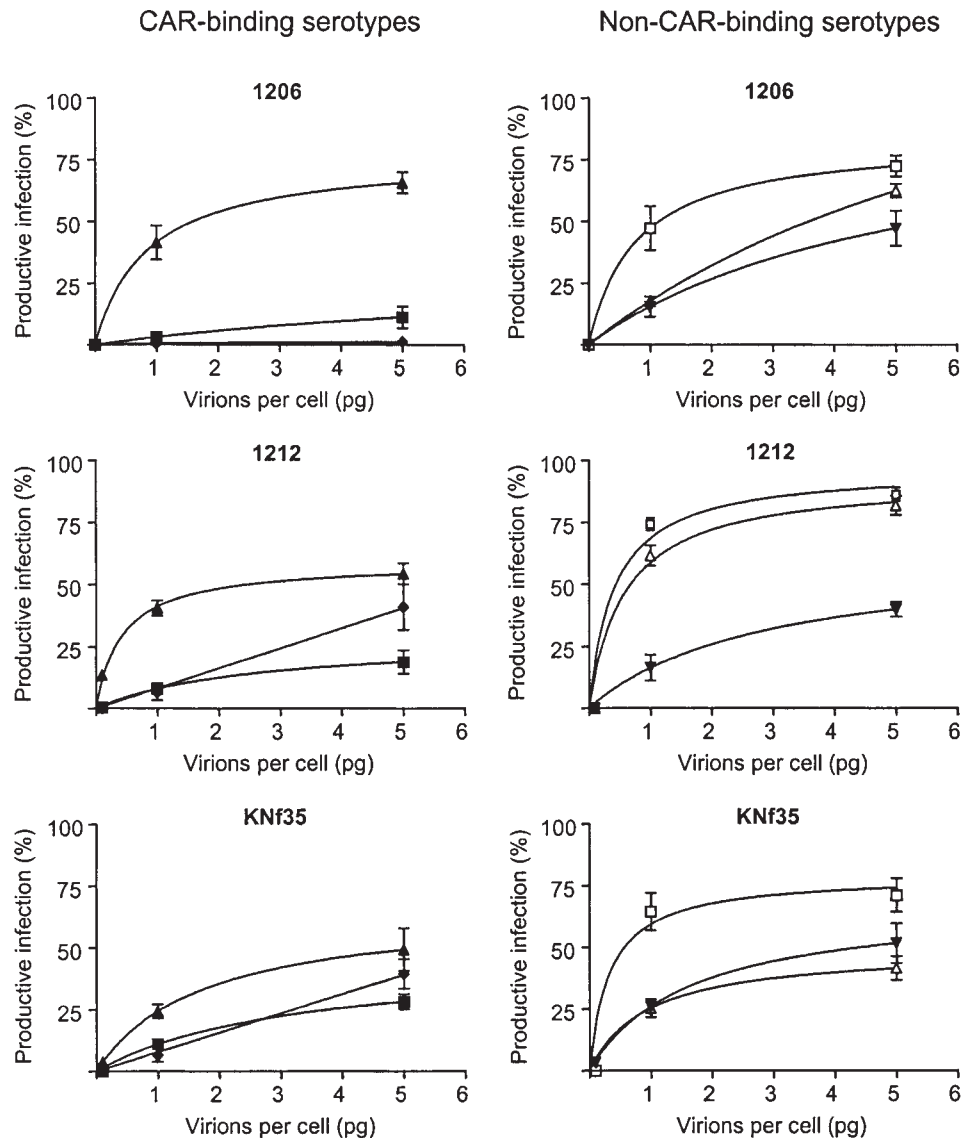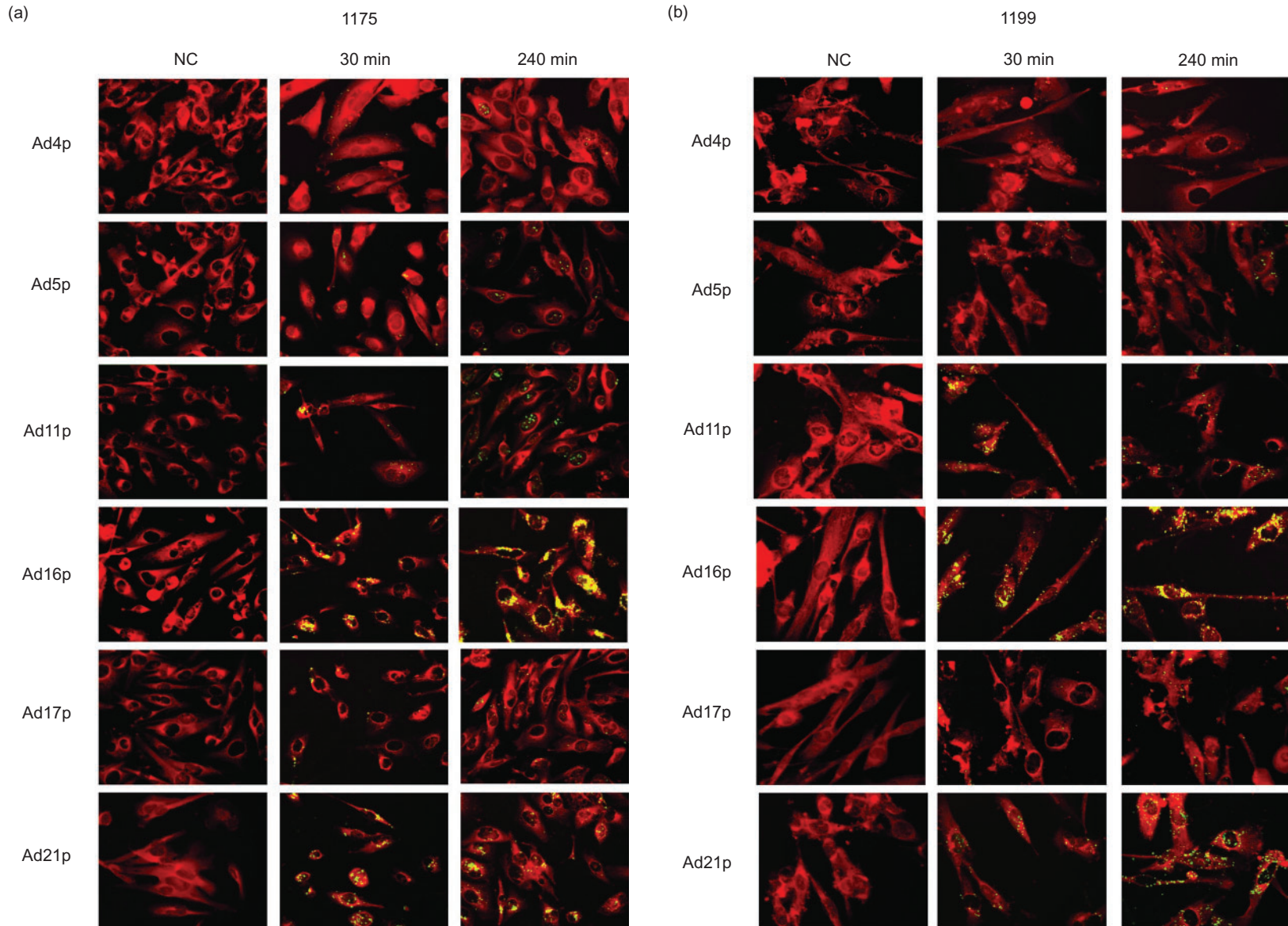
Fig. 2. *In situ* hybridization. The glioma cell lines 1175 (a) and 1199 (b), permissive and non-permissive, respectively, for infection by CAR-binding serotypes, were infected with 10 pg virions per cell. Thirty and 240 min p.i., cells were fixed and hybridized using a DIG-labelled adenovirus-genomic 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,
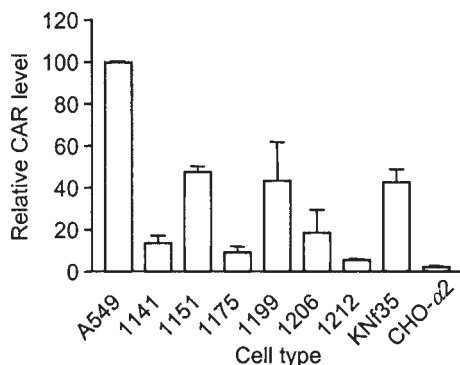
there was no clear correlation between the level of CAR expression and permissiveness to infection in the glioma cells (Fig. 4).

