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Current Pharmaceutical Biotechnology, 2013, *14*, 000-000

Developing Immunologically Inert Adeno-Associated Virus (AAV) Vectors for Gene Therapy: Possibilities and Limitations

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Abstract: Gene therapy has become a clinical reality as demonstrated by remarkable benefits seen in Phase I/II clinical trials for hemophilia B, lipoprotein lipase deficiency and Leber's congenital amarousis. The choice of, and the improved understanding in vector characteristics have contributed significantly to this success. The adeno-associated virus (AAV) vectors used in these trials have been long known to be relatively safe and efficacious. However, certain factors, most notably host immunity to the vector, prevent their widespread use. In patients who have pre-existing antibodies to AAV, these vectors will be rapidly cleared. Administration of a relatively high initial dose of vector to achieve and sustain a higher margin of therapeutic benefit is limited by concerns of vector dose-dependent T cell response. Frequent vector administration necessitated by the non-integrating nature of the virus is difficult due to the variable, yet significant host immunological memory. Thus generation of AAV vectors that are immunologically inert is pivotal for the long-term success with this promising vector system. Several strategies, that aim targeted disruption of antigenic sites or those that chemically modify the vectors have been proposed for host immune evasion. While these approaches have been successful in the pre-clinical model systems, this continues to be a field of intense experimentation and constant improvisation due to limited information available on vector immunology or data from human studies. This review forms a comprehensive report on current strategies available to generate immunologically inert AAV vectors and their potential in mediating long-term gene transfer.

Keywords: Adeno-associated virus, B cell response, Gene therapy, Immune evasion, Immune response, Stealth vector, T cell response.

INTRODUCTION

Even though a variety of methods have been developed for introducing the defective gene (exogenous DNA sequences) into a target cell, viruses have generally been efficient for this purpose. Among the currently available viral vector approaches, gene delivery based on the non-pathogenic adeno-associated virus (AAV) is thought to be potentially safer than more commonly used retroviral and adenoviral vectors [1, 2]. Recombinant AAV vectors have been successfully used to transduce a variety of genes in a number of cell types *in vitro* and the ability of these vectors to mediate persistent transgene expression has been proven in a number of small and large animal models *in vivo* [3]. AAV serotype 2 (AAV2) is the prototype AAV vector that has been extensively studied [4, 5]. Although ~80% of the human population is sero-positive for AAV2 [6], to date, no known disease has been associated with AAV2 infection in humans. AAV vectors have been successful in phase I/II studies for hemophilia B, cystic fibrosis, alpha-1 anti-trypsin deficiency, Parkinson disease, Duchenne muscular dystrophy and Leber's congenital amaurosis [7-12]. However, it is also known that relatively large vector doses are needed to enhance the therapeutic benefit, which triggers an immune response against the transgene or the viral capsid. An exception to this is AAV mediated ocular gene transfer, where relatively low doses of vectors are efficient and the immune privileged status of the eye reduces the risk of immune response as shown during RPE65 gene transfer in non-human primates or in humans [13-15]. Several reports have shown correlation between vector re-administration and loss of transgene expression *via* the generation of neutralizing antibodies and T cell mediated destruction of transduced host cells [16] (Fig. **1**). Recombinant AAV offers immense potential in gene therapy if the immune response blockade can be resolved or minimized; this has also led to search of non human AAV serotypes [17] and studies on different naturally occurring serotypes from AAV1-9 [18]. In addition, a variety of molecular tools have been applied to generate vectors with immune evasion properties [19]. This review provides a comprehensive analysis of strategies that have been employed for generating immunologically inert AAV vectors and their potential use in gene therapy applications.

