Adeno-associated virus (AAV)-3-based vectors transduce haematopoietic cells not susceptible to transduction with AAV-2-based vectors

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Introduction

Adeno-associated viruses (AAVs), small, non-enveloped, single-stranded DNA viruses of the family Parvoviridae, are classified as dependoviruses because they replicate efficiently only in the presence of a co-infecting helper adeno- or herpesvirus (Berns et al., 1995). AAVs have been isolated from a variety of different species, including primates, dogs, cows, horses, sheep and chickens (Berns et al., 1995), where they appear to be non-pathogenic. To date, five primate AAVs have been described (plus AAV-6, which is probably a variant of AAV-1 and has not been tested with antisera; Rutledge et al., 1998), distinguished serologically by their antigenically distinct capsid proteins. Isolates of AAV-2, AAV-3 and AAV-5 have been obtained directly from human clinical specimens, and man appears to be the natural host. In contrast, neither AAV-1 nor AAV-4 has been isolated from clinical specimens, but serum antibodies to AAV-1 and AAV-4 have been detected in non-human primates (Chiorini et al., 1999), suggesting that they are simian viruses (Parks et al., 1970; Xiao et al., 1999). Until recently, only AAV-2 had been characterized at the genomic level, but AAV-3 (Muramatsu et al., 1996), AAV-4 (Chiorini et al., 1997), AAV-5 (Chiorini et al., 1999) and AAV-6 have now been cloned and sequenced (Rutledge et al., 1998).

Much current interest in AAVs stems from their potential...
use as vectors for gene therapy; virtually all studies have employed recombinant (r) AAV-2. Although AAV-2 undergoes a lytic infection in the presence of helper virus, in the absence of helper virus, wild-type virus can integrate into the host cell genomic DNA. In addition, viral particles are heat stable and resistant to solvents and detergent, withstand a wide range of pH and temperature change and can easily be concentrated (Arella et al., 1990). AAV-2-based vectors have been shown to transduce both dividing and non-dividing cells (Podsakoff et al., 1994) and cells transduced by AAV-2-based vectors can express functional proteins for many months after a single treatment (Monahan et al., 1998). However, while AAV-2 has a broad host range, not all cells can be infected with AAV-2, possibly due to the absence of the AAV-2 cellular receptor (Mizukami et al., 1996; Ponnazhagan et al., 1996). We have shown previously that AAV-2 and AAV-3 do not compete with each other in cell-binding studies (Mizukami et al., 1996) and we hypothesized that they bind to different cellular receptors and should have different cell tropisms. We therefore constructed rAAV-2 and rAAV-3 vectors expressing green fluorescent protein (GFP) and determined their relative ability to transduce hematopoietic cells. In addition, heparan sulphate and virus-overlay binding studies were performed to study further the differences between rAAV-2 and rAAV-3 vectors.

**Methods**

**Cells and viruses.** COS-7 cells, Chinese hamster ovary (CHO)-K1 and heparin-deficient mutant cell lines (pgsB-618, pgdE-606, pgsA-745 and pgsD-677) were obtained from the ATCC (Manassas, VA, USA) and maintained in Dulbecco’s modified minimal essential medium (DMEM, Gibco) or in Ham’s F12 medium (Gibco) with 10% foetal calf serum (FCS, Life Technologies). UT-7/Epo cells (provided by N. Komatsu, Jichi Medical School, Japan) were maintained as described previously (Komatsu et al., 1993). Suspension cultures of HL-60, U937, THP-1, KG1, KG1a, HEL, K562, TF-1, Molt-3, Meg-01, Jurkat, CESS, CEM, JM-1, RS cells and viruses.

**Plasmids.** In order to facilitate subcloning, an EcoRI site was introduced into pAAV3 (the infectious AAV-3 plasmid) by PCR mutagenesis (QuackJen, Stratagene) between the inverted terminal repeat and the Rep coding region (nt 182–187). Similarly, an Xbal site was introduced at the 3’ end of the genome, before the inverted terminal repeat (nt 4512–4517). The complete coding sequence, excluding terminal repeats, was excised with EcoRI and Xbal and subcloned into pBluescript to produce the rAAV3-3 packaging vector (pAAV3-pack).

**Fig. 1.** Schematic representation of the plasmids used to produce rAAV-3. (a) rAAV3-GFP/neo plasmid, containing intact GFP and neomycin resistance (Neo) genes. The GFP gene was inserted between HindIII and Xbal sites. BHG, Bovine growth hormone. (b) AAV-3 packaging plasmid, containing the AAV-3 Rep gene and Cap (VP) genes inserted between the EcoRI and Xbal sites.

