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RESEARCH ARTICLE

Genetic modifications of the adeno-associated virus type 2 capsid reduce the affinity and the neutralizing effects of human serum antibodies

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The high prevalence of human serum antibodies against adeno-associated virus type 2 (AAV) vectors represents a potential limitation for in vivo applications. Consequently, the development of AAV vectors able to escape antibody binding and neutralization is of importance. To identify capsid domains which contain major immunogenic epitopes, six AAV capsid mutants carrying peptide insertions in surface exposed loop regions (I-261, I-381, I-447, I-534, I-573, I-587) were analyzed. Two of these mutants, I-534 and I-573, showed an up to 70% reduced affinity for AAV antibodies as compared to wild-type AAV in the majority of serum samples. In addition, AAV mutant I-587 but not wild-type AAV efficiently transduced cells despite the presence of neutralizing antisera. Taken together, the results show that major neutralizing effects of human AAV antisera might be overcome by the use of AAV capsid mutants.
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Keywords: *adeno-associated virus; immunogenic epitopes; AAV antibodies; neutralizing antibodies; human serum samples*

Introduction

The human parvovirus adeno-associated virus type 2 (AAV) is a promising vector for human somatic gene therapy. Recombinant AAV vectors (rAAV) have many advantages in comparison to other vector systems, including the ability to transduce both dividing and nondividing cells, long-term gene expression *in vitro* and *in vivo*, and the apparent lack of pathogenicity. AAV has a broad host range and transduces a wide variety of tissues, including muscle, lung, liver, brain, and hematopoietic cells.[1–7] In a potential model of the infection process, AAV first interacts with its primary receptor heparan sulfate proteoglycan (HSPG), mediating the attachment of the virions to the host cell membrane.[8] In addition, two types of coreceptors, $\alpha_v\beta_5$ integrin and fibroblast growth factor receptor 1 (FGFR), have been suggested, which seem to be involved in the subsequent internalization process.[9,10] However, conflicting results were reported about the contribution of these coreceptors.[11,12] Following receptor binding, AAV enters the cell via a dynamin-dependent endosomal pathway.[13,14] After acidification of endosomes, viral particles are released into the cytoplasm and rapidly transported to the nucleus, involving microfilaments and microtubules, before they enter the nucleus.[15–17]

The capsid of wild-type AAV (wtAAV) harbors a linear, single-stranded DNA genome of 4.7 kb, which contains two open reading frames (ORF), *rep* and *cap*, flanked by the inverted terminal repeats (ITR). The 5′ ORF encodes the four nonstructural proteins (Rep78, Rep68, Rep52, and Rep40), which are required for replication, transcriptional control and site-specific integration. The three structural proteins, designated VP1, VP2, and VP3, are encoded in the 3′ ORF and transcribed from the same promoter (p40) using alternate splicing and different translational initiation codons. The small icosahedral capsid, which is only 25 nm in diameter, is composed of 60 subunits with a relative stoichiometry of about 1:1:8 for VP1, VP2, and VP3.[18] Recently, the atomic structure of AAV has been determined.[19] Each subunit has a β-barrel motif, which is highly conserved among parvoviruses. Between the strands of the β-barrel core large loop insertions are found that constitute the majority of the capsid surface. These loop insertions seem to interact with antibodies and cellular receptors and are highly variable among related parvoviruses. The most prominent features of the capsid are the threefold-proximal peaks, which cluster around the threefold axis.[19] Positions on the AAV capsid where interaction with HSPG takes place have been determined.[20,21] They map to a positively charged region on the side of the threefold-proximal peaks.[19] Moreover, epitopes of monoclonal antibodies (mAb) which interfere with the AAV infection process (A20 and C37-B) have also been mapped to this threefold axis.[19,22]

Although knowledge is rising about basic AAV biology, there are still obstacles for the application of AAV as vector system for somatic gene therapy. One major problem is the high prevalence of AAV specific

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antibodies (Ab) in the human population. In all, 50–96% are seropositive for AAV Ab, and 18–67.5% of them have neutralizing Ab, depending on age and ethnic group.[23–25] Especially these pre-existing neutralizing Ab have profound implications for the application of AAV in human gene therapy. Animal experiments have shown that neutralizing Ab greatly reduce or even prevent transgene expression after readministration of the vector.[7,26,27] Different mechanisms for neutralization of viral infections have been described:[28] (i) aggregation of viral particles, (ii) induction of conformational changes in the capsid, (iii) interference with receptor attachment, and (iv) inhibition of uncoating due to virion stabilization. In related parvoviruses immunogenic sites are formed by highly variable and accessible domains on the capsid surface, which can be generally found in the threefold spike region (Figure 1): on the tip and the shoulder of the spike, and between the twofold dimple and the spike.[29] In canine parvovirus (CPV), two dominant neutralizing epitopes, which are both conformational epitopes, are found on the shoulder of the threefold spike (epitope A: residues 93, 222, 224, 426 and epitope B: residues 299, 300, 302 of VP2).[30] Similarly, the epitopes of two monoclonal neutralizing Ab of B19 parvovirus have also been mapped to the threefold spike region.[31] Therefore, it is possible that immunogenic sites of AAV are also located in variable regions of the threefold spike region.