PRINCIPLE OF VIRAL IMMUNE EVASION

Viral genomes are known to contain overlapping protein coding sequences and reading frames. This gene overlap is present in many viruses such as Papilloma virus, Simian Immunodeficiency Virus, Human Immunodeficiency Virus (HIV) and Hepatitis B virus [20]. This genomic design has

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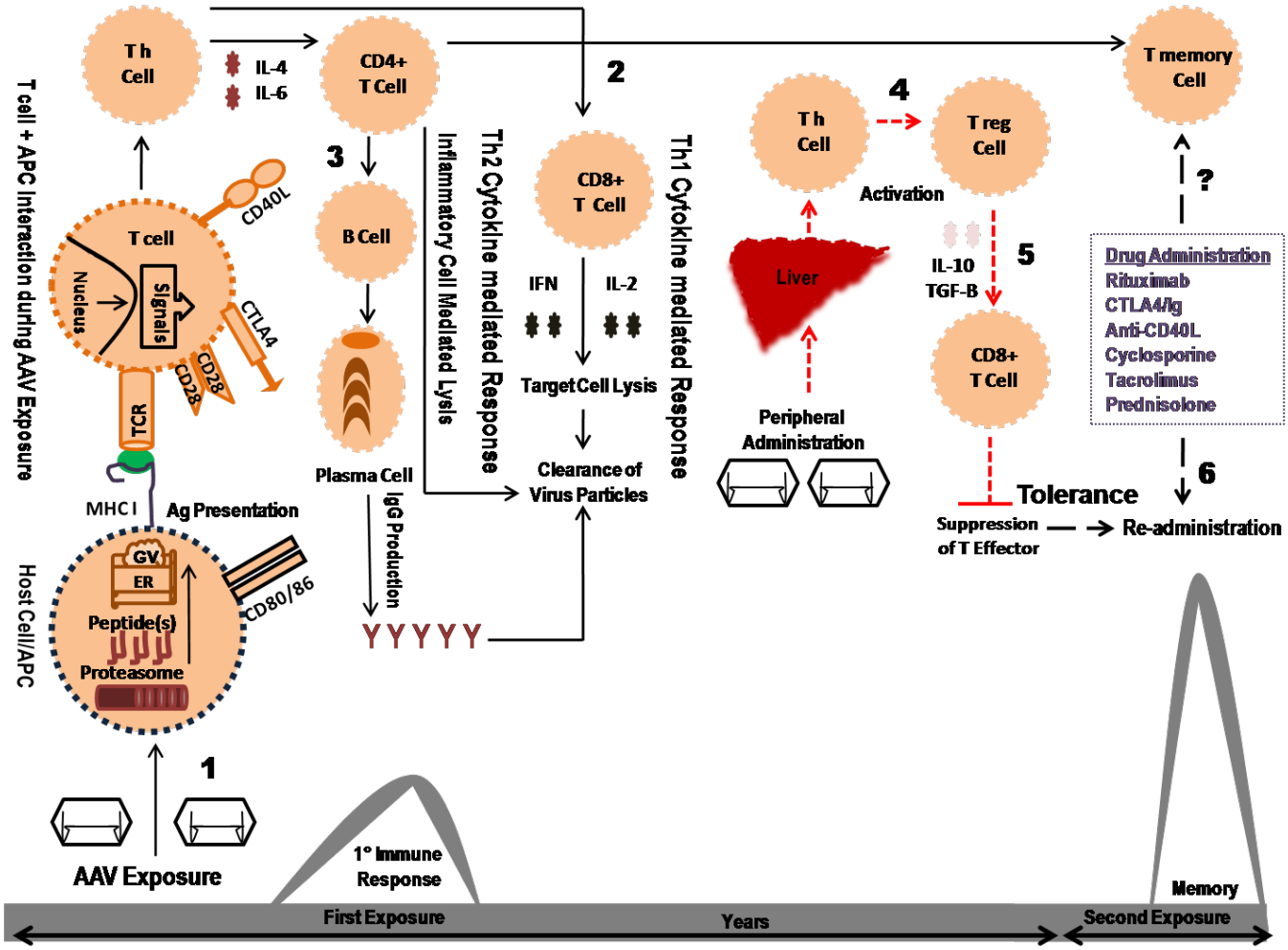