A pAAV3-lacZ plasmid was produced by insertion of the human cytomegalovirus (CMV) promoter, a β-galactosidase gene and the simian virus 40 (SV40) poly(A) sequence (nt 2–641 and 833–4506; pCMVbeta, Clontech) into the EcoRI/Xbal site of the mutated pAAV3 vector (Fig. 1a). The pAAV3-GFP-neo plasmid was then constructed by replacing the β-galactosidase gene in pAAV3-lacZ with the GFP gene and the neomycin resistance gene. A 1.8 kb fragment was deleted from pAAV3-lacZ by digestion with EcoRI and EcoRV followed by blunt-ending with Klenow enzyme and self-ligation. The SalI site in the backbone sequence of the plasmid was deleted and a new SalI site was introduced at the ‘5′ region of the SV40 poly(A) sequence by PCR mutagenesis, changing GGCTAC to GTCGA (pAAV3-GFP/Sal). The 736 bp fragment that contains the GFP gene was obtained from pGFP-S65T (Clontech) by digestion with HindIII and Xbal and subcloned into pcDNA3 (Invitrogen). The resulting plasmid (pDNA-GFP) was then digested with MluI and SalI to release a 3.7 kb fragment that contained the GFP gene with the bovine growth hormone poly(A) signal driven by the CMV promoter and the neomycin resistance gene with an SV40 poly(A) signal driven by the SV40 promoter (GFP cassette). This GFP cassette was cloned into previously produced pAAV3-lacZ/Sal so as to replace the β-galactosidase gene.
Table 1. Transduction of haematopoietic cells by rAAV-2–GFP or rAAV-3–GFP

<table>
<thead>
<tr>
<th>Cell line</th>
<th>rAAV-2</th>
<th>rAAV-3</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Day 2</td>
<td>Day 5</td>
</tr>
<tr>
<td>Myeloid</td>
<td></td>
<td></td>
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<tr>
<td>HL-60</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>U937</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>THP-1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>KG1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>KG1α</td>
<td>5.2 ± 1.4</td>
<td>4.1 ± 0.9</td>
</tr>
<tr>
<td>K562</td>
<td>52.0 ± 11.1</td>
<td>48.2 ± 3.4</td>
</tr>
<tr>
<td>Lymphoid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mol-3</td>
<td>12.2 ± 0.5</td>
<td>11.2 ± 0.9</td>
</tr>
<tr>
<td>Jurkat</td>
<td>6.2 ± 2.4</td>
<td>5.0 ± 0.3</td>
</tr>
<tr>
<td>CESS</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>CEM</td>
<td>0.9 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>JM-1</td>
<td>2.2 ± 0.5</td>
<td>1.9 ± 0.9</td>
</tr>
<tr>
<td>RS4:11</td>
<td>3.1 ± 0.6</td>
<td>2.7 ± 0.6</td>
</tr>
<tr>
<td>Raji</td>
<td>4.0 ± 0.7</td>
<td>4.1 ± 0.9</td>
</tr>
<tr>
<td>D1.1</td>
<td>3.6 ± 0.8</td>
<td>3.3 ± 1.2</td>
</tr>
<tr>
<td>Primary lymphocytes</td>
<td>11.0 ± 2.2</td>
<td>NT</td>
</tr>
<tr>
<td>Erythroid/megakaryocytoid</td>
<td></td>
<td></td>
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<tr>
<td>HEL</td>
<td>—</td>
<td>—</td>
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<tr>
<td>TF-1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Meg-01</td>
<td>—</td>
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<tr>
<td>UT-7/Epo</td>
<td>—</td>
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<tr>
<td>Mo-7e</td>
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</tbody>
</table>

To produce the rAAV-2 packaging vector (pAAV-2-pac), an Xhol fragment containing the whole protein coding sequence of AAV-2 was obtained from pSub201 (a gift of J. Samulski, Samulski et al., 1989) and subcloned in the Xhol site of pBluescript. The pAAV-2-GFP-neo plasmid was constructed by using pSub201, with replacement of the coding sequence of AAV-2 by the GFP cassette. For this purpose, an EcoRI fragment was deleted from pAAV3-lacZ by digestion with XbaI and subcloned into pAAV3-lacZ by blunt-ending with Klenow enzyme and self-ligation (pAAV3-lacZ/del). The 3.5 kb fragment containing the GFP cassette was obtained by digestion of pAAV3-GFP-neo plasmid with MluI and BamHI and subcloned into pAAV3-lacZ/del. The resulting plasmid was then digested with EcoRI and BamHI and the 4.2 kb fragment was cloned into pSub201/Eco using EcoRI–BamHI adapters, in order to produce the plasmid pAAV2-GFP-neo.

