Gene Therapy (2014), 1–6

www.nature.com/gt

SHORT COMMUNICATION

AAVrh.10 immunogenicity in mice and humans. Relevance of antibody cross-reactivity in human gene therapy

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Simian adeno-associated virus (AAV) serotype rh.10 is a promising gene therapy tool, achieving safe, sustained transgene expression in the nervous system, lung, liver and heart in animal models. To date, preexisting immunity in humans has not been confirmed, though exposure is unexpected. We compared the humoral immune response with serotypes AAVrh.10 and AAV9 in mice, and AAVrh.10, AAV9 and AAV2 in 100 healthy humans. Mice, injected-intravenously, raised significantly more anti-AAV9 than anti-AAVrh.10 IgG (immunoglobulins), and sera demonstrated greater neutralizing capacity, correspondingly. Antibody cross-binding studies in mice showed negligible cross-recognition between AAVrh.10, AAV9 and AAV2. In humans, IgG prevalence against the most common human serotype, AAV2, was 72%; AAV9, 47% and AAVrh.10, a surprising, 59%. Yet, neutralizing-antibody seroprevalences were 71% for AAV2, 18% for AAV9 and 21% for AAVrh.10. Thus, most anti-AAV9 and anti-AAVrh.10 IgG were nonneutralizing. Indeed, sera generally neutralized AAV2 more strongly than AAVrh.10. Further, all samples neutralizing AAVrh.10 or AAV9 also neutralized AAV2, suggesting antibody cross-recognition. This contrasts with the results in mice, and highlights the complexity of tailoring gene therapy to minimize the immune response in humans, when multiple-mixed infections during a lifetime evoke a broad repertoire of preexisting antibodies capable of cross reacting with non-human serotypes.

Gene Therapy advance online publication, 20 November 2014; doi:10.1038/gt.2014.103

INTRODUCTION

Adeno-associated virus (AAV) vectors do not provoke a strong innate immune response as dendritic cells are poorly transduced. Consequently, markers of innate immunity tend not to be upregulated, though evidence is growing, concerning their relevance to the outcome of AAV-mediated gene transfer.[1] More overtly problematic is the challenge posed by preexisting anti-AAV antibodies present in patients' serum before therapy. These arise with prior exposure, given AAVs are widespread in humans (reviewed by Calcedo and Wilson[2]). In addition, generation of antibodies and T-cell responses against the transgene product, particularly a foreign protein, may depend on the AAV serotype and its capacity to infect antigen-presenting cells.[3–5] Thus, selecting the appropriate viral capsid is critical when planning a therapeutic approach using AAV vectors, since it determines tropism[6] and has major implications regarding the host immune response.

We focus on AAVrh.10, a rhesus macaque serotype,[7] hypothesizing that humans are less likely to be exposed, hence preexisting antibodies should be minimal. Recombinant AAVrh.10 shows promise for gene therapy. It stably-transduces neurons and to a lesser extent oligodendrocytes, after intracranial or intrathecal administration, demonstrating capacity to revert different central- and peripheral-nervous system pathologies in mouse models such as late-infantile neuronal ceroid lipofuscinosis,[8] metachromatic leukodystrophy,[9] diabetic neuropathy,[10] and amyotrophic lateral sclerosis[11] among other diseases. Recently rAAVrh.10 was approved for two clinical trials to treat late-infantile neuronal lipofuscinosis (NCT01161576) and Sanfilippo Type A syndrome.[12] Furthermore, intravenous administration of AAVrh.10 efficiently transduces liver, heart and dorsal root ganglia and reverses cardiomyopathy in a mouse model of Friedreich's ataxia.[13]

Several studies report the prevalence of neutralizing antibodies (NAbs) against AAV serotypes 1, 2, 5, 6, 7, 8, 9 and the capsid hybrid rh32.33.[14,15] However, no data on preexisting antibodies to AAVrh.10 in the general human population, nor on the immunogenicity of AAVrh.10 among serotypes have been reported. Here we show the humoral immune response to AAVrh.10 is significantly weaker than AAV9 in mice. Yet, in humans, immunoglobulin G (IgG) prevalence against AAVrh.10 is greater than against AAV9. However, these immunoglobulins are a mixed population of NAbs and non-NAbs. Further characterization suggests cross-reactivity with abundant-preexisting antibodies raised against AAV2.

RESULTS AND DISCUSSION

We previously demonstrated serotype-dependent differences in the titers of NAbs generated after intrasciatic administration in mice. Three weeks post injection of AAV1, 2 or 8, AAV8-transduced animals had the lowest titers.[16] In addition, using AAVrh.10, we have observed less NAbs were raised after intrathecal injection to mice compared with AAV serotypes 1, 8 and 9 (unpublished data). Here, we compared the immunogenicity of AAVrh.10 and AAV9 in mice first by quantifying the total anti-AAV IgG raised 3 weeks after intravenous injection of either virus, and then testing the sera's capacity to neutralize the virus using a luciferase reporter. We chose AAV9 for comparison as it crosses the blood-brain barrier and is therefore a popular candidate for efficient central

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Received 5 June 2014; revised 7 October 2014; accepted 10 October 2014

nervous system gene delivery to treat neurological diseases with somatic involvement, such as lysosomal storage diseases.[17,18]