In a previous study, we had generated six AAV capsid mutants bearing an integrin-specific peptide ligand (L14) insertion at position 261, 381, 447, 534, 573, or 587 of the AAV VP1 protein.[32] These positions were originally selected based on structural alignments with the related parvoviruses CPV, B19 and feline panleukopenia virus (FPV), with regard to flexible, highly variable, and immunogenic domains of these viruses (see Figure 1 and Nicklin *et al*[33]). Indeed, we and others could show that the insertion sites are displayed on the capsid surface[21,32–34] and the recently published atomic structure of AAV confirmed the localization of the selected positions in surface exposed loop regions.[19] Moreover, binding studies with the neutralizing mAb A20 and C37-B demonstrated that the mutations impaired their affinities for the AAV capsid. This indicated that immunogenic domains had been affected by the capsid mutations (see Figure 2a and Wobus *et al*[22]). In this paper, we analyzed these six AAV capsid mutants with polyclonal human serum samples in binding and neutralization assays. This allowed us to characterize immunogenic and neutralizing regions on the AAV capsid.

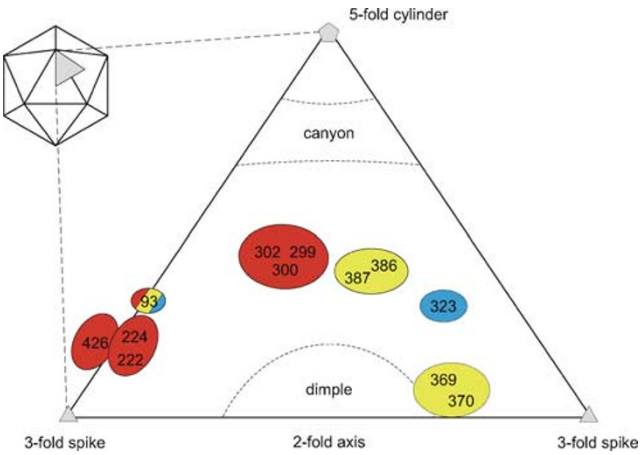

Figure 1 *Map of major antigenic regions in CPV, FPV and B19. The triangle represents one asymmetric subunit of the CPV major capsid protein. The positions of the five-, three- and two-fold symmetry axis are indicated. The amino-acid positions of major antigenic determinants in CPV (red), B19 (yellow), and FPV (blue) are given (aligned to the CPV capsid protein).*[29]

Results

AAV antibodies from human serum samples have a reduced affinity for AAV insertion mutants

To determine major antigenic domains involved in the humoral immune response of humans against the capsid of AAV, we analyzed the ability of human antisera to recognize AAV capsid mutants carrying a 14 amino-acid (aa) peptide (L14, QAGTFALRGDNPQG) of the laminin fragment P1 inserted at positions 261, 381, 447, 534, 573, or 587.[32]

In a first step, the capsid morphology of the six different VP3 mutants was characterized by electron microscopy (EM). All viral preparations showed an EM morphology similar to wtAAV and contained predominantly intact particles (data not shown).

The presence of antibodies against wtAAV was then analyzed in 65 human serum samples. In an ELISA, 43 out of 65 serum samples (66%) were positive for AAV antibodies. Out of these 43 seropositive samples, 29 sera with a high titer of AAV-specific antibodies (OD > 0.6

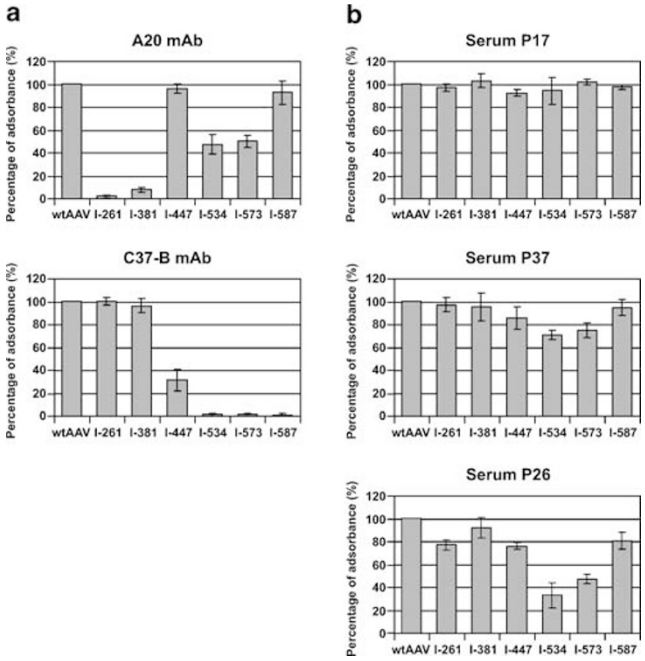

Figure 2 *Binding of A20 and C37-B monoclonal antibody (a) and human serum samples (b) to wtAAV and AAV capsid mutants as determined by ELISA. Microtiter plates were coated with identical particle amounts of wtAAV and AAV insertion mutants and incubated with hybridoma supernatants of either A20 or C37-B mAb or with serum samples of human patients as described in Materials and methods. Binding of antibodies to wtAAV was set at 100% (y-axis). Each experiment was repeated independently at least three times; the figure shows the mean values and s.d. (indicated by the error bars).*

after subtraction of background) were selected for further analysis. The binding affinity of these 29 serum samples to the six AAV insertion mutants (I-261, I-381, I-447, I-534, I-573, and I-587) was analyzed in an ELISA and compared to wtAAV. Identical numbers of particles of the respective insertion mutants and of wtAAV, as determined by EM (Table 1) and confirmed by Western blotting, were coated on the ELISA plates. Binding of serum Ab to wtAAV was set as 100%, and the change in serum binding to the AAV mutants was determined. Several patterns of interaction of human sera with AAV mutants could be distinguished (Table 2). One group of serum samples, designated *class A*, reacted with all six AAV mutants in a way similar to wtAAV (eg serum P17, Figure 2b). This was the case for 10 out of 29 sera (34%). A second group of serum samples (12 out of 29, 42%), designated *class B*, displayed a reduced binding affinity only to mutants I-534 and I-573 (eg serum P37, Figure 2b). The average reduction for both, I-534 and I-573, was 31% as compared with wtAAV. A third and smaller group of serum samples (seven out of 29, 21%), *class C*, additionally showed a reduced affinity for the other four capsid mutants (eg serum P26, Figure 2b). On average, binding to these four mutants was decreased by 7% (I-381) to 26% (I-447) (Table 2). Nevertheless, insertions at positions 534 and 573 reduced binding of serum