Production and purification of rAAV-2 and rAAV-3. Vector plasmids (10 µg) containing GFP and the appropriate packaging plasmid (2 µg) were co-transfected into COS-7 cells (10⁵ cells in 400 µl) by electroporation (170 V, 500 F; Gene Pulser, Bio-Rad). The cells were infected with Ad5 (m.o.i. = 5) 24 h later, and the cells were harvested after 40 h. The monolayer was washed and adherent cells were dislodged by scraping, resuspended in PBS and lysed by six cycles of freezing and thawing. After ultrasonication, sodium deoxycholate was added to a final concentration of 0.5% and allowed to incubate for 30 min at 37 °C. CsCl was added to a density of 1.4 g/cm³ and rAAV and adeno-virus were separated by ultracentrifugation at 35000 g for 40 h. The visible rAAV band was removed and a second CsCl purification was performed. To remove the GCl, rAAV was dialysed against PBS for 24 h and the virus was stored at −80 °C until use.

Particle titres were measured for both rAAV-2 and rAAV-3 by DNA dot-blot hybridization. Filters were prepared by applying GFP-containing plasmids, rAAV-2 and rAAV-3 that had been denatured in 10 M NaOH, onto nylon membranes, baking for 2 h in a vacuum oven and hybridizing with a random-primed 32P-labelled probe (GFP segment obtained from double restriction enzyme digestion of the GFP/Neo plasmid with HindIII and Xhol). Genomic titres were calculated by comparison with dilutions of the GFP/Neo plasmid.

All preparations were tested for contamination by wild-type AAV-2 and AAV-3 by PCR amplification (data not shown) and were routinely negative. The biological infectious titre of preparations was calculated by using COS-7 cells: cells were infected with different concentrations of rAAV and the number of GFP-positive cells detected by fluorescence microscopy was measured. The number of cells infected was calculated as one AAV infectious particle per cell.

Virus titration and infection of cell lines. Cells (10⁵) were cultured in medium with 2% FCS to avoid overgrowth. After 6 h culture, the medium was removed from adherent cell lines and the monolayers were overlaid with virus-containing medium (m.o.i. of 5 or 100). For suspension lines, cells were pelleted by centrifugation and resuspended in...
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Fig. 2. COS-7 cells infected with rAAV-2 (a) and rAAV-3 (b) at m.o.i. = 100. All of the cells demonstrated green fluorescence, indicating GFP expression, at day 2. Some of the cells are out of focus. The brighter yellow cells indicate higher expression of GFP.

virus-containing medium. After incubation with virus for 2 h at 4 °C, the cells were transferred to a 37 °C incubator for 15 min, fresh medium (100 µl) was added to the cells and the cells were cultured for 7 days in 96 well culture plates (Costar). The plates were examined for GFP-positive cells by fluorescence microscopy and the number of positive cells was calculated.

- Inhibition of transduction by heparin. To test the ability of heparin to compete with cell binding, COS-7 cells were grown in RPMI 1640 and rAAV-2 and rAAV-3 (m.o.i. = 5) were added directly with different concentrations of heparin (Sigma). After 7 days incubation, the number of GFP-positive cells was estimated and inhibition was calculated.

- Virus-overlay binding assay. Virus-overlay assays with iodinated AAV-2 and AAV-3 were performed as described previously (Mizukami et al., 1996). Briefly, membrane proteins derived from HeLa S3 cells were separated by SDS–PAGE and transferred to nitrocellulose and non-specific binding sites were blocked with 5% BSA in PBS–Tween prior to incubation with 125I-labelled AAV-2 and AAV-3 (10^11 particles/ml). After extensive washing, virus binding was visualized by exposure to a phosphorimager screen (Molecular Dynamics) or X-ray film.

Results

Production of rAAV-3

By using a double plasmid transfection method, we were able to produce both rAAV-2 and rAAV-3 vectors. They had similar transduction efficiencies: 200 gene copies per cell was equivalent to an infectious m.o.i. of 1 on COS-7 cells. When rAAV-3 at an m.o.i. of 5 was used to transduce COS-7 cells, more than 70% of the cells expressed GFP, and 100% of cells expressed GFP after transduction at an m.o.i. of 100 (Fig. 2). To ensure that the ability to detect GFP was not due to direct transfection of GFP-containing plasmid (pseudotransduction), plasmid was mixed with COS-7 cells and the cells were examined for GFP production. No GFP-positive cells were detected with either plasmid preparation (data not shown).