Figure 1a, shows that all the animals injected with AAV9 ($n = 9$) raised more anti-AAV IgG than the animals injected with AAVrh.10 ($n = 8$). Mean total IgG of each group (AAV9 = 2659 ± 326 μg ml^{-1} and AAVrh.10 = 692 ± 97 μg ml^{-1}) was significantly different ($P < 0.05$). Note, in normal-mouse sera the typical IgG concentration range is 0.7–5 mg ml^{-1}.[19] Thus, our quantification of the anti-AAV IgG subpopulation is physiologically compatible. Figure 1b demonstrates that sera with antibodies raised against AAV9 are more neutralizing than with antibodies against AAVrh.10. The percentage of infection-inhibition dropped significantly for AAVrh.10 over a series of serum dilutions, whereas for AAV9 it remained nearly 100% throughout. The mean percentage of infection-inhibition, comparing AAVrh.10 and AAV9, at 1:200 serum dilution is seen in Figure 1c which may be directly compared with anti-AAV IgG (Figure 1a). Pearson-correlation analysis showed a significant positive-correlation between IgG and NAbs (0.776, $P = 0.0002$, $n = 17$). Supplementary Figure 1 shows similar correlation with green fluorescent protein as a reporter gene, using sera from mice injected with various AAV serotypes, (0.852, $P = 0.0004$, $n = 12$).

When choosing a vector for gene therapy, immunogenicity (here referring to the capacity to raise NAbs) is a relevant consideration. The presence of NAbs does not necessarily preclude-successful transduction, provided that a critical threshold is not reached or depending on the tissue transduced or the administration route. In this vein, persistent expression of the transgene in both mice and non-human primates was achieved after administering AAVrh.10 interpleurally despite sustained presence of NAbs in the sera.[20] This was also observed for AAV9 delivered intrathecally to non-human primates,[21] although these results are controversial;[22] as NAbs in the sera prevented transduction after intravenous administration.[21,22] Our results indicate, regarding the raising of NAbs in mice, AAVrh.10 is a more favorable choice of vector than AAV9, evoking fewer antibodies in the context studied.

We also performed antibody cross-binding studies in mice *via* indirect enzyme linked immunosorbant assay, using sera with IgG raised against either AAVrh.10, 9 or 2, to see if these antibodies would bind to the viral capsid of other serotypes. Table 1 shows that antibody binding to other capsids was negligible, results resembling naive control levels in all cases, indicating the absence of significant cross-reactivity of IgG against these three AAVs in mice. This is not strain specific, as we obtained similar results with C57bl/6 mice with AAV2 (data not shown). Lack of serological cross-reactivity across certain serotypes in serum raised against AAVs in animal models has been previously reported.[8,23] Nevertheless, there is relatively high homology between the VP1-capsid protein among AAVs and recent reports show capsid antibodies to different AAV serotypes bind common regions.[24,25] Indeed to reduce such problems, directed evolution[26] or other

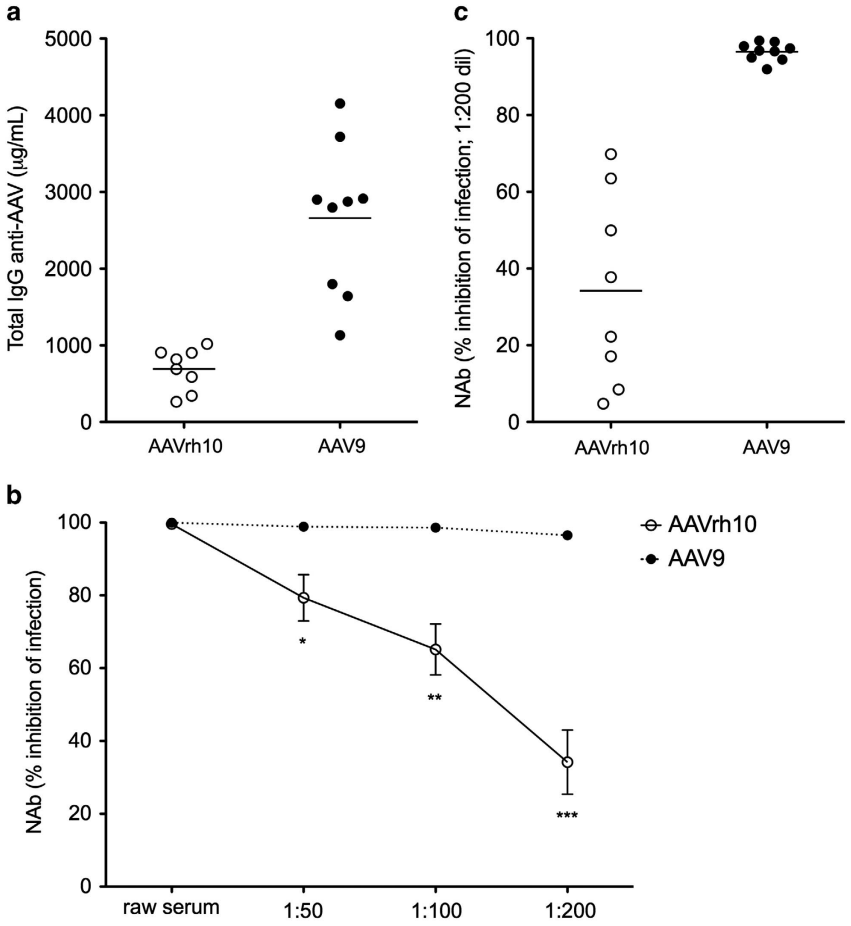

Figure 1. AAV9 is more immunogenic than AAVrh.10 in mice. Sera from 6–8 week-old mice, 21 days post-intravenous injection of 1x10^{11} vg ml^{-1} of either AAV9 ($n = 9$) or AAVrh.10 ($n = 8$) in a total volume of 150 μl, comparing (**a**) total anti-AAV IgG μg ml^{-1} determined by indirect enzyme linked immunosorbant assay, '—' denotes the mean; (**b**) Neutralizing antibodies (NAbs) expressed as percentage of inhibition of adeno-associated virus (AAV) infection of HEK293 cells by the sera over a dilution range from 0 to 1:200, determined using luciferase reporter. Two-tailed *t*-tests: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; and (**c**) scatter plot of NAb data at 1:200 dilution from (**b**), '—' denotes the mean. All samples in A, B and C were tested in duplicates.