Table 1 Titers of AAV stocks

Virus stocks	*Physical particles per ml*[a]	*Infectious particles per ml*[b]
wtAAV	4.9×10^{12}	7×10^{9}
I-261	1.0×10^{12}	1×10^{4}
I-381	1.0×10^{12}	$<1 \times 10^{2}$
I-447	2.2×10^{12}	1×10^{6}
I-534	3.5×10^{12}	$<1 \times 10^{2}$
I-573	3.1×10^{12}	$<1 \times 10^{2}$
I-587	2.5×10^{12}	6×10^{6}
rAAV	2.5×10^{12}	3×10^{9}
rAAV-587/L14	3.0×10^{11}	4×10^{6}
rAAV-587/MecA	1.3×10^{12}	2×10^{8}

[a]Physical particle titers were determined by EM for *rep*/*cap*-containing particles and by dot-blot analysis for recombinant vectors.
[b]Determined on HeLa cells by immunofluorescence for preparations containing a *rep* gene, and by FACS analysis for GFP encoding particles.

Table 2 Binding of seropositive human sera to AAV insertion mutants compared to wtAAV

Class[a]	*Serum*	*I-261*	*I-381*	*I-447*	*I-534*	*I-573*	*I-587*
A	P1	–	–	–	–	–	–
	P2	–	–	–	–	–	–
	P6	–	–	–	–	–	–
	P14	–	–	–	–	–	–
	P17	–	–	–	–	–	–
	P19	–	–	–	–	–	–
	P24	–	–	–	–	–	–
	P31	–	–	–	–	–	–
	P47	–	–	–	–	–	–
	P60	–	–	–	–	–	–
	Reduction[b]	0.3% ± 6.6%	0.2% ± 4.7%	7.5% ± 5.5%	0.0% ± 7.4%	3.9% ± 8.3%	−2.3% ± 5.4%
B	P5	–	–	–	↓	↓↓	–
	P7	–	–	–	↓↓↓	↓↓	–
	P16	–	–	–	↓↓	↓	–
	P27	–	–	–	↓↓↓	↓↓	–
	P29	–	–	–	↓	↓	–
	P32	–	–	–	–	↓	–
	P33	–	–	–	↓	↓	–
	P37	–	–	–	↓↓	↓↓	–
	P48	–	–	–	↓↓	↓↓	–
	P51	–	–	–	↓	↓↓	–
	P53	–	–	–	↓	↓↓	–
	P59	–	–	–	↓	↓	–
	Reduction	3.4% ± 5.8%	1.8% ± 8.9%	9.2% ± 5.5%	30.9% ± 13.5%	30.7% ± 7.2%	4.6% ± 3.5%
C	P3	↓	–	↓↓	↓↓↓	↓↓↓	–
	P26	↓	–	↓	↓↓↓	↓↓↓	↓
	P35	–	–	↓↓	↓↓	↓↓	–
	P40	–	↓↓	–	↓↓	↓↓	–
	P54	–	–	↓	↓↓↓	↓↓	–
	P57	↓	↓	↓↓	↓↓	↓↓	↓
	P65	↓	–	↓	↓↓↓	↓↓↓	↓
	Reduction	12.5% ± 10.6%	6.8% ± 9.9%	25.8% ± 11.7%	51.3% ± 10.9%	44.8% ± 5.0%	8.8% ± 9.1%
mAb	A20	↓↓↓↓↓	↓↓↓↓↓	–	↓↓↓	↓↓↓	–
	C37-B	↓↓↓↓↓	–	↓↓↓	↓↓↓↓↓	↓↓↓↓↓	↓↓↓↓↓

The symbols –, ↓, ↓↓, ↓↓↓, and ↓↓↓↓↓ illustrate a reduction in affinity in comparison to wtAAV of 0-14, ⩾15, ⩾25, ⩾50, and 100%, respectively.
[a]Classification of the serum samples: A, affinity to insertions mutants like to wtAAV; B, reduced affinity to I-534 and I-573; C, reduced binding with I-534, I-573 and other capsid mutants.
[b]The mean reduction and standard deviations are given.

antibodies to the capsid more than insertions at other positions, that is by 51 and 45%, respectively (Table 2). Interestingly, binding of *class C* sera to these two capsid mutants was more affected than binding of *class B* serum samples. The observed differences in binding affinity did not significantly change when using various serum dilutions.

Strikingly, mutations at positions 534 or 573 had an effect on the affinity of human antisera in 19 of 29 cases. In some cases, serum binding was reduced up to 70%. In seven serum samples, mutations at position 261, 381, 447, or 587 also resulted in a reduced binding affinity, albeit to a smaller extent. Based on these findings, we conclude that insertions in positions 534 or 573 affected major antigenic determinants of the humoral immune response against the AAV capsid.

