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RESEARCH ARTICLE

Combinatorial engineering of a gene therapy vector: directed evolution of adeno-associated virus

Luca Perabo[1,3†*]
Jan Endell[1,3†]
Susan King[2]
Kerstin Lux[1]
Daniela Goldnau[1,3]
Michael Hallek[1,2,3,4]
Hildegard Büning[1,3,4]

[1]*Gene Center, Ludwig-Maximilians-University of Munich, Germany*

[2]*GSF-National Research Center for Environment and Health, Munich, Germany*

[3]*Clinic I for Internal Medicine, University of Cologne, Germany*

[4]*Center for Molecular Medicine, University of Cologne, Germany*

*Correspondence to: Luca Perabo, Gene Center of the Ludwig-Maximilians-University of Munich, Feodor-Lynen Str. 25, 81377 Munich, Germany.
E-mail: perabo@lmb.uni-muenchen.de

†These authors contributed equally to this work.

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Abstract

Background Viruses are being exploited as vectors to deliver therapeutic genetic information into target cells. The success of this approach will depend on the ability to overcome current limitations, especially in terms of safety and efficiency, through molecular engineering of the viral particles.

Methods Here we show that *in vitro* directed evolution can be successfully performed to randomize the viral capsid by *error prone PCR* and to obtain mutants with improved phenotype.

Results To demonstrate the potential of this technology we selected several adeno-associated virus (AAV) capsid variants that are less efficiently neutralized by human antibodies. These mutations can be used to generate novel vectors for the treatment of patients with pre-existing immunity to AAV.

Conclusions Our results demonstrate that combinatorial engineering overcomes the limitations of rational design approaches posed by incomplete understanding of the infectious process and at the same time offers a powerful tool to dissect basic viral biology by reverse genetics.

Keywords AAV vectors; directed evolution; combinatorial library; pre-existing immunity; human gene therapy; capsid modification

Introduction

The viral families (e.g. adeno-, retro-, herpes- and adeno-associated viruses) used as gene transfer vectors for human gene therapy [1] all have limitations that need to be addressed. Common concerns are safety, viral tropism (target specificity), immunogenicity, ability to elicit strong and stable transgene expression, and the possibility to produce the vector at high titers [2,3]. Recently, the adeno-associated virus of type 2 (AAV-2) has received increasing attention as a vector [4]. AAV-2 is non-pathogenic in humans, does not induce a strong immune response, and can transduce both dividing and quiescent cells. Viral particles are stable and can be produced at high titers. Current efforts to improve AAV vectors aim to control the tropism of the vector and to overcome barriers to infection such as neutralization by human antibodies [5–10]. Although unveiling of the atomic structure of AAV-2 [11] boosted these efforts, the manipulation of the virions by rational design remains a difficult task due to our still incomplete knowledge of the capsid biology. Therefore, the goal of engineering tailored viral vectors could be achieved more easily if one could take advantage of combinatorial techniques that have been extensively used to optimize protein function in the past decade [12–19].

Recently, two reports demonstrated that AAV peptide-display libraries can be generated by insertion of randomized peptides at a specific capsid location [20,21]. These viral libraries could be screened for the selection of receptor-specific clones that infected wild type (wt) AAV-2-resistant cell types. However, ultimate optimization of gene therapy vectors will require manipulation of the whole capsid to be able to control different features of viral biology. In other studies, different strains of murine leukemia virus (MLV) were bred by DNA shuffling of their envelope genes to modify their tropism and stability [22,23]. Here we describe a novel *directed evolution* approach based on randomization of the viral capsid by *error prone polymerase chain reaction (PCR)*. To demonstrate the potential of this technology we applied it for the selection of AAV vectors that escape neutralization by human antibodies.