Table 1. Anti-AAV IgG to AAVrh.10, 9 and 2 do not cross-react in mice

Sera from mice injected with AAV	*Mean OD*	*% Binding to capsid*	*Anti-AAV IgG (μg ml^{-1})*	*Mean OD*	*% Binding to capsid*	*Anti-AAV IgG (μg ml^{-1})*	*Mean OD*	*% Binding to capsid*	*Anti-AAV IgG (μg ml^{-1})*
AAVrh10	*Anti-AAVrh.10*			*Anti-AAV9*			*Anti-AAV2*		
Animal 1	0.338 ± 0.017	100	1444 ± 54	0.004 ± 0.001	1.2	91 ± 8	0.014 ± 0.003	4.1	203 ± 22
Animal 2	0.328 ± 0.020	100	1412 ± 63	0.021 ± 0.004	6.4	262 ± 28	0.006 ± 0.004	1.8	112 ± 49
Animal 3	0.394 ± 0.023	100	1624 ± 75	0.007 ± 0.000	1.8	139 ± 0	0.002 ± 0.003	0.5	55 ± 44
Animal 4	0.347 ± 0.018	100	1471 ± 56	0.005 ± 0.003	1.4	101 ± 38	0.004 ± 0.003	1.2	89 ± 45
AAV9	*Anti-AAVrh.10*			*Anti-AAV9*			*Anti-AAV2*		
Animal 1	0.007 ± 0.003	1.4	135 ± 36	0.508 ± 0.013	100	2005 ± 48	0.005 ± 0.006	1.0	91 ± 80
Animal 2	0.009 ± 0.001	1.1	161 ± 11	0.855 ± 0.013	100	2588 ± n/a	0.005 ± 0.003	0.6	107 ± 44
Animal 3	0.008 ± 0.000	1.5	150 ± 0	0.528 ± 0.018	100	2076 ± 63	0.003 ± 0.001	0.6	81 ± 18
Animal 4	0.006 ± 0.001	1.1	120 ± 7	0.526 ± 0.001	100	2068 ± 2	0.004 ± 0.001	0.8	91 ± 8
AAV2	*Anti-AAVrh.10*			*Anti-AAV9*			*Anti-AAV2*		
Animal 1	0.003 ± 0.001	0.5	73 ± 10	0.002 ± 0.001	0.3	47 ± 36	0.644 ± 0.016	100	2523 ± 65
Animal 2	0.005 ± 0.001	1.0	106 ± 7	0.001 ± 0.001	0.2	24 ± 13	0.519 ± 0.006	100	2043 ± 20
Animal 3	0.003 ± 0.001	1.0	68 ± 31	0.001 ± 0.001	0.3	12 ± 0	0.309 ± 0.001	100	1351 ± 2
Animal 4	0.008 ± 0.003	1.5	147 ± 34	0.004 ± 0.000	0.7	99 ± 0	0.548 ± 0.001	100	2148 ± 2
Naive control	0.014 ± 0.006	4.0	199 ± 49	0.010 ± 0.006	1.7	162 ± 63	0.005 ± 0.003	1.0	101 ± 38

Abbreviations: AAV, adeno-associated virus; IgG, immunoglobulin G; OD, optical density; n/a = not available: one duplicate out of standard curve range. Percentage binding to capsid normalized from OD. Percentage binding for control OD calculated using pooled mean OD of four animals at 100% binding for each viral capsid. Results are means of duplicates ± s.e.m.

capsid-engineering methods[27] are being explored to design AAV-gene therapy vectors with greater resistance to NAbs.

The human scenario concerning preexisting antibodies to AAVs is far more complex, as exposure to wild types, particularly AAV2, is common. At 3 years of age, over 20% of children already have NAbs against AAV2.[28] However, we hypothesized humans would not have been exposed to a simian serotype, and would therefore not harbor NAbs against AAVrh.10. With AAVrh.10 and AAV9, we included AAV2 in the study as the most common human serotype, and to compare with other published results. In our sample of 100 healthy human adults from Catalonia, Spain, the seroprevalence of anti-AAV IgG was 72% for anti-AAV2, 47% for anti-AAV9 and a surprising 59% for anti-AAVrh.10, whereas 28% of donors were negative for IgG against any of the viruses (Figure 2a). The results for AAV2 and 9 are highly consistent with the seroprevalences in France,[14] 72% and 47%, respectively. Concerning NAbs, 71% of our serum samples neutralized AAV2, 18% AAV9 and 21% AAVrh.10 at the lowest serum dilution used (1:20; Figure 2b). Hence, many serum samples with anti-AAV9 and anti-AAVrh.10 IgG were nonneutralizing, particularly those low for anti-AAVrh.10 IgG. However, all these sera neutralized AAV2. Other authors also reported a drop between the percentage of donors with anti-AAV IgG and those with neutralizing capacity for the same virus; particularly evident for AAV5, AAV8 and, to a lesser extent, AAV9.[14] We found no effect of sex (Mann–Whitney tests) or age (Spearman correlation) in the results for IgG or NAbs for any of the viruses.