Transduction of HeLa cells by rAAV-587/L14 is not inhibited by pre-existing neutralizing antibodies in human serum samples

A detailed understanding of major immunogenic domains on the AAV capsid is not only important with regard to the binding of serum antibodies to the virus and its subsequent neutralization by the immune system, but also with regard to the existence of neutralizing antibodies that directly inhibit infection of the target cells by AAV vectors. To analyze the interference of different human antisera with AAV transduction, we used a recombinant AAV vector carrying the L14 ligand at position 587 (rAAV-587/L14) to determine whether this modification would block the neutralizing ability of human antisera. Unfortunately, only rAAV-587/L14 could be purified to sufficiently high titers to perform these studies, therefore the other insertion mutants were not further tested (Table 1).

First, we determined the presence of neutralizing Ab in human serum samples. The 43 positive serum samples were tested in a neutralization assay with an AAV vector coding for GFP, which carried the wild-type capsid (rAAV). rAAV was incubated with serial dilutions of serum samples prior to transduction of HeLa cells. Thereafter, the number of GFP expressing cells was assessed by FACS analysis. Neutralizing titers were defined as the serum dilution where transduction was reduced by 50% (N_{50}). Serum samples were considered as neutralizing when the N_{50} was 1:320 or higher. 31 of these 43 serum samples (72%) contained neutralizing Ab against AAV, in agreement with previously published data.[23]

Of these 31 serum samples, equally distributed over the above-mentioned three classes (5 *class A*, 6 *class B*, and 4 *class C*), 15 were selected for further analysis. The effect of these serum samples on the transduction of HeLa cells by rAAV-587/L14 as compared with rAAV was determined (Figure 3a). In addition, the neutralizing mAb C37-B and an anti-L14 serum (see Materials and methods) were tested. For these experiments, identical transducing particle numbers of rAAV-587/L14 and rAAV were used. Both vectors were incubated with serial dilutions of neutralizing serum samples prior to transduction of HeLa cells. For all serum samples tested, transduction by rAAV-587/L14 was eight up to 64-fold (mean 15-fold) less reduced than transduction by rAAV. In 13 out of 15 serum samples, transduction by rAAV-587/L14 was only slightly impaired, with neutralizing titers of 1:80 or lower, demonstrating the ability of rAAV-587/L14 to escape the effects of neutralizing Ab (Figure 3a). Strikingly, rAAV-587/L14 was able to escape the neutralizing Ab in serum P47 at any dilution tested, and serum samples P17, P31, and P37 reduced transduction only at a dilution of 1:20, where unspecific interactions could not be excluded. Figure 3b and c shows one representative experiment with serum P35, which completely inhibited transduction by rAAV at a 1:80 dilution (Figure 3b). In marked contrast, transduction by rAAV-587/L14 was not affected (Figure 3c). Only two serum samples (P16 and P48) were able to neutralize rAAV-587/L14 transduction efficiently, with a N_{50} of 1:320. We assume that this was due to the high neutralizing Ab content in these serum samples, because transduction by rAAV-587/L14 still remained less affected than transduction by rAAV. As an additional control, the mAb C37-B was tested. C37-B is a neutralizing Ab that inhibits binding of AAV to the host cell.[22] It failed to bind I-587 in an ELISA, therefore it should not interfere with rAAV-587/L14 transduction. As expected, rAAV-587/L14 transduction was not neutralized by C37-B, while rAAV transduction could be totally inhibited by this antibody (data not shown). In marked contrast, anti-L14 serum, which was generated against the L14 ligand, neutralized

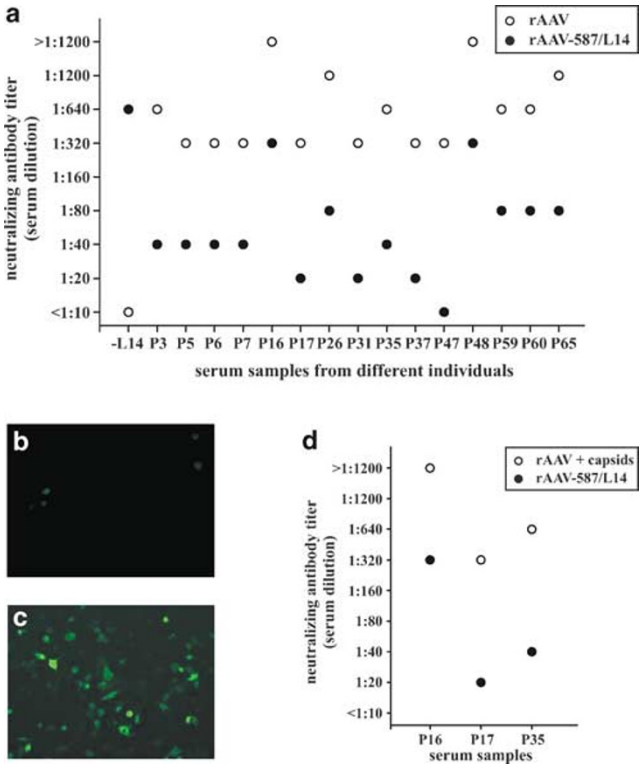

Figure 3 *Neutralization assay on HeLa cells. (a) Neutralizing antibody titers against rAAV and rAAV-587/L14. Serial dilutions (1:10–1:1200) of 15 neutralizing human serum samples were analyzed on HeLa cells. As a control, rabbit serum directed against the inserted L14-ligand (α-L14) was tested. The neutralizing titers (N_{50}) are expressed as the dilution at which transduction was 50% reduced compared to the positive control. rAAV (b) and rAAV-587/L14 (c) were incubated with serum P35 (1:80) prior infection of HeLa cells. GFP expression was monitored 48 h postinfection. (d) Empty capsids were added to rAAV to adjust physical particle numbers. The N_{50} was determined as described above.*

rAAV-587/L14 transduction completely at a 1:160 dilution, while rAAV transduction remained unaffected (Figure 3a). To rule out the possibility that these observations were due to different numbers of physical particles used for rAAV and rAAV-587/L14, we performed additional control experiments, where neutralization assays were performed with identical numbers of physical particles for both AAV vectors. For these experiments, empty capsids were added to the rAAV preparation and neutralization assays were performed with three selected serum samples (P16, P17, P35). As seen in Figure 3d, these experiments yielded identical results. Taken together, these results demonstrate that the mutant rAAV-587/L14 is able to escape pre-existing neutralizing Ab in human serum samples.