Materials and methods

Generation of a randomized AAV library and rAAV mutants

Error prone PCR was performed (primers: 5′-AAT GAT TAA CCC GCC ATG CT-3′ and 5′-GGT ACG ACG ACG ATT GCC-3′) on the fragment of the *cap* gene of AAV-2 coding for amino acids 353 to 767 (VP1 numbering). Target DNA (150 ng) was amplified in a 50-µl PCR using 0.2 mM dNTPs, 0.4 mM primers, 1 unit of Mutazyme II and 5 µl reaction buffer (GeneMorph II, Stratagene). The reaction was conducted as follows: 95 °C/2 min; 35 cycles × (95 °C/30 s, 48.5 °C/30 s, 72 °C/100 s); 72 °C/10 min. The amplified DNA was digested with BsiWI and SnaBI restriction enzymes and cloned in an AAV-2 genome-containing plasmid (pUC-AAV2 [6]) to replace the corresponding wt sequence. A DNA library of approx. 2.5×10^7 clones was obtained by electroporation into XL1-Blue MRF' *E. coli*. The entire mutagenized region of 96 clones was sequenced to determine the average rate of mutations (5.7 mutations/clone) and to verify the random distribution of amino acid substitutions. Viral library and rAAV mutants were produced and titrated as previously described [20,24]. Empty particles were generated by transfecting 293 cells with a 1 : 2 molar ratio of pXX6 (helper plasmid) and a Rep-Cap-containing plasmid which is devoid of AAV-packaging signals (inverted terminal repeats, ITRs).

Selection protocol

HeLa cells (2×10^6) were seeded on 150-mm Petri dishes 24 h before infection. The 29 human sera were obtained with informed consent from the Klinikum Großhadern (Munich, Germany), and tested for their ability to neutralize AAV-2 infection [5]. A strongly neutralizing serum (10 µl) [5] and 2×10^{10} genomic particles of the viral library were incubated in 10 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) for 2 h at 4 °C. The amount of viral particles used corresponded to an multiplicity of infection (MOI) of 50 and allowed us to apply approximately up to 1000 copies of each viral clone increasing the possibility that efficient clones could really infect at least one cell, whilst keeping the risk fairly low of generating chimeric viruses with no correspondence between genotype and phenotype. The solution was diluted in an additional 10 ml of DMEM/10% FCS and used to incubate the cells in the presence of adenovirus (700 plaque-forming units (pfu)/cell). Forty-eight hours post-infection cells were collected by centrifugation and resuspended in lysis buffer (150 mM NaCl, 50 mM Tris-Cl, pH 8.5). Cells were lysed by three rounds of freeze/thawing and debris was removed by centrifugation. Adenovirus was heat-inactivated (60 °C, 30 min) and the viral progeny-containing supernatant was used for further selection rounds. After each round, viral DNA was extracted from 200 µl of the lysate and sequenced.

Sequence analysis

Sequencing was performed (Agowa GmbH, Berlin, Germany) after DNA extraction and PCR amplification from cultured bacteria (DNA library) or from viral preparations (viral library and viral pool after selections). Single viral genomes were obtained by cloning amplified DNA into pUC-AAV2 and electroporation into *E. coli* (XL1-Blue MRF'). For each analysis >70 clones were sequenced.

Infection assays

HeLa cells (2×10^4) were seeded in 48-well plates 24 h before infection. Identical numbers of transducing (MOI of 5) and total particles (6.5×10^8, adding wt AAV-2 empty particles when needed) were incubated with serial dilutions (1 : 40 to 1 : 2560 in phosphate-buffered saline (PBS)) of human sera for 2 h at 4 °C in a total volume of 40 µl. Before addition to the cells, DMEM/10% FCS (240 µl) was added to each sample. At least 5000 cells were analyzed by fluorescence-activated cell sorting (FACS) (Beckman Coulter XL/MCL) 48 h post-infection to determine the amount of green fluorescent protein (GFP)-expressing cells. For determination of the decoy effect of wt and double mutant capsids, 2×10^4 HeLa cells were seeded in 48-well plates 24 h before infection. No empty particles, or 3.9×10^8 of either empty wt or double mutant particles, were added to identical amounts of double mutant total particles (1.7×10^7). Incubation, infection and FACS analysis were performed as described above.

Heparin inhibition assay

Twenty-four hours prior to infection, 2×10^4 HeLa cells were seeded per well in 48-well plates. Equal amounts of

infectious particles (MOI of 5) were incubated in a total volume of 200 µl of DMEM/10% FCS in the presence or absence of soluble heparin (85 U/ml) at 37 °C for 30 min. This solution was then used to infect cells. Forty-eight hours post-infection the percentage of transduced cells was determined by FACS analysis.