Transduction of myeloid cells

Initial experiments were done with an m.o.i. of 5. rAAV-2 and rAAV-3 had similar abilities to transduce K562 (~ 25% of cells expressed GFP on day 2) and KG1a (~ 3% of cells expressed GFP on day 2; data not shown) cells. However, other myeloid cell lines were negative. The cells were therefore tested at a higher m.o.i. to ensure that virus concentration was not limiting. At an m.o.i. of 100, both vectors transduced the K562 cell line, with 50% of the cells expressing GFP on day 2 (Table 1). Similarly, both vectors transduced the CD34 antigen-positive cell line KG1a, but at lower efficiency (5% of cells positive on day 2). However, neither vector was able to transduce the other cell lines tested, even at an m.o.i. of 100 (Table 1). To ensure that the inability to detect GFP was not due to a non-functional CMV promoter, all cells were also tested by direct transfection of the GFP-containing plasmid and all clearly gave GFP-positive cells (data not shown).

Transduction of lymphoid cells

Lymphoid cell lines with either B or T cell markers were also tested for their ability to be transduced with the AAV vectors. As with the myeloid cell lines, all cells were tested at low and high m.o.i. At an m.o.i. of 100, rAAV-2 was able to transduce all lymphoid cells tested, although with lower efficiency than COS-7 cells; in contrast, the rAAV-3 vector only transduced the Jurkat (a T cell leukaemia line) and Raji (a Burkitt’s lymphoma line) cell lines. Cells from lines Molt-3, CESS, CEM, JM-1, RS4:11 and D1.1 could be transduced with rAAV-2 but not rAAV-3 (Table 1). In addition, primary peripheral lymphocytes stimulated by IL-2 were tested with both rAAV-2 and rAAV-3: GFP-positive cells were detected with both vectors.

Transduction of erythroid/megakaryoblastoid cells

rAAV-2 was unable to transduce two erythroid cell lines, HEL and TF-1, and two megakaryoblastoid cell lines, Meg-01 and UT-7/Epo, even at high m.o.i. (Table 1). In contrast, rAAV-3 transduced HEL, TF-1, Meg-01 and UT-7/Epo cells.
Fig. 3. UT-7/Epo cells infected with rAAV-3. UT-7/Epo cells (10⁴) were seeded in microtitre plate wells and infected with rAAV-3 (m.o.i. = 100). Cells were examined on day 2 by fluorescence microscopy. Two GFP-positive cells are shown in a background of 400 cells.

even at an m.o.i. of 5 (Fig. 3). Neither vector transduced Mo-7e cells at either virus concentration.

**Role of heparan sulphate**

Heparan sulphate has been reported to act as a receptor for AAV-2, and we examined whether the difference in transduction efficiencies was due to different binding to glycosaminoglycans. At an m.o.i. of 5, rAAV-2 was able to transduce all the mutant CHO-K1 cells lines tested, including cell lines that had no glycosaminoglycan on the cell surface (pgsA-745 and pgsD-677; Table 2). In contrast, rAAV-3 was unable to transduce pgsA-745 cells, which have a defect in xylosyltransferase and do not produce glycosaminoglycan or heparin sulphate (Esko et al., 1985, 1988), or pgsE-606 cells, which are heparan sulphate sulphotransferase deficient and produce an undersulphated form of heparan sulphate (Bame & Esko, 1989) (Table 2).

As reported previously, the addition of heparin to cell cultures inhibited rAAV-2 transduction markedly. A similar inhibition was seen with rAAV-3, but the dose response was different, with a much steeper inhibition curve with rAAV-2 (Fig. 4). The ID₅₀ for heparin was calculated to be 1 ng/ml for rAAV-2, but 10-fold higher for rAAV-3 (Fig. 4).

**AAV-2- and AAV-3-binding proteins**

Virus-overlay binding assays with AAV-2 demonstrated binding to a 150 kDa membrane protein, as reported previously (Mizukami et al., 1996). However, when AAV-3 was used, there was no binding to the 150 kDa protein, but instead...
there was binding to a protein band of ~42 kDa (Fig. 5). Similar bands were detected in other permissive cell lines (KB and 293 cells; data not shown).