Neutralizing sera do not interfere with the L14-mediated tropism of rAAV-587/L14 on B16F10 cells

Insertion of the integrin-specific L14 peptide in 587 expands the tropism of AAV to nonpermissive B16F10 cells.[32] To determine if rAAV-587/L14 was able to retain its ability to infect the target cell line B16F10 via the inserted ligand L14 in the presence of neutralizing antisera, we performed additional experiments with selected serum samples. rAAV-587/L14 was incubated with serial dilutions of P35 serum before transduction of irradiated B16F10 cells. After 72 h GFP expression was measured. rAAV-587/L14 efficiently transduced B16F10 cells despite incubation with P35 at a 1:80 dilution, whereas anti-L14 serum completely inhibited transduction at this dilution (Figure 4b and c). When testing P37 and P26, the same neutralizing titers as determined on HeLa cells were obtained (data not shown). These findings showed that the AAV L14 targeting vector could escape neutralizing antibodies in human sera while retaining its retargeting ability.

Ability of rAAV-587 to escape neutralizing sera does not depend on the inserted L14 ligand

To exclude that the escape from neutralizing antisera was caused by a specific ligand, we tested another insertion mutant, rAAV-587/MecA that carries a 7 aa ligand (GENQARS) at position 587. This mutant has been selected by AAV-display on Mec1 cells and efficiently transduces Mec1 cells and primary B cells from chronic lymphocytic leukemia patients in a receptor-specific manner.[35] rAAV-587/MecA and rAAV were incubated with the serum P35 before Mec1 cells were infected. Transduction of Mec1 cells by rAAV-587/MecA was not affected by the neutralizing Ab of serum P35 (1:80 dilution). In contrast, rAAV transduction was almost completely inhibited by this serum (Figure 5). Experiments with other neutralizing serum samples provided identical results (data not shown). In additional control experiments the neutralizing Ab A20 was able to inhibit transduction by rAAV-587/MecA, while C37-B had no effect (data not shown).

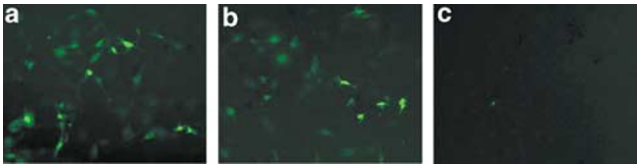

Figure 4 *Neutralization assay on B16F10 cells. Infection of irradiated B16F10 cells with rAAV-587/L14 alone (a) or after coincubation with P35 serum (b) or anti-L14 serum (c) at a 1:80 serum dilution. Cells were analyzed for GFP expression after 72 h.*

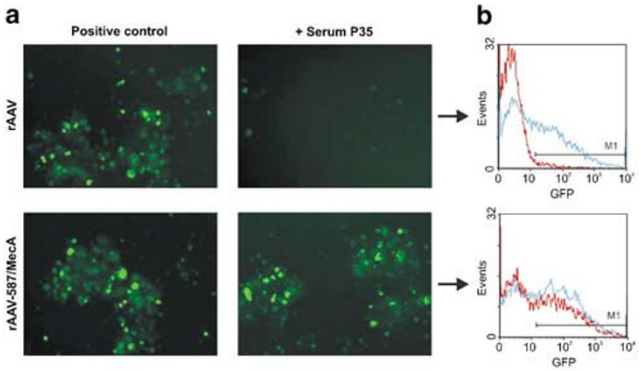

Figure 5 *Effect of neutralizing antisera on rAAV-587/MecA transduction. (a) After infection with adenovirus, Mec1 cells were infected with rAAV (top row) and rAAV-587/MecA (bottom row) alone (positive control) or after coincubation with serum P35 at a 1:80 dilution (+ serum P35). Note that more physical particles were used for rAAV to achieve similar transduction. (b) FACS analysis of rAAV (top row) and rAAV-587/MecA (bottom row) incubated with serum P35 (red line) in comparison to their positive controls (blue line). GFP expression was determined 48 h postinfection.*

Taken together, the results demonstrate that the insertion of different heterologous ligands at position 587 allows escape from pre-existing neutralizing antibodies. Targeting properties of these vectors are retained in these capsid mutants, even in the presence of neutralizing antisera.

Discussion

As a result of the high prevalence of antibodies against AAV in the population, it is essential in gene therapy approaches to understand the immunogenic determinants of the AAV capsid and to develop strategies to circumvent antibody binding and neutralization of AAV vectors. In this study, we analyzed six AAV capsid mutants (I-261, I-381, I-447, I-534, I-573, and I-587) with a 14 aa peptide ligand inserted into the VP3 part of the capsid protein to identify immunogenic domains on the AAV capsid. We showed that peptide insertions at position 534 or 573 reduced binding of human antisera in 66% of the analyzed samples, indicating that these regions might be preferentially recognized by human AAV antibodies. In addition, we analyzed AAV vectors modified at position 587 to study the potential of AAV capsid mutants to escape the neutralizing effects of human antisera with regard to the transduction efficiency. We demonstrated that these modified vectors were able to escape neutralizing Ab in human antisera without losing their ability to infect cells via the targeted receptors. In marked contrast, transduction of AAV carrying the unmodified capsid was significantly reduced or inhibited. These findings demonstrate that the insertion of peptide ligands at site 587 reduces the ability of AAV antibodies in human blood to neutralize the transduction by rAAV vectors.