Results

Production of a library of AAV-2 particles with random capsid mutations

A library of 2.5×10^7 capsid variants with scattered point mutations throughout the capsid protein gene (*cap*) of AAV-2 was obtained by *error prone PCR* (Figure 1). The PCR primers allowed us to target 82% of the amino acids expressed on the viral surface, minimizing at the same time mutagenesis of the structural core of the capsid subunit [11] in order to reduce loss of biodiversity after packaging. Sequence analysis confirmed that after PCR the mutations were evenly distributed along the *cap* gene. As expected, after packaging of the DNA pool into viral particles, a major amount of biodiversity was lost and the average number of amino acid mutations per clone dropped from 5.7 to 0.9, suggesting that clones with impaired assembly were eliminated. In addition a preferential localization of the mutations on surface epitopes was observed (data not shown). This can be explained by a greater tolerance of the surface structure, mainly composed of flexible loops [11], to structural modifications.

Selection of capsid variants that escape human serum neutralization

Depending on age and ethnic group, between 50 and 96% of the human population is seropositive for AAV-2, and 18–67.5% of these individuals have neutralizing antibodies [25–27]. Animal experiments have shown that neutralizing antibodies may reduce or even prevent AAV-2 transduction *in vivo* [28,29]. For this reason it is likely that application of AAV-2 vectors to patients with pre-existing immunity would require higher viral doses. Although other AAV serotypes could prove useful to escape neutralization by anti-AAV-2 antibodies, different AAV serotypes often have different tropism, restricting this possibility. Moreover, since it is likely that even immune-escaping mutants will trigger the production of neutralizing antibodies, it is desirable to increase the number of capsid alternatives at our disposal for repeated applications of the vector.

Therefore, we designed a biopanning protocol for the selection of viral mutants that are less efficiently neutralized by human antibodies (Figure 1). The randomized library was pre-incubated with an AAV-2 neutralizing human serum and applied to a cell line (HeLa) that supports AAV-2 replication. In a control experiment we pre-incubated the library with an AAV-2-seropositive but non-neutralizing human serum. In both cases, to support AAV replication, cells were coinfected with the maximum amount of Ad that allowed infection of cells without inducing cytotoxic effects after 48 h, therefore maximizing the probability that the helper effect is provided to all AAV-infected cells. Viral progenies were harvested 48 h post-infection and applied to new cells for further selection rounds. Thus the amplification of the clones carrying successful mutations occurs during the virus infection cycle obviating the need to introduce artificial re-amplification steps. These experimental conditions apply a selective pressure to the initial viral population and the pool is progressively enriched with mutants that are better able to infect the cells despite the presence of neutralizing antibodies. It is important to note that in this way viruses are not only selected for their ability to escape antibody binding, but also for their overall biological fitness (e.g. efficiency of infection and progeny production).

Characterization of neutralization-escaping mutants

After each selection round the viral progeny DNA was extracted from cell lysates and single viral clones were analyzed by sequencing. After three selection rounds, three point mutations occurring at two different amino acid positions were strongly selected (Figure 2A): an arginine to glycine mutation (R459G according to VP1 numbering) was found in 9 of 94 sequenced clones, an arginine to lysine mutation (R459K) was found in 3 clones, and an asparagine to aspartate mutation (N551D) was found in 17 clones. Two clones carried the double mutation R459K-N551D. Mutations of 22 other residues occurred only once. Of the 31 clones carrying a mutation in either of the two frequently mutated sites, 25 did not carry any additional amino acid substitution at other sites. Strikingly, although these two residues are separated by 92 amino acids in the primary sequence of the protein, they are located very closely in the three-dimensional structure of the viral particle (Figure 2B). Moreover, these residues are located on the capsid surface and are therefore accessible to antibody binding, suggesting their importance as immunogenic residues. Although N551 has lower surface exposure than R459 [11], it has been previously reported that even amino acids that are buried in the inner VP3 protein structure can nonetheless influence the surface topology and biology of the virus [9,30]. Moreover, once the N is exchanged with a D, this latter residue could find its way through the structure and become more exposed on the surface, where it could impair the binding of a pre-existing antibody.