A feasible explanation for our results is that we are witnessing cross-reactivity between serotypes.[14,28] It is noteworthy that the 21 sera which neutralized AAVrh.10 had significantly higher IgG against AAV2 than against AAVrh.10 (paired *t*-test, $P < 0.001$) and consequently, all neutralized AAV2 more strongly than AAVrh.10 (Table 2). In addition, sera neutralizing AAV9 had significantly more IgG against AAV2 than AAV9 (Table 2; paired *t*-test, $P < 0.05$). Note, not all AAVrh.10-positive sera neutralized AAV9 or *vice-versa* and a paired *t*-test on those sera which neutralized both viruses was statistically nonsignificant for both IgG and NAb, though a few sera were very high for anti-AAV9 IgG. To characterize this further, we undertook profiling studies comparing neutralizing capacity for the three viruses over several serum dilutions. Figure 2c shows, in all cases analyzed, AAVrh.10 was never the virus that was most strongly inhibited by NAbs.

These results suggest antibody cross-recognition particularly from antibodies raised against AAV2. Indeed, almost all sera containing anti-AAV2 IgG (98.6%) neutralized this virus, indicating that these are highly specific antibodies, while only 38.3 and 35.6% of the sera containing anti-AAV9 and anti-AAVrh.10 IgG, respectively, were neutralizing (comparing Figures 2a and b). This suggests that the latter are not specific antibodies and could have been raised against AAV2 but recognize epitopes present in the AAVrh.10 capsid *via* homology with other serotypes. However, we cannot discard that our *in vitro* assay has limited sensitivity as previously described[29] and optimized *in vivo* assays are needed to confirm the results.

Along these lines, we highlight recent results from epitope mapping of naturally occurring antibodies to AAV2, 5, 8 and 9 in sheep, showing that animals harbor antibodies to both unique and common capsid epitopes.[25] Interestingly, since the antibodies detected recognized surface and internal or buried-capsid peptides, the authors premise that immunity is raised to intact capsids, as well as, to capsid epitopes revealed after proteolysis. This multiplies the potential repertoire of preexisting antibodies in species where natural infection occurs.

Our results stress several key considerations for AAV vector choice in gene therapy. First, serotypes differ in their propensity to raise antibodies, exemplified by AAVrh.10 being less immunogenic than AAV9 in mice. Second, the vector immune response in mice may not predict the response in humans as seen in our cross-binding studies. Finally, the serological response to an AAV is of much greater breadth in humans, beyond the immune response raised by a single vector administration. This is probably because of multiple, mixed exposure to AAVs during a lifetime, as well as, concomitant molecular evolution of the virus.[15] This combination of factors stimulates a broad repertoire of preexisting antibodies, both neutralizing and nonneutralizing, with differing affinity to capsids of other serotypes. Thus, despite promising results so far, the simian origin of AAVrh.10 does not guarantee safe passage from preexisting antibodies for use in human gene therapy, as there may be cross-reactivity with anti-human AAV antibodies. This is particularly evident for human sero-positivity to AAV2, which might be used as a flag for potential reactivity to other serotypes.

MATERIALS AND METHODS

Injection of mice with AAVs

Antibodies were raised against the capsid of AAV serotypes by injecting 6–8 week-old naive ICR (imprinting control region) male