The atomic structures of related parvoviruses like CPV, FPV, and B19 have been resolved during the past decade and antigenic sites have been determined (see Figure 1).[29,30,31,36–38] At the beginning of our studies the capsid structure of AAV was still unknown. Alignments of these related parvoviruses with AAV led to hypothetical models of the AAV capsid, and systematic mutagenesis helped to map functional sites on the capsid.[20,21,32] Based on our structural alignments, six sites on the AAV capsid, selected with regard to flexible, highly variable loops and immunogenic domains of related parvoviruses (Figure 1), were identified to accept the insertion of an integrin-specific RGD ligand (L14, QAGTFALRGDNPQG).[32] Immunological analysis demonstrated the surface localization of the inserted L14 peptide.[32] Characterization of other AAV serotypes revealed that the selected positions are also within highly variable regions among these serotypes.[39] The recent unveiling of the atomic structure of AAV[19] broadly confirmed the flexible loop regions predicted by our initial structural model.[32] When mapping the six insertion sites used in this report on the three-dimensional structure of AAV, they can all be found on the capsid surface within the threefold spike region (Figure 6).

The high prevalence of AAV-specific Ab causes substantial problems for human gene therapy. Different approaches have been pursued to map epitopes on the AAV capsid. Moskalenko *et al*[25] used small overlapping peptides (15 aa) spanning the VP1 protein and human antisera to screen the AAV capsid protein by peptide scan. They identified several linear epitopes presented on the capsid surface, among them sites mapping to I-261 and I-447. However, some of the identified peptides might block Ab binding unspecifically or might not be displayed on the capsid surface.[19,22] Moreover, conformational epitopes cannot be identified by this method. Wobus *et al*[22] used murine mAb, which recognize conformational epitopes (D3, C37-B, C24-B, and A20), to identify epitopes on the AAV capsid. Immunological analysis of these mAb with our six AAV insertion mutants helped mapping the epitopes of these antibodies on the AAV capsid and provided information about regions involved in receptor attachment. However, murine mAb cannot mimic the polyclonal Ab repertoire after an infection in humans. We therefore analyzed the ability of human antisera to recognize the six AAV insertion mutants in order to determine major antigenic domains of AAV involved in the humoral immune response. By using an ELISA, we could demonstrate that the majority of the serum samples had a reduced affinity towards two insertion mutants, I-534 and I-573. Although at 39 aa distance from each other on the primary sequence, these sites are found in close proximity in the assembled capsid, on the side of the peaks at the threefold rotation axis.[19] These data indicate the importance of the threefold-proximal peak region in the recognition by the humoral immune response, as it has been already shown for B19 or CPV.[29–31] It remains to be elucidated whether these mutations interfere with Ab binding directly or indirectly due to structural changes in adjacent regions.

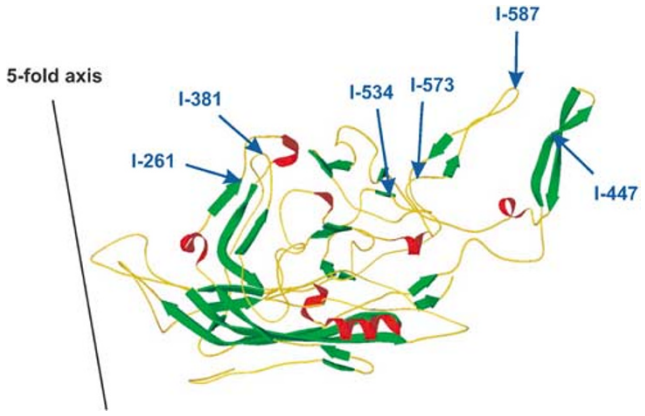

Figure 6 *Model of the AAV major capsid protein according to the atomic structure by Xie et al[19] (taken from The Protein Data Bank). The sites of insertion are marked with arrows.*

Insertions at sites 261, 381, 447, and 587 affected binding of serum Ab only in a minority of serum samples. This was surprising, because mutations at position 261, 381, and 587 abolished binding of murine mAb A20, and C37-B, respectively (Figure 2a). Moreover, amino acids corresponding to positions 261, 381, and 447 are part of the major antigenic determinants in CPV.[30] Different explanations are conceivable for this minor effect of these mutations on human Ab binding. (i) It is possible that epitopes, especially linear epitopes, which are adjacent to these insertion sites, have not been affected and that they are responsible for the remaining reactivity of serum Ab towards these mutants. (ii) In contrast to CPV, AAV residues 261 and 381 are located in the valley between two peaks of the threefold symmetry axis and this region might be less accessible for Ab binding, or less relevant for inducing a humoral immune response. (iii) Serum samples consist of a polyclonal Ab population. Thus, epitopes which only induce a weak Ab response might not have been detected in this binding assay, although they were affected by the mutations. For the same reason, we also did not expect a complete inhibition of binding of the polyclonal Ab, as observed with the monoclonal Ab A20 with I-261, I-381, and C37-B with I-534, I-573, and I-587. (iv) In addition, the specificity of Ab for a given antigen is dependent on the B-cell repertoire, T-cell repertoire and the major histocompatibility complex (MHC), and is therefore different at the species and individual level. Murine Ab generated against viral antigens may differ in their targeted sequences to those generated in humans. All six insertions are directly at or close to the spike region. It is likely that in individuals the positions of the major antigenic determinants of AAV are different. In this case, differences in human serum Ab binding were only seen if the major antigenic determinants were close to the spike region. This might also explain why serum samples of *class A* displayed no reduced affinity for any of the six insertion mutants, but this does not exclude that different mutants would impair binding of these sera. Consequently, it is reasonable to assume that other immunogenic determinants exist, which have not been identified so far.