Remarkably, mapping all the other (less frequently occurring) selected sites on the three-dimensional structure shows that 73% of all the recovered mutation sites clustered in the same capsid region and are

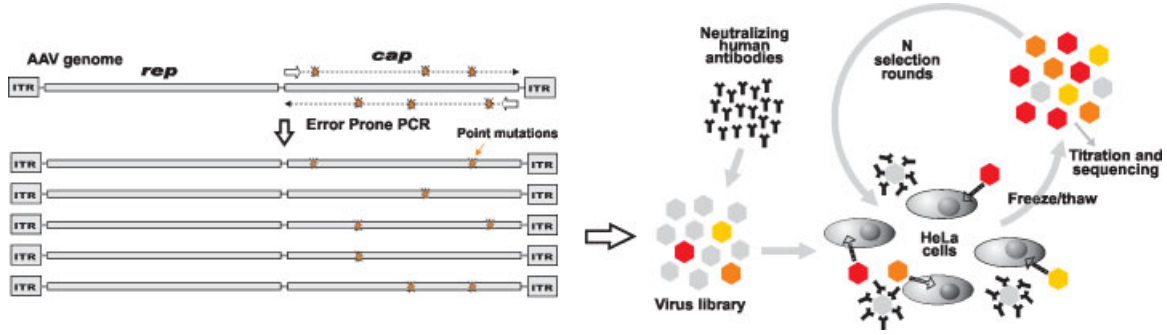

Figure 1. Production of a library of viral particles by *error prone PCR* of the AAV-2 capsid gene (*cap*) and biopanning protocol. ITRs (inverted terminal repeats) are the packaging signals of the AAV genome. The *rep* gene codes for four proteins involved in viral replication