Integrin expression

The A549 cells contained very small amounts of $\alpha_v\beta_3$ integrins and showed relatively high expression of $\alpha_v\beta_5$ integrins (Fig. 5). Both 1151 and 1206 cells manifested very high expression of $\alpha_v\beta_3$ integrins, about 30 times more than the permissive A549 cell line. All the brain tumour cells contained more $\alpha_v\beta_3$ integrin and less $\alpha_v\beta_5$ integrin than the A549 cells. The $\alpha_v\beta_5$ 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 $\alpha_v\beta_3$ was not associated with a high level of $\alpha_v\beta_5$. 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).
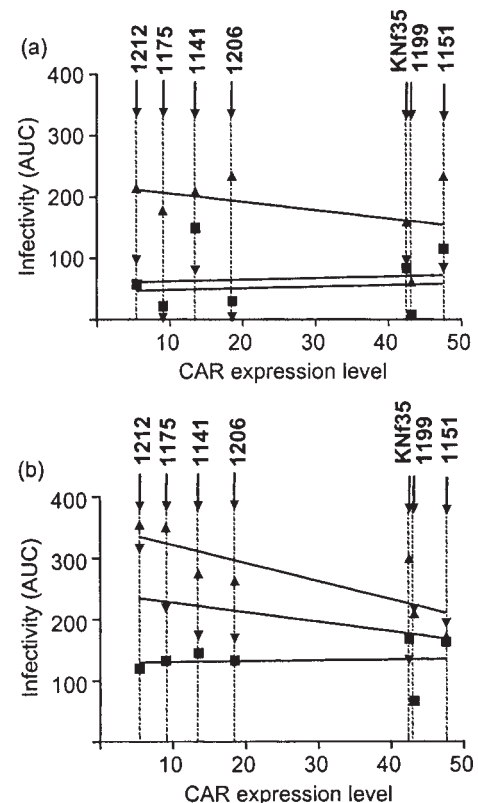
Fig. 4. Correlation between the level of CAR expression and permissiveness for infection. The area under the infection curve (AUC, calculated from Fig. 1) is plotted against the level of CAR expression relative to A549 cells (Fig. 3). Results for both CAR-binding serotypes (a) [Ad4p (■), Ad5p (▲), Ad17p (◆)] and non-CAR-binding serotypes (b) [Ad11p (▼), Ad16p (□), Ad21p (△)] are plotted.

Fig. 3. Level of CAR expression on different glioma cells relative to the positive-control A549 cells. CAR was stained using anti-CAR Ab (Rmcb) and analysed by flow cytometry. The geometric mean value of fluorescence was measured and the value for A549 cells was set to 100. The result is presented as the mean ± SEM of at least three independent experiments.

Fig. 5. Expression level of $\alpha_v\beta_3$ integrins (a) and $\alpha_v\beta_5$ integrins (b) on different glioma cells relative 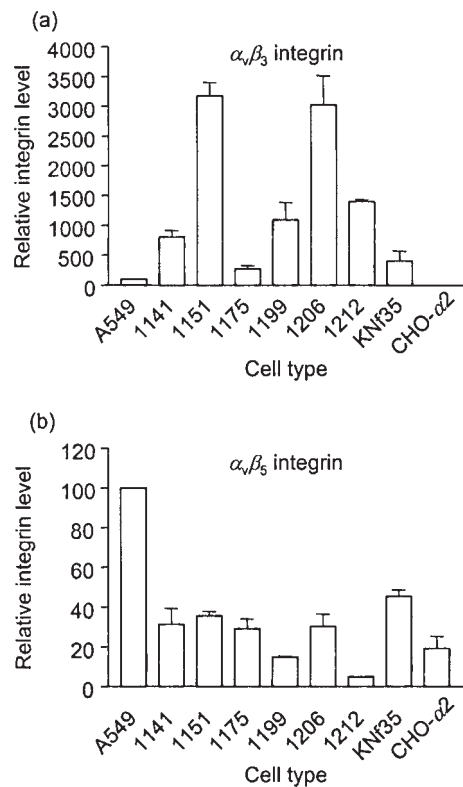to permissive A549 cells. Cells were analysed by flow cytometry. The geometric mean value of the fluorescence was measured and the value for the A549 cells was set to 100. The result is presented as the mean ± SEM of at least three independent experiments.