For the *in vivo* application of AAV, epitopes which interact with neutralizing antibodies are of particular importance. We, therefore, investigated the ability of AAV vectors with insertion of different peptide ligands at 587 to escape the pre-existing neutralizing Ab in human antisera. Such vectors have previously been

shown to efficiently retarget infection to wtAAV-resistant cells.[32,33] Moreover, Wu *et al*[21] demonstrated that this 587 region is involved in binding to the primary attachment receptor HSPG. In total, 15 neutralizing serum samples were analyzed for their ability to neutralize rAAV-587/L14 transduction in comparison to rAAV. Strikingly, rAAV-587/L14 could escape the neutralizing effects exerted by 13 of the 15 neutralizing serum samples and efficiently transduced various cell lines. The targeting properties of rAAV-587/L14 were not affected, and escape could also be observed with a second ligand (MecA) that differed in size and sequence, demonstrating that the escape did not depend on the particular L14 insertion. Unfortunately, infectious titers of the other insertion mutants were not sufficient to perform neutralization assays. However, it is possible that insertion of different peptide ligands yield higher infectious titers, as they strongly depend on the peptide size and sequence (compare MecA with L14, and Shi *et al*[40]). Nevertheless, our results do not allow the conclusion that insertions at position 587 are the only site to generate mutants, which are able to escape the neutralizing effects of human antisera.

It seems contradictory that I-587 only slightly impaired binding of serum samples in the ELISA, while it had such strong effects on the neutralizing capacity of human antisera. At least two explanations are possible: Human sera consist of a polyclonal Ab population directed against various epitopes on the AAV surface, but only a small amount of these Ab are capable of neutralizing AAV transduction. Therefore, in an ELISA these neutralizing Ab may not have a noticeable effect on the overall Ab binding, whereas they inhibit virus transduction in a neutralization assay. The three-dimensional structure of AAV shows that the sites for interaction with the viral receptor HSPG are located within the peaks of the threefold axis, proximate to residue 587.[19,21] Furthermore, the neutralizing Ab C37-B, which inhibits binding of wtAAV to the host cell, has its epitope adjacent to this site in the assembled capsid. Therefore, this region seems to be critical for receptor binding. It is very likely that neutralizing Ab are preferentially directed against this region and explains why the action of these Ab is affected by insertions at this region.[19,22] Whether these modifications directly interfere with neutralizing epitopes at position 587 or antigenic determinants on neighboring loops within the threefold proximal peaks are affected due to conformational changes remains to be elucidated. Additional experiments with point mutations or small deletions at 587, for example, could help to map such epitopes in the 587 region more precisely. Another explanation is that neutralizing Ab, which have been generated against the wtAAV capsid, only block the wt capsid mediated transduction. After insertion of a targeting ligand at position 587 the virus mutant can use a different uptake route than wtAAV, which does no longer depend on HSPG binding (rAAV-587/L14 via the integrin receptor).[32,33] Therefore, it is supposable that these neutralizing Ab cannot block the interaction of these AAV mutants with alternative cell-surface receptors. Of course, these explanations are not mutually exclusive, and due to the complexity and various mechanisms of the neutralization process other explanations are also possible. However, it is reasonable to assume that capsid modifications at position 587 might not only allow to alter the tropism of AAV but also to generate immune escape variants. All data presented here were generated *in vitro*. Animal experiments are now needed to corroborate the utility of this concept, not only with regard to neutralization by antibodies, but also with regard to transduction efficiency and tropism of these genetically modified vectors. In addition, *in vivo* studies could help to clarify if insertion of such peptide ligands possibly promotes the generation of new epitopes, for example, the fusion region where the peptide is inserted.

Taken together the results indicate that the threefold-proximal peaks on the AAV capsid are major antigenic determinants for antibody binding as well as for neutralization of AAV transduction. Moreover, our results demonstrate that modifications at site 587 could allow to generate AAV vectors with the ability to escape neutralization by human antisera. Importantly, these modified vectors retain their ability to transduce specific target cells. These findings might be useful for the production of AAV vectors suitable for repeated administration in human gene therapy.

Materials and methods

Cell culture

HeLa, 293, and B16F10 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). Mec1 cells were cultivated in Iscove's modified DMEM supplemented with 10% FCS, penicillin–streptomycin and L-glutamine. Cells were maintained at 37°C in a 5% CO_2 humidified incubator.

Antibodies and human serum samples

B1, A20 and C37-B were generated by immunization of mice with purified AAV capsid proteins and synthetic peptides, respectively, followed injection with AAV empty particles.[22,41] 76/3 was generated by immunization with purified Rep protein as described.[41] Serum against the L14 ligand was obtained after immunization of a rabbit with L14 peptide.[32] Serum samples from human patients were kindly provided by the Klinikum Großhadern in Munich, Germany.