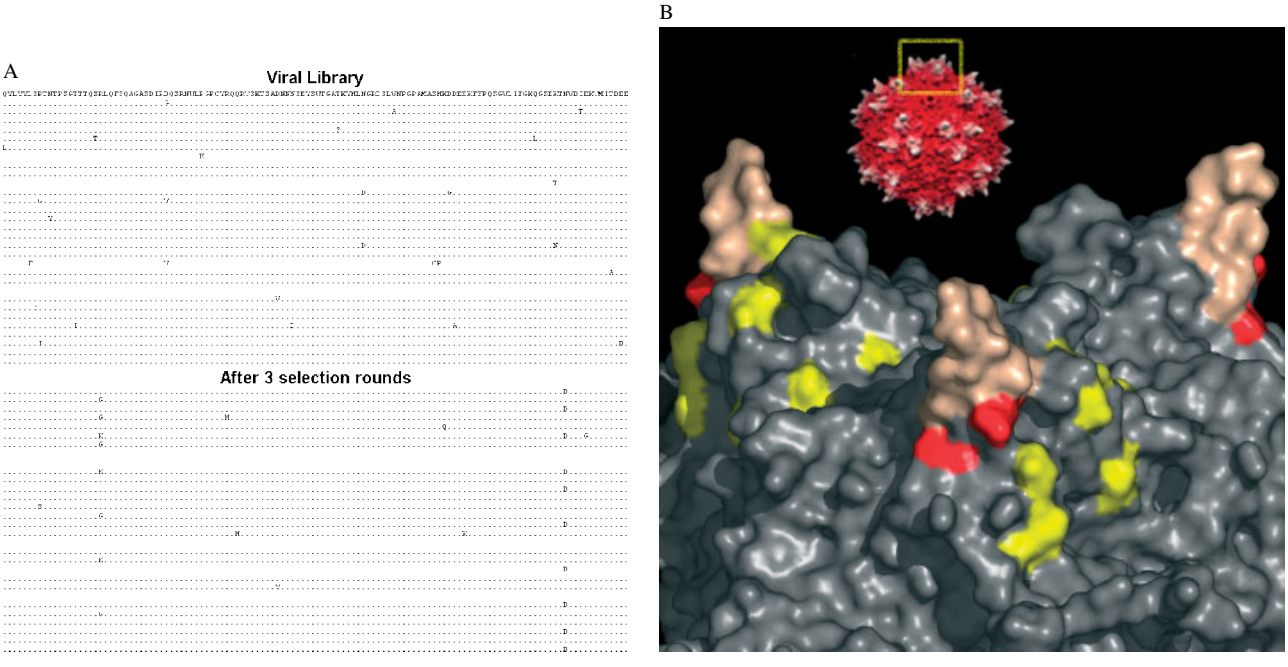

Figure 2. (A) Selection of neutralization-escaping viral mutants. The sequence of 30 representative clones is presented to show the biodiversity of the viral library and of the viral pools after selection in the presence of a neutralizing human serum. (B) Mapping of the selected amino acid mutations on the capsid structure. Red: frequently occurring mutations (positions 459 and 551). Yellow: other selected mutations mapping in the same region; these appeared only once out of 94 sequenced clones and represent 73% of the overall number of identified mutations. The three peaks are symmetrical and coloured to facilitate the visualization of the capsid structure. The yellow box on the complete viral particle (adapted from Xie *et al.* [11], Copyright 2002 National Academy of Sciences, USA) depicts the magnified region

expressed on the capsid surface (Figure 2B). A previous epitope-scanning study failed to identify this region as immunogenic [31]. Another study described an immunogenic epitope that only partially co-localizes with this region and does not include N551 [25]. The residues identified by our approach are part of a quaternary structure that is composed of protein loops contributed by different capsid subunits. This structure cannot be mimicked by a single peptide fragment, suggesting why previous studies could have failed to identify it.

Eight of nine mutations in this cluster involved electrostatically charged amino acids where substitutions resulted in loss of the charge. The only exceptions were the three R459K mutations, which conserved the positive charge. Interestingly, however, in two cases the R459K mutation was combined with the N551D mutation, a combination which showed a clearly increased fitness in comparison to the single mutants (see below). After the first round of selection, the R459G mutation appeared in 9 of 96 examined sequences. One clone carried a R459W mutation that could not be recovered after three selection rounds. The N551D mutation appeared only in three clones. Two of these latter clones carried an R459G mutation at the same time. This double mutant was not recovered after the third selection round. Although no experiment was conducted to investigate the reason for this, we can speculate that when expressed in conjunction with the N551D mutation, the R459G mutation is not as well tolerated by the capsid as the R459K mutation, which could represent a milder structural rearrangement.

Understanding these properties of the viral particle and fine tuning capsid mutations to optimize the phenotype by rational design would have required huge experimental efforts.

A control selection performed with the library in the presence of a non-neutralizing human serum yielded wt AAV-2 clones in 68 of 79 cases (data not shown). Each of the 11 mutants carried a single point mutation. The 11 mutations were scattered throughout the capsid sequence and occurred only once; only three of them involved a charge shift. These results suggest that the wt capsid (that has already been subject to natural selection pressure) is the structure that is most fitted to the task of performing a productive infection on HeLa cells in the presence of an AAV-2 non-neutralizing human serum. This control also demonstrates that the selection of mutants in the presence of neutralizing antibodies is not due to other factors (e.g. the presence of adenovirus). To exclude that immune-escaping viral mutants can be generated through spontaneously occurring mutations (in the absence of capsid modification by error prone PCR), wt AAV-2 was applied to HeLa cells in the presence of neutralizing antibodies and three selection rounds were performed (same experimental conditions as for the real selection). None of 30 sequenced viral clones was found to carry a single point mutation.

To confirm that immune-escaping viral mutants had been selected, recombinant GFP-expressing AAV particles carrying the mutations R459G, N551D or the R459K-N551D double mutation were produced. Genomic and particle titers of the mutants showed no significant difference to wt vector titers (Table 1). This was expected because the selection process should yield clones with good packaging efficiency since mutations interfering with efficient packaging are assumed to be negatively selected when the viral progeny is generated. However, to demonstrate that this kind of selection really applies, evaluation of titers obtained from a larger repertoire of mutants would be required. Infectious titers showed a better infectivity of the double mutant in comparison to wt and single mutant clones (Table 1). This could reflect the selective pressure applied to the pool during the selection procedure. An increase in the infection efficiency (e.g. ability to bind to cellular receptors or to perform post-entry steps) would contribute to the amplification of a mutant additionally to its ability to escape neutralization. The selected mutations could not only allow escape from antibody neutralization, but at the same time improve the generic efficiency of the infection process. However, further investigations are required to confirm this observation and this hypothesis.