DISCUSSION

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).

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
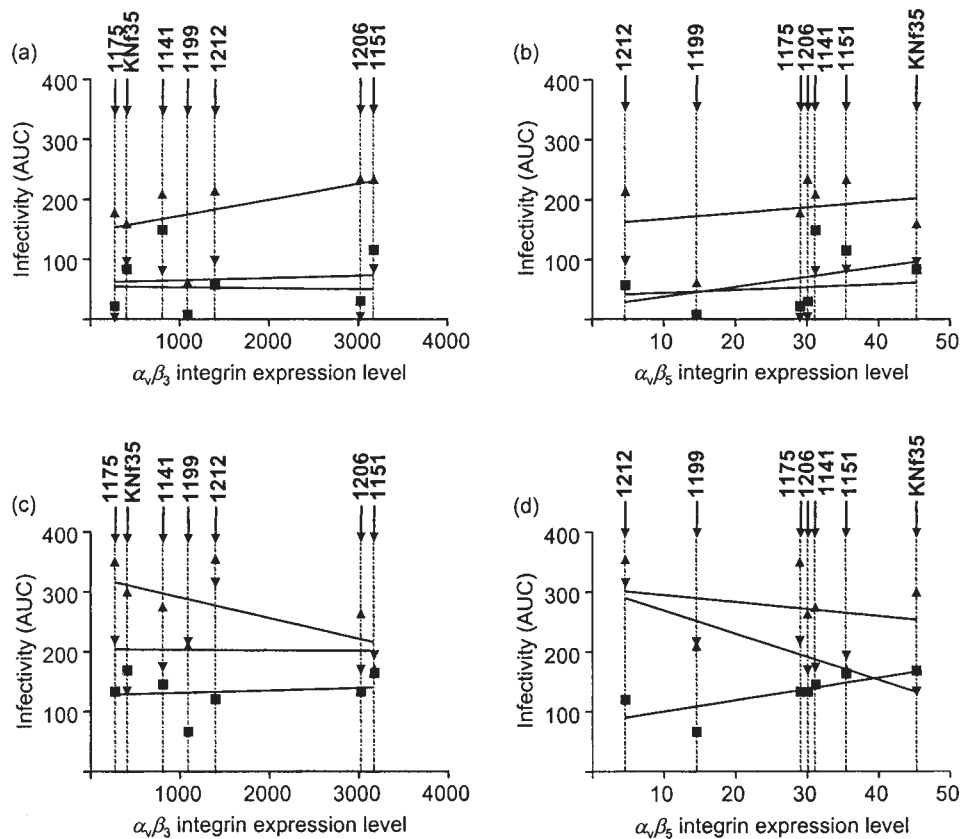
Fig. 6. Correlation between the expression level of $\alpha_v\beta_3$ integrins (a, c) and $\alpha_v\beta_5$ integrins (b, d) and permissiveness of glioma cells for infection by six different adenovirus serotypes [(a, b) CAR-binding serotypes Ad4p (■), Ad5p (▲) and Ad17p (▼); (c, d) non-CAR-binding serotypes Ad11p (■), Ad16p (▲) and Ad21p (▼)]. The area under the curve (AUC, calculated from Fig. 1) is plotted against the level of integrins.

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 $\alpha_v\beta_5$ integrin, but expresses larger amounts of $\alpha_v\beta_3$ 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 $\alpha_4\beta_1$ 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 α_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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