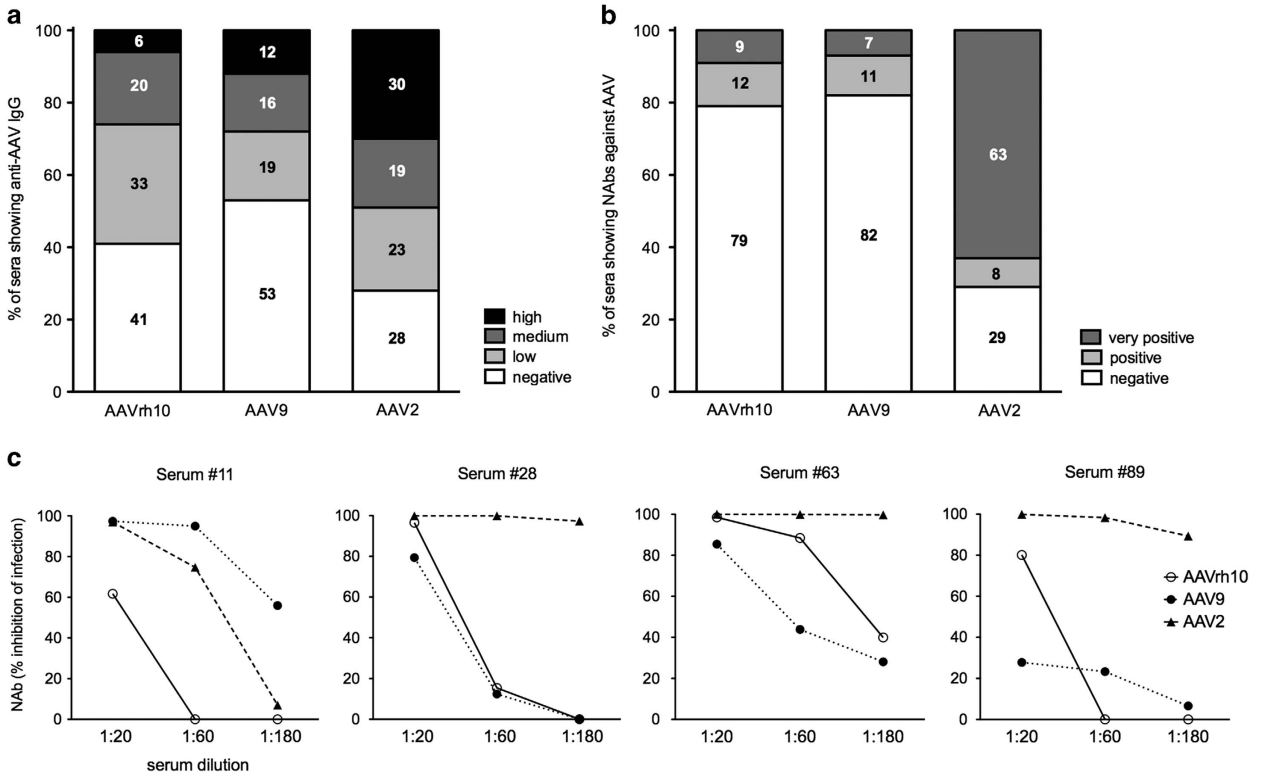

Figure 2. Pre-existing immunity to AAV serotypes rh.10, 9 and 2 in healthy human sera from Catalonia, Spain ($n = 100$). (**a**) Distribution percentage of donors with titers of anti-AAV immunoglobulin G (IgG) determined by indirect enzyme linked immunosorbant assay. Titers classified using optical density (OD) at 450 nm: high (OD > 1.600), medium (OD ⩽ 1.600 and > 1.000), low (OD ⩽ 1.000 to negative cut-off) and negative when OD readings were similarly low over a range of dilutions in duplicate (1:20, 1:60, 1:180). Negative cut-off was the mean OD value for 25 such samples, +3 s.d. (**b**) Distribution percentage of donors with titers of NAbs against each AAV, according to, if serum dilutions of 1:20 inhibited vector transduction by ⩾90%, very positive; ⩾50 and <90%, positive; or <50%, negative. All samples were tested in duplicates using a luciferase reporter. (**c**) Neutralization profiles of sera from four donors (#11, 28, 63 and 89) showing percentage inhibition of adeno-associated virus (AAV) infection of HEK293 cells by the sera at 1:20, 1:60 and 1:180 dilutions in duplicate using a luciferase reporter.

mice intravenously in the tail vein with $1x10^{11}$ vg ml^{-1} of AAV9, AAVrh.10 or AAV2 coding for green fluorescent protein in a total volume of 150 µl, plus one phosphate-buffered saline-injected control. Animals were killed 3 weeks post injection for serum collection. All experimental procedures were approved by the Universitat Autònoma de Barcelona (UAB) Animal Experimentation Committee.

Human serum samples

Serum samples from 100 healthy adult donors were obtained from the Catalan 'Banc de Sang i Teixits' (BST), approved by the Human and Animal Experimentation Ethics Committee (UAB), the Clinical Investigation Ethics Committee at Vall d'Hebron Hospital and the Scientific Committee of the BST Biobank.

AAV vector production

AAV vectors were produced by the Viral Production Unit, UAB (VPU) (http://sct.uab.cat/upv), following standard operating procedures.[30] Briefly, HEK293AAV cells (Stratagene, Carlsbad, CA, USA) were co-transfected with pXX6 providing helper virus functions; pRep2CapX packaging plasmid expressing the *rep* gene of AAV2 and the *cap* genes of either AAV2, AAV9 or AAVrh.10 (provided by Dr JM Wilson, University of Pennsylvania), and pAAV-ITR containing luciferase or green fluorescent protein as reporter genes driven by the cytomegalovirus (CMV) promoter between AAV2 ITRs. Recombinant vectors were clarified after benzonase treatment (50 U ml^{-1}, Novagen, Madison, WI, USA) and polyethylene glycol (PEG 8000, Sigma, St Louis, MO, USA) precipitation. Viruses were then purified on an iodixanol density gradient (Optiprep, Axis-Shield, Oslo, Norway).[31] Viral genomes per ml (vg ml^{-1}) were quantified by picogreen (Invitrogen, Carlsbad, CA, USA; M Chillon, manuscript submitted). Strictly speaking, recombinant AAVs are classified as pseudotypes, while distinct wild-type AAVs are serotypes. However, for the sake of simplicity we shall use serotype to refer to both recombinant and wild type throughout the text.

Total anti-AAV IgG in human and mouse sera

Indirect enzyme linked immunosorbant assays were set up on the basis of previously published reports.[14,32] Maxisorp microwell plates (Nunc A/S, Roskilde, Denmark) were coated with $1x10^9$ vg per well of virus. For the standard curve (mice only), mouse IgG (Sigma-Aldrich, St Louis, MO, USA) was coated in serial dilutions. Secondary antibodies were conjugated with peroxidase (rabbit anti-human IgG (wholemolecule)-peroxidase (Sigma A8792, Sigma-Aldrich) for humans; and ECL sheep anti-mouse IgG, horseradish peroxidase-linked species-specific whole antibody (GE Healthcare (NA931), Little Chalfont, UK; for mice). Detection was *via* 3,3′,5,5′tetramethylbenzidine (TMB) substrate reagent (BD biosciences, Franklin Lakes, NJ, USA). Absorbance was measured at 450 nm in a Bio-tek power wave reader linked to KC4 software program version 3.3 (BioTek, Winooski, VT, USA).