Equal infectious titers were used to infect HeLa cells in the presence of serial dilutions of the same neutralizing human serum that was used for the selection (Figure 3). In addition, amounts of total viral particles were normalized by addition of AAV-2 empty capsids in order to exclude the influence of particle titers on antibody sequestration. The amount of serum needed to halve the number of transduced cells was defined as N_{50}. N_{50} values for wt virus and mutants were determined using Figure 3. The values obtained for R459G, N551D and R459K-N551D were respectively 4.1-, 3.3- and 5.5-fold higher than the corresponding N_{50} values obtained for wt, demonstrating that the selected mutations improved the ability of the virus to escape neutralization. Similar results were obtained after pre-incubation with AAV-2-neutralizing sera obtained from seven different donors: all tested sera showed a weaker neutralization of the double mutant in comparison to wt (single mutants were not tested). N_{50} values of R459K-N551D were 1.3- to 5.3-fold higher than for wt AAV-2 (data not shown), demonstrating that the ability to escape neutralization was not limited to the particular serum used for the selection.

Additionally, to further prove that the selected mutations diminish antibody recognition of the viral capsids, the ability of wt and mutant empty particles to act as decoys for human neutralizing antibodies was tested by infecting HeLa cells with the R459K-N551D mutant in the absence or presence of wt or R459K-N551D empty particles (Figure 4). When empty particles carrying the double mutation were added, the N_{50} value was only slightly increased (+35%). However, the N_{50} value was substantially increased (+190%) if the same amount of empty wt particles was added, showing that wt capsids were better at sequestering the neutralizing antibodies which hindered transduction. The ratio between these values calculates to a 5.4-fold higher increase after addition of wt empty particles compared to the addition of double mutant empty particles. This is in good agreement with the 5.5-fold value obtained from Figure 3. No differences in infectivity were detectable in the infections performed with additional empty capsids

Table 1. Viral titers per μl of GFP-expressing rAAV-2 and selected mutants

	Genomic titer	Infectious titer	Particle titer	Heparin inhibition
wt	1.2 × 10(8)	5.9 × 10(5)	1.49 × 10(9)	99.2%
R459G	3.45 × 10(8)	4.9 × 10(5)	1.65 × 10(9)	98.7%
N551D	4.18 × 10(8)	8.8 × 10(5)	2.2 × 10(9)	99.4%
Double mutant	2.36 × 10(8)	1.8 × 10(6)	1.36 × 10(9)	99.4%
AAV2 Empty particles	–	–	1.25 × 10(9)	n.d.
Double mutant Empty particles	–	–	1.58 × 10(9)	n.d.

Titers in this table refer to the viral preparations used for the infection experiments shown in figures 3 and 4. All mutants have been packaged at least twice resulting in titers consistant with those provided here.

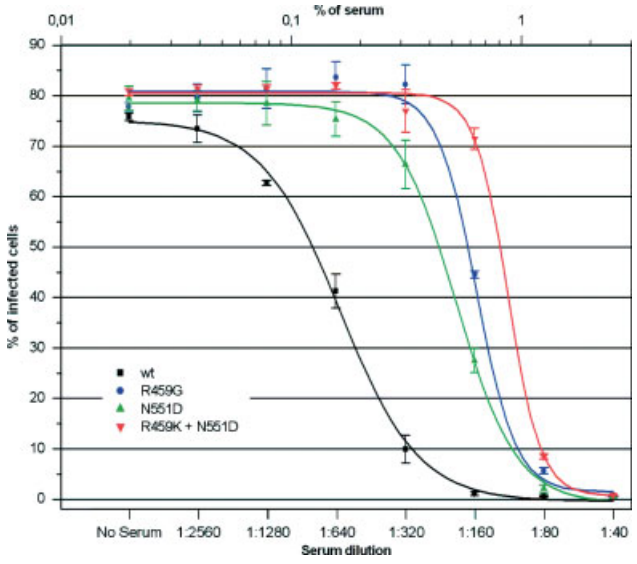

Figure 3. Transduction of HeLa cells in the presence of an AAV-2-neutralizing serum. Cells were infected with same infectious titers of GFP-expressing rAAV virions. Particle titers were adjusted by the addition of wt AAV-2 empty capsids. Black: wt rAAV-2; blue: R459G mutant; green: N551D; red: double mutant (R459K + N551D). Results are expressed as mean and standard error of triplicate values. Serum concentrations are expressed on the x-axis as dilution factor (below) or as percentage to facilitate calculation of N_{50} values (above)