Plasmids

The plasmid pUC-AV2 contains the full-length AAV2 genome and was constructed as described in Girod *et al*.[32] The plasmids pI-261, pI-381, pI-447, pI-534, pI-573, pI-587 are derived from pUC-AV2, with the L14-encoding sequence inserted in the cap gene. The AAV2-based helper plasmids pRC, pRC(I-587), and pRC(587/MecA) contain the AAV2 rep- and cap-encoding regions but lack the viral ITRs.[32,35] pRC(587/MecA) contains a DNA fragment coding for the MecA ligand inserted between amino-acid position 587 and 588. The pGFP plasmid is an AAV2-based vector plasmid in which the AAV ITR sequences flank the hygromycin selectable marker gene controlled by the thymidine kinase promoter and the enhanced GFP gene regulated by the cytomegalovirus promoter.[42] The adenovirus helper plasmid pXX6.[43] was kindly provided by RJ Samulski.

Preparation of virus stocks

The AAV stocks were generated as described previously[42] with the following modifications. 293 cells seeded at 80% confluence in plates with 15 cm of diameter were transfected with a total of 37.5 μg of vector plasmid (pGFP) and packaging plasmid (pRC for wt capsid, pRC(I-587) for L14 carrying capsid, and pRC(587/MecA) for MecA carrying capsid) and adenoviral plasmid (pXX6) at a 1:1:1 molar ratio. For viruses containing an AAV *rep* and *cap* gene, the pUC-AV2 plasmid or mutated plasmids were transfected with pXX6 in a 2:1 M ratio. After 48 h, cells were collected and pelleted by centrifugation. Cells were resuspended in 150 mM NaCl, 50 mM Tris-HCl (pH 8.5), lysed by repeated freeze–thaw cycles, and treated with Benzonase (50 U/ml) for 30 min at 37°C. Cell debris was removed by centrifugation and the supernatant was loaded onto an iodixanol gradient as described.[44] After centrifugation at 69 000 rpm for 1 h at 18°C, the AAV containing iodixanol phase was harvested.

Evaluation of AAV titers

Particle titers of virus stocks containing *rep*/*cap* were determined by EM and confirmed by Western blotting. EM was performed at the DKFZ (Heidelberg). Iodixanol gradient purified viral particles were adsorbed onto Formvar-carbon-coated copper grids and negatively stained with uranyl acetate. Titers were calculated in comparison to a known viral standard.[45] Western blot analysis was performed to confirm the titers obtained by electron microscopy. Equal numbers of AAV particles were separated on a 10% SDS-PAGE and blotted on nitrocellulose membrane using standard protocols. Capsid proteins were detected by B1 hybridoma supernatant, followed by incubation with a peroxidase-coupled secondary antibody (Sigma) and visualized by enhanced chemiluminescence (Pierce). For recombinant vectors encoding the GFP protein genomic titers were quantified by dot-blot analysis as described.[32] Briefly, serial dilutions of the AAV preparations were first incubated in 2 M NaOH, then blotted onto a nylon membrane, and finally hybridized with a random-primed *gfp* probe by standard methods. Infectious particle titers of the GFP encoding virus stocks were determined by infecting irradiated HeLa cells (100 Gy from a ^{137}Cs gamma irradiation source) with serial dilutions of the AAV preparation in a 12-well plate. After 48 h, cells were harvested and assayed for GFP expression by fluorescence-activated cell sorting (FACS). Infectious titers on B16F10 and Mec1 cells were performed accordingly by coinfection with adenovirus 5 (AdV). Titers of AAV stocks carrying the *rep* and *cap* gene were determined by infection of HeLa cells after AdV coinfection and detection of the viral Rep proteins with Cy3-labeled 76/3 monoclonal antibody (Cy3 mono-Reactive Dye Pack, Amersham, according to the manufacturer's protocol).

ELISA

Identical particle amounts (5×10^8 per well) as determined by EM of wtAAV and AAV insertion mutants were coated onto microtiter plates (MaxiSorp; Nunc Nalgene International) in PBS overnight at 4°C. After blocking with 3% BSA/5% sucrose in washing buffer (PBS/0.05% Tween 20) wells were incubated with A20- or C37-B-hybridoma supernatant or human serum diluted 1:50 to 1:400 in blocking buffer for 1 h at room temperature. After washing, wells were incubated with a biotin-conjugated anti-human or anti-mouse secondary antibody (Dianova) diluted in washing buffer for 1 h. Detection and quantification was performed as described previously.[32] Serum samples were considered as seropositive for AAV antibodies when the measured OD was 0.2 or higher at a 1:300 dilution after subtraction of background.

Neutralization assay

HeLa cells or B16F10 cells were seeded in 96-well plates (5×10^3 cells per well) and infected with AdV (MOI 5) or irradiated 2 h prior to infection with AAV, respectively. Identical transducing particle numbers (MOI 5) of rAAV (wt capsid) and rAAV-587/L14 were incubated with serial dilutions (1:10 to 1:1200) of human serum in PBS for 2 h at 4°C in a total volume of 30 μl. Before addition of the AAV/serum-mixture medium was replaced by 100 μl of fresh medium. At 48 h (HeLa) or 72 h (B16F10) after infection GFP-positive cells were detected by FACS analysis and fluorescence microscopy. Similarly, Mec1 cells were seeded at 5×10^4 cells per well, infected with AdV followed by infection with rAAV or rAAV-587/MecA, which had been incubated with serial dilutions of human serum as described above. The neutralizing titers are expressed as the dilution at which transduction was 50% reduced compared to the positive control (N_{50}). Serum samples were considered as neutralizing when the N_{50} was 1:320 or higher.

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