Discussion

Haematopoietic cells are important potential targets for gene therapy because of their ready accessibility and the potential to treat congenital disorders, such as haemoglobinopathies, immunodeficiencies and metabolic storage disorders, by correction of stem and/or progenitor cells and to modify the immune response to infections and cancer through genetic alteration of lymphocyte function. For these reasons, haematopoietic cells are to date the most popular target cells in human gene therapy because of their ready accessibility and the potential to treat congenital disorders, such as haemoglobinopathies, immunodeficiencies and metabolic storage disorders, by correction of stem and/or progenitor cells and to modify the immune response to infections and cancer through genetic alteration of lymphocyte function. 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In both studies, cell lines showed a decreased efficiency of transduction in the order K562 > MOLT-3 > Raji, with U-937 cells appearing non-permissive. Whether HL-60 cells are permissive for AAV-2 but below the sensitivity of our assay is unclear, but even after positive selection with G418, as performed by Itou et al. (1998), less than one cell per well appeared to be neomycin resistant (consistent with background). However, in contrast to Itou et al. (1998), we could not transduce Meg-01 cells with rAAV-2 vectors under our conditions, possibly reflecting either the use of different clones of Meg-01 or the variant culture conditions used to grow the cells. Both passage number and culture conditions can be critical for the expression of some cell surface antigens (Veomett et al., 1989). For example, the cell line UT-7/Epo is permissive for parvovirus B19 in our laboratory (Shimomura et al., 1993) and yet, in a different laboratory, the same cell line has been reported to be non-permissive (Leruez et al., 1994).

The inability to transduce all haematopoietic cells with rAAV-2 led to our investigation of other potential AAV-based vectors (Muramatsu et al., 1996). We previously determined the complete nucleotide sequence of AAV-3 and showed not only that AAV-3 was serologically distinct from AAV-2 but that it also differed in transcription control elements, including the lack of a typical promoter sequence at position p40 and the presence of the consensus sequence for adenovirus-related transcription factor E4F binding within the upstream region of the p5 promoter (Muramatsu et al., 1996). In addition, we have shown previously that binding of AAV-3 to cells was not competed for by excess AAV-2 virions, indicating that the two viruses use different antigens as their host cell receptor (Mizukami et al., 1996). In the present study, using GFP expression, we confirm that both rAAV-2 and rAAV-3 transduce haematopoietic cells but that there is a difference between the vectors, with rAAV-2 transducing lymphoid cells preferentially and rAAV-3 transducing megakaryoblastoid cells preferentially. In addition, we show that there is a difference in the binding of the two viruses to both heparin and related molecules and to membrane proteins in the virus-binding overlay assay. The nature of the AAV-3-binding 42 kDa protein is currently unknown, but it is not fibroblast growth factor receptor or integrin β5, both described as putative co-receptors for AAV-2 (Qing et al., 1999; Summerford et al., 1999).

There is currently much interest in trying to transduce stem cells, including haematopoietic stem cells or CD34 cells, with gene therapy vectors. However, we were unable to transduce several myeloid cell lines, including primary CD34 cells, with either vector. Although some have reported that rAAV-2 can transduce CD34 cells (Fisher-Adams et al., 1996; Zhou et al., 1994; Goodman et al., 1994; Chatterjee et al., 1999), we and others have subsequently not been able to confirm these results (Alexander et al., 1997) and transduction of CD34 cells may be particularly inefficient, requiring high titres of virus. However, although further studies are required, our results suggest that

![Fig. 5. Virus-overlay binding assays of HeLa S3 membrane protein fraction with iodinated AAV virions. Membrane proteins (20 µg) were electrophoresed on an 8% SDS–PAGE gel, transferred to a nitrocellulose membrane and then reacted with 10¹¹ particles/ml ¹²⁵I-labelled AAV-2 and AAV-3 for 1 h. The signals were visualized by exposure to a phosphorimager screen (Molecular Dynamics).](image-url)
rAAV-3 is unlikely to offer any benefit over rAAV-2 vectors as a vehicle for the delivery of genetic material to human CD34 cells.

In a report concerning a different isolate of AAV-3, non-haematopoietic adherent cell lines were tested with rAAV-3 carrying an alkaline phosphatase marker (Rutledge et al., 1998). No marked difference (≤1 log) in transduction efficiency was noted between rAAV-2 and rAAV-3, although it was shown that the rAAV-3 vector could be used in the presence of anti-AAV-2 sera, suggesting that AAV-3 may have a role as a vector for readministration of genes in the presence of antibody to AAV-2. This is significant, as a recent study reported that 80% of healthy blood donors had anti-AAV-2 antibody and that 70% of them had the neutralizing antibody to AAV-2 (Erles et al., 1999). Neutralizing antibody efficiently reduces the transduction rate of AAV-2 (Erles et al., 1999), and one potential use of rAAV-3 vectors may be to evade this neutralization effect. In addition, our results, especially the finding that rAAV-3 vectors transduce haematopoietic cells that are resistant to standard rAAV-2-based vectors, including myeloid and megakaryocytic cells, potentially broaden the utility of the rAAV vectors.

References


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