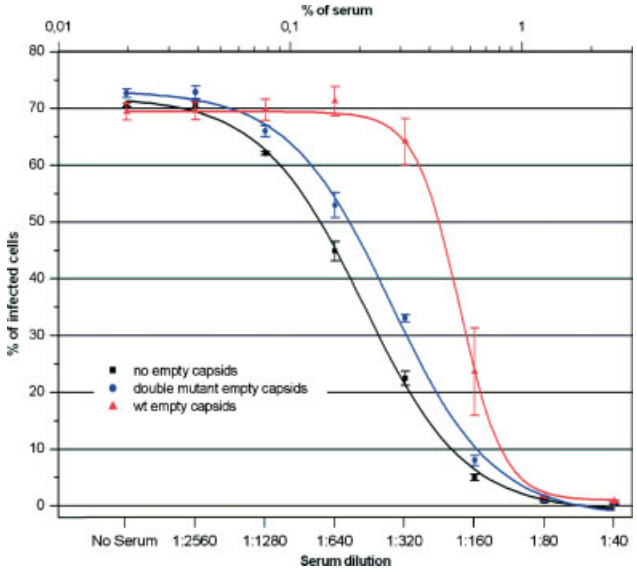

Figure 4. Decoy activity of wt and mutated empty capsids. HeLa cells were infected with R459K-N551D in the presence of neutralizing serum without addition of empty particles (black line) or after addition of double mutant empty particles (blue line) or wt empty capsids (red line). Serum concentrations are expressed on the x-axis as the dilution factor (below) or as percentage (above)

without serum, showing that the differences observed using wt and mutant empty particles were not due to a different level of cellular receptor competition for the viral attachment or any other infection step. In conclusion, these experiments demonstrate that the mutations reduce the affinity of neutralizing antibodies for the viral capsids.

Finally, we investigated if the selected capsid mutations had an influence on the viral tropism. We used soluble heparin, an analogue of the AAV-2 primary receptor heparin sulfate proteoglycan (HSPG), to show that cell transduction could be blocked in a similar manner and to a similar level to wt AAV-2 (Table 1). Moreover, tropism of the immune-escaping clones was unchanged in comparison to wt AAV-2 when tested on HeLa (Table 1), a very permissive cell line for wt AAV-2, and on M-07e (data not shown), a wt AAV-2-resistant cell line [20].

Discussion

These results demonstrate that randomized mutagenesis of the viral capsid can be successfully applied for the generation of improved viral vectors for gene therapy. The described protocol for the production of a viral library is based on transfection of cells with a pool of viral genomes. Since it is possible that some cells are transduced by more than one AAV genome, this procedure yields a certain amount of chimeric viral particles where the genotype is not coupled to phenotype, as demonstrated by the presence of viral DNA sequences carrying stop codons, an event that can be explained only by genotype-phenotype uncoupling. Although this could initially lead to a loss of biodiversity of the viral library in comparison to the DNA library, after the first selection round no stop codons could be detected in the viral pool demonstrating that, as expected, the selection process eliminates such chimeric particles. The initial loss of biodiversity could be avoided by initially infecting permissive cells with AAV hybrid (wt and mutant) virions at low MOI [21]. Although it was not necessary to perform this step for the experiments presented here, this solution could be recommended for screenings where the biodiversity could represent a limiting factor for the success of the procedure.