Results were expressed as OD readings related to serum dilutions (humans), or as total IgG anti-AAV in µg ml^{-1} for mice. For humans, individuals were considered negative for IgG antibodies against the AAV of interest when OD readings were similarly low over a range of dilutions (1:20, 1:60, 1:180). A cut-off OD for negative samples was determined, taking the mean OD value for a minimum of 25 such samples, +3 s.d. (cut-off

Table 2. Co-prevalence of neutralizing antibodies to AAVs rh.10, 9 and 2 and their abundance (optical density in enzyme linked immunosorbant assay) for the 21 human serum samples (from $n = 100$) neutralizing AAVrh.10

Human serum #	*Neutralizes AAVrh.10*	*% Inhibition of AAVrh10*[a]	*Anti-AAVrh. 10 IgG*[a]	*Neutralizes AAV9*	*% Inhibition of AAV9*[b]	*Anti-AAV9 IgG*[b]	*Neutralizes AAV2*	*% Inhibition of AAV2*[c]	*Anti-AAV2 IgG*[c]
1	Pos	75	1.013 ± 0.020	Pos	56	0.896 ± 0.045	V pos	98	1.896 ± 0.030
11	Pos	62	1.116 ± 0.059	V pos	97	2.011 ± 0.004	V pos	97	1.138 ± 0.065
15	V pos	98	1.535 ± 0.008	V pos	99	1.918 ± 0.011	V pos	98	1.881 ± 0.005
16	Pos	78	0.904 ± 0.011	Neg	< 50	0.534 ± 0.054	V pos	100	1.406 ± 0.009
25	Pos	89	1.122 ± 0.024	Neg	< 50	0.676 ± 0.010	V pos	98	1.830 ± 0.022
26	V pos	98	1.847 ± 0.147	V pos	99	1.885 ± 0.086	V pos	100	1.728 ± 0.098
28	V pos	97	1.452 ± 0.033	Pos	79	1.236 ± 0.030	V pos	100	1.812 ± 0.001
29	Pos	63	1.027 ± 0.028	Neg	< 50	0.512 ± 0.006	V pos	100	1.822 ± 0.023
32	Pos	74	1.030 ± 0.023	Neg	< 50	0.420 ± 0.011	V pos	100	1.773 ± 0.018
39	Pos	87	1.191 ± 0.046	Neg	< 50	0.871 ± 0.047	V pos	98	1.976 ± 0.028
53	V pos	97	1.157 ± 0.023	V pos	95	1.698 ± 0.066	V pos	100	2.016 ± 0.018
54	Pos	58	0.828 ± 0.001	Pos	57	1.035 ± 0.004	V pos	100	1.494 ± 0.044
63	V pos	99	1.888 ± 0.028	Pos	85	1.485 ± 0.001	V pos	100	1.942 ± 0.078
65	Pos	88	1.562 ± 0.045	V pos	99	2.318 ± 0.178	V pos	95	1.494 ± 0.045
67	V pos	98	1.652 ± 0.092	V pos	97	1.009 ± 0.115	V pos	100	2.016 ± 0.059
69	Pos	65	1.264 ± 0.021	Pos	70	0.587 ± 0.008	V pos	100	1.567 ± 0.040
76	V pos	97	1.361 ± 0.037	Neg	< 50	0.315 ± 0.011	V pos	100	1.905 ± 0.136
87	V pos	98	1.365 ± 0.033	V pos	96	1.419 ± 0.022	V pos	98	2.075 ± 0.009
89	Pos	80	1.680 ± 0.030	Neg	< 50	0.450 ± 0.001	V pos	100	1.562 ± 0.009
94	Pos	77	1.261 ± 0.016	Pos	59	0.563 ± 0.054	V pos	98	1.962 ± 0.014
99	V pos	97	1.603 ± 0.020	Pos	57	1.459 ± 0.045	V pos	98	1.982 ± 0.003
33 (control)	Neg		0.179 ± 0.001	Neg		0.218 ± 0.004	V pos		1.375 ± 0.050
20 (baseline)	Neg		0.160 ± 0.006	Neg		0.101 ± 0.001	Neg		0.161 ± 0.018

Abbreviations: v pos = very positive >90% inhibition of transduction, pos = positive 50–90% inhibition, neg = negative < 50% inhibition. Results are means of duplicates ± s.e.m. Neutralization assays at 1:20 dilution. Paired *t*-test for equivalence of means: [a]$P < 0.001$ anti-AAVrh.10 versus anti-AAV2, [b]$P > 0.05$ anti-AAVrh.10 versus anti-AAV9 (positive samples only), [c]$P < 0.05$ anti-AAV9 (positive samples only) versus anti-AAV2.

AAVrh.10 = 0.575, AAV9 = 0.665 and AAV2 = 0.471). IgG titer based on the OD was established as: high = OD > 1.600, med = OD ⩽ 1.600 and >1.000, and low = ⩽1.000 to the cut-off (See Supplementary Figure 2 for examples).