The mutants described here carry amino acid substitutions that involve changes in charged amino acids and might therefore alter regional surface electron potential, local p*K*a, or even local quaternary structure, and might affect a significantly larger region of the AAV capsid surface. Based on this, it is currently unclear whether the strongly selected amino acid mutations correspond to immunogenic sites or if their substitution exerts influence on nearby capsid regions, maximizing phenotypic change per single genotypic change. However, the clustering of the selected mutations suggests an immunogenic site to be located on the external side of the three-fold symmetry peak.

Despite increased ability to escape neutralization, the mutants generated can still be inactivated by relatively high sera dilutions and further optimization will be required for clinical application. However, the double mutant was more efficient than single mutants suggesting that better variants could be generated by combining several additional mutations on one capsid at the same time. This is also suggested by the observation that

polyclonal antibodies contained in human serum are probably able to interact with different epitopes on the viral capsid. Complete escape from neutralization will require engineering of all these distinct epitopes. Given the limited size of the library described here, and considering the low average number of mutations per clone, combinations of multiple mutations are unlikely to be selected. Application of increasing selective pressure or performing further selection rounds could have led to the identification of some of these mutants. However, these will be more easily isolated by screening libraries obtained by consecutive rounds of mutagenesis and selection or by gene shuffling of previously mutants used as parental strains [14,19]. High-throughput breeding of different retrovirus strains yielded viral vectors with increased stability and with modified tropism in previously reported experiments [22,23]. This interesting approach could be also applied to AAV. It is noteworthy that this procedure usually yields complex chimera derived from several parental strains, from which it is difficult to understand how the selected mutations contribute to the phenotype. It should be also noted that despite a clear selection for the two described mutations, the number of recovered wt clones after the third selection round was still high. Additionally, the same experimental setup allowed us to recover viral clones after three rounds of selection applied to wt AAV-2. This was possible because the selective pressure was kept low by the high serum dilution used in these experiments. Moreover, the ability of the selected mutants to escape neutralization could be proven for all the seven AAV-2 neutralizing human sera tested but ranged quantitatively, suggesting that the pool of neutralizing antibody differs from one individual to the other. Taken together, these observations suggest that introduction of the refinements proposed here to the library, in parallel with application of a stronger selective pressure (increasing serum concentration and/or pooling of different sera), should result in the selection of capsid variants with greater ability to escape antibody neutralization. In addition, due to the statistical nature of the process, it is likely that other important immune-escaping mutations could be identified simply by repeating the selection experiment described here. These speculations are also supported by the observation that we only identified a maximum of two alternative amino acids for every selected mutation site while it is reasonable that other amino acid substitutions could generate clones with similar or even improved phenotype. Moreover, the identified amino acid substitutions originated in all cases from the same codon types (no alternative codons for the same amino acid were found). These observations are in agreement with statistical expectations from a library of 10^7 clones that cannot represent all the possible combinations of 400 positions and 20 amino acids and once more underline the importance of upgrading the selection protocols with repeated mutagenesis and DNA shuffling.

Heparin-binding phenotypes and the infection experiment performed on a cell line (M-07e) that is not permissive to wt AAV-2 infection suggest that the mutations described here do not influence the tropism of the mutants. However, it cannot be excluded that tropism alterations could be detected on other cell types or induced by different immune-escaping mutations that could be identified with error prone PCR technology. In this case, protocols should be applied to specifically select mutations that do not interfere with the desired viral tropism. This can be achieved by alternating infections in the presence of neutralizing antibodies and infections on target cells.

It remains to be elucidated whether these novel mutants would generate neutralizing human antibodies after their first application. However, even in this case, one or more immune-escaping clones could provide patients with pre-existing immunity with the chance to receive at least one or a few successful vector applications. The pool of capsid mutations required for this goal could be generated by combined efforts that employ different mutagenesis techniques. Finally, successful escape from neutralization *in vivo* will require the resolution of a number of additional factors. Some of these complications can be addressed by the further development of capsid randomization technology and by setup of selection procedures that more closely mimic the *in vivo* environment. However, the production of ideal vectors is likely to be achieved by designing procedures that exploit combinations of several different engineering approaches including educated guesswork.

We anticipate that further development of this technology could yield virus variants with modified tropism, increased genome size capacity, and reduced toxicity. Finally, the study of viral biology will benefit from the use of such combinatorial techniques as reverse genetics.

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