Anti-AAV IgG cross-binding to different viral capsids

We performed enzyme linked immunosorbant assays, as described above, using sera from AAV9, AAVrh.10 and AAV2-intravenously injected mice (four animals per group) and naive serum as a control. Sera were tested in duplicate for antibodies to the capsid of the injected virus and to the uninjected AAV9, AAVrh.10 and/or AAV2 capsid, as appropriate. OD reading at 450 nm was considered as 100% binding (maximum OD) for sera containing IgG binding to the same AAV capsid that the antibodies were raised against. OD readings for binding to other capsids were expressed as a percentage of the maximum OD signal. ODs for naive serum were consistently negligible for all capsids that were tested.

AAV neutralizing assays

Serum from heat-inactivated human serum samples or mouse sera (non-heat inactivated) was serially diluted with infection medium (Dulbecco's modified Eagle's medium+2% *fetal bovine serum*+1% PenStrep, PAA; (GE Healthcare, Buckinghamshire, UK)), incubated for 30 min at 37 °C with virus to then infect HEK293QB cells (QBiogene, Lachine, QC, Canada; 20,000 cells/well) at $1x10^9$ vg per well for AAV9 and AAVrh.10, and $2x10^7$ vg per well for AAV2 to achieve similar transduction as determined by titration. Vector-transgene expression was quantified after 48 h, lysing the cells according to manufacturer's instructions (Pierce Firefly Luciferase Flash Assay kit (ThermoFisher Scientific, Waltham, MA, USA)). Luminescence was read in VICTOR3 (PerkinElmer, Waltham, MA, USA). Transduction efficiency was expressed as luminescence, normalized by amount of protein per well (Pierce BCA Protein Assay kit, (ThermoFisher Scientific)), giving final values of luminescence per µg protein. Serum samples were considered positive for NAbs if they inhibited reporter gene expression by >50% compared with the maximum signal (average of three maximum values for negative sera, considered 100% transduction). If inhibition was >90% the serum was considered very positive.

Statistical analysis

Data analysis was performed with IBM SPSS statistics software (Armonk, NY, USA). Values are expressed as mean ± s.e.m. Differences between mean values were compared using two-tailed *t*-tests and one tailed paired *t*-tests, with $P < 0.05$ considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank the Vector Production Unit at CBATEG (Universitat Autònoma de Barcelona) for producing AAV vectors, the LLEB (UAB) for the luminescence measurements, Dr James M. Wilson (University of Pennsylvania) for providing AAV9 and AAVrh.10 RepCap plasmids and Dr Lorena Ariza (CBATEG, UAB) for experimental advice. We are also grateful to the Catalan Banc de Sang i Teixits (BST) for the human samples. GP was recipient of predoctoral fellowship from the Generalitat de Catalunya (2009FI_B00219). This work was supported by the Generalitat de Catalunya (2014 SGR 1354), the Instituto de Salud Carlos III (PS09720) and the Marató TV3 (110432) to AB.

REFERENCES

1 Mingozzi F, High KA. Immune responses to AAV vectors: overcoming barriers to successful gene therapy. *Blood* 2013; **122**: 23–36.
2 Calcedo R, Wilson JM. Humoral immune response to AAV. *Front Immunol* 2013; **4**: 341.
3 Mays LE, Vandenberghe LH, Xiao R, Bell P, Nam HJ, Agbandje-McKenna M *et al*. Adeno-associated virus capsid structure drives CD4-dependent CD8+ T cell response to vector encoded proteins. *J Immunol* 2009; **182**: 6051–6060.

4 Ciesielska A, Hadaczek P, Mittermeyer G, Zhou S, Wright JF, Bankiewicz KS *et al.* Cerebral infusion of AAV9 vector-encoding non-self proteins can elicit cell-mediated immune responses. *Mol Ther* 2013; **21**: 158–166.
5 Samaranch L, San Sebastian W, Kells AP, Salegio EA, Heller G, Bringas JR *et al.* AAV9-mediated expression of a non-self protein in nonhuman primate central nervous system triggers widespread neuroinflammation driven by antigen-presenting cell transduction. *Mol Ther* 2014; **22**: 329–337.
6 Grieger JC, Samulski RJ. Adeno-associated virus vectorology, manufacturing, and clinical applications. *Methods Enzymol* 2012; **507**: 229–254.
7 Gao G, Alvira MR, Somanathan S, Lu Y, Vandenberghe LH, Rux JJ *et al.* Adeno-associated viruses undergo substantial evolution in primates during natural infections. *Proc Natl Acad Sci USA* 2003; **100**: 6081–6086.
8 Sondhi D, Hackett NR, Peterson DA, Stratton J, Baad M, Travis KM *et al.* Enhanced survival of the LINCL mouse following CLN2 gene transfer using the rh.10 rhesus macaque-derived adeno-associated virus vector. *Mol Ther* 2007; **15**: 481–491.
9 Piguet F, Sondhi D, Piraud M, Fouquet F, Hackett NR, Ahouansou O *et al.* Correction of brain oligodendrocytes by AAVrh.10 intracerebral gene therapy in metachromatic leukodystrophy mice. *Hum Gene Ther* 2012; **23**: 903–914.
10 Homs J, Pages G, Ariza L, Casas C, Chillon M, Navarro X *et al.* Intrathecal administration of IGF-I by AAVrh10 improves sensory and motor deficits in a mouse model of diabetic neuropathy. *Mol Ther Method Clin Dev* 2014; **1**: 7.
11 Wang H, Yang B, Qiu L, Yang C, Kramer J, Su Q *et al.* Widespread spinal cord transduction by intrathecal injection of rAAV delivers efficacious RNAi therapy for amyotrophic lateral sclerosis. *Hum Mol Genet* 2014; **23**: 668–681.
12 Tardieu M, Zerah M, Husson B, de Bournonville S, Deiva K, Adamsbaum C *et al.* Intracerebral administration of adeno-associated viral vector serotype rh.10 carrying human SGSH and SUMF1 cDNAs in children with mucopolysaccharidosis type IIIA disease: results of a phase I/II trial. *Hum Gene Ther* 2014; **25**: 506–516.
13 Perdomini M, Belbellaa B, Monassier L, Reutenauer L, Messaddeq N, Cartier N *et al.* Prevention and reversal of severe mitochondrial cardiomyopathy by gene therapy in a mouse model of Friedreich's ataxia. *Nat Med* 2014; **20**: 542–547.
14 Boutin S, Monteilhet V, Veron P, Leborgne C, Benveniste O, Montus MF *et al.* Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. *Hum Gene Ther* 2010; **21**: 704–712.
15 Calcedo R, Vandenberghe LH, Gao G, Lin J, Wilson JM. Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. *J Infect Dis* 2009; **199**: 381–390.
16 Homs J, Ariza L, Pages G, Udina E, Navarro X, Chillon M *et al.* Schwann cell targeting via intrasciatic injection of AAV8 as gene therapy strategy for peripheral nerve regeneration. *Gene Ther* 2011; **18**: 622–630.
17 Foust KD, Nurre E, Montgomery CL, Hernandez A, Chan CM, Kaspar BK. Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nat Biotechnol* 2009; **27**: 59–65.
18 Duque S, Joussemet B, Riviere C, Marais T, Dubreil L, Douar AM *et al.* Intravenous administration of self-complementary AAV9 enables transgene delivery to adult motor neurons. *Mol Ther* 2009; **17**: 1187–1196.
19 Lieberman R, Stiffel C, Asofsky R, Mouton D, Biozzi G, Benacerraf B. Genetic factors controlling anti-sheep erythrocyte antibody response and immunoglobulin synthesis in backcross and F2 progeny of mice genetically selected for 'high' or 'low' antibody synthesis. *J Exp Med* 1972; **136**: 790–798.
20 Chiuchiolo MJ, Kaminsky SM, Sondhi D, Hackett NR, Rosenberg JB, Frenk EZ *et al.* Intrapleural administration of an AAVrh.10 vector coding for human alpha1-antitrypsin for the treatment of alpha1-antitrypsin deficiency. *Human gene therapy. Clin Dev* 2013; **24**: 161–173.
21 Gray SJ, Nagabhushan Kalburgi S, McCown TJ, Jude Samulski R. Global CNS gene delivery and evasion of anti-AAV-neutralizing antibodies by intrathecal AAV administration in non-human primates. *Gene Ther* 2013; **20**: 450–459.
22 Samaranch L, Salegio EA, San Sebastian W, Kells AP, Foust KD, Bringas JR *et al.* Adeno-associated virus serotype 9 transduction in the central nervous system of nonhuman primates. *Hum Gene Ther* 2012; **23**: 382–389.
23 Riviere C, Danos O, Douar AM. Long-term expression and repeated administration of AAV type 1, 2 and 5 vectors in skeletal muscle of immunocompetent adult mice. *Gene Ther* 2006; **13**: 1300–1308.
24 Gurda BL, DiMattia MA, Miller EB, Bennett A, McKenna R, Weichert WS *et al.* Capsid antibodies to different adeno-associated virus serotypes bind common regions. *J Virol* 2013; **87**: 9111–9124.
25 Tellez J, Van Vliet K, Tseng YS, Finn JD, Tschernia N, Almeida-Porada G *et al.* Characterization of naturally-occurring humoral immunity to AAV in sheep. *PLoS One* 2013; **8**: e75142.
26 Bartel M, Schaffer D, Buning H. Enhancing the clinical potential of AAV vectors by capsid engineering to evade pre-existing immunity. *Front Microbiol* 2011; **2**: 204.
27 Louis Jeune V, Joergensen JA, Hajjar RJ, Weber T. Pre-existing anti-adeno-associated virus antibodies as a challenge in AAV gene therapy. *Hum Gene Ther Methods* 2013; **24**: 59–67.
28 Calcedo R, Morizono H, Wang L, McCarter R, He J, Jones D *et al.* Adeno-associated virus antibody profiles in newborns, children, and adolescents. *Clin Vaccine Immunol* 2011; **18**: 1586–1588.
29 Wang L, Calcedo R, Wang H, Bell P, Grant R, Vandenberghe LH *et al.* The pleiotropic effects of natural AAV infections on liver-directed gene transfer in macaques. *Mol Ther* 2010; **18**: 126–134.
30 Zolotukhin S. Production of recombinant adeno-associated virus vectors. *Hum Gene Ther* 2005; **16**: 551–557.
31 Zolotukhin S, Byrne BJ, Mason E, Zolotukhin I, Potter M, Chesnut K *et al.* Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Ther* 1999; **6**: 973–985.
32 Treleaven CM, Tamsett TJ, Bu J, Fidler JA, Sardi SP, Hurlbut GD *et al.* Gene transfer to the CNS is efficacious in immune-primed mice harboring physiologically relevant titers of anti-AAV antibodies. *Mol Ther* 2012; **20**: 1713–1723.

Supplementary Information accompanies this paper on Gene Therapy website (http://www.nature.com/gt)