Fig. (1). Mechanisms of immune response directed against AAV vectors. Immune response due to AAV infection differs in intensity (shown by peak of time scale drawn below) during initial/primary and secondary immune response events. Variability in immune response to AAV also depends on route of administration [62]. **(1)** AAV infection results in host cell/APC mediated capsid antigen presentation to T cells and T cell activation in target tissue. **(2)** CD4+ T cell and CD8+ T cell activation is mediated by cytokine secretion [98] [99] which causes cyto-toxicity towards AAV transduced cells. CD8+ T cell mediated IL-2 and IFN-Y secretion results in acute Th1 responses [90] [100] [101]. CD8+ T effector cells undergo rapid proliferation and migration to peripheral sites of infection, where CD8+ T cell receptor (TCR) engagement with antigen plus MHCI molecules results in recognition and destruction of transduced cells [102]. CD4+ T cell mediated IL-4 and IL-6 secretion helps in chronic Th2 responses and T memory cell generation. **(3)** T lymphocyte dependent B cell activation leads to immunoglobulin class switching, affinity maturation and production of IgG subclasses [103] [104] [105] via plasma cells [98]. **(4)** T memory cells are evoked faster than T naïve cells upon re-exposure to AAV; CD4+ T cell help is critical for this memory and immune surveillance**.** Regulatory T cells (Tregs), a CD4+ T cell subset develop and result in active suppression of adaptive immune effectors [103] [75] in absence of necessary pro-inflammatory cytokine milieu. The pre-dominant subclass Tregs express high CD25 and transcription factor Foxp3 (CD4+CD25+FoxP3+). **(5)** Treg can actively suppress CD8+ T effector cells by secretion of TGF-B and/or IL-10 and inhibit T response development [99], this results in tolerogenic response of liver to AAV re-administration. **(6)** Due to limitations encountered in complete abrogation of immune response, recent AAV administration reports have used immuno-suppressive regimens (as shown) to overcome T and B cell mediated virus destruction [70] but their effect on immunological memory has not been elucidated. APC-Antigen presenting cell, GV-Golgi vesicle, ER-Endoplasmic reticulum, Th-T Helper cell, Tc-T Cytotoxic cell, Treg-T Regulatory cell, IL-Interleukin, IFN-Interferon, IgG-Immunoglobulin. The arrows and numbers represent pathways and corresponding events, respectively.

benefits and flaws, in using compact nucleotide sequences for different proteins, but with structural restrictions for the protein encoded. Any mutations in closely spaced genes might result in sterically or functionally affected proteins. It thus becomes important to understand resultant epitopic changes that contribute to alterations in structural or functional properties of the virus as well as any evolutionary advantage conferred. In context of animal viruses, immune escape phenotype variants have been envisioned as an error-proof mechanism to encounter the barriers posed by immune system at various stages of host-pathogen interactions. The route of entry and subsequent infection by viral particles are the nodal points of evasion machinery for a virus, to overtake the host machinery and to create a fine balance between the

replication cycle and cellular metabolism for its propagation. HIV is known as an efficient virus with the property to rapidly evade virus specific CD8+ T lymphocyte response [21, 22]. This inherent property is due to the error prone reverse transcriptase activity during acute and chronic HIV infection which results in cytotoxic lymphocyte escape mutations. These mutations are intra-epitopic and affect HLA binding or TCR interactions, hence leading to decreased CTL response. Interference with antigen processing may result in reduced peptide generation and peptide/MHCI complex formation for T cell activation. CTL recognition escape mutations may also be due to changes in proteasomal processing or N-terminal trimming in epitope-flanking regions. Cardinaud *et al.* [23] have shown a correlation between mutations on HIV-1 genome and the viral fitness; this proof of concept study has also shown absence of correlation between redundancy in codon usage and viral replication. Taking cue from such examples of highly selective yet flexible viral adaptive mechanisms, the concept of viral immune escape mutations has begun to emerge in conventional and translational gene therapy research.

It must be noted that the cellular events that contribute to an immune response and the principle on which they operate against any invading pathogen are generally universally applicable. For eg. activation of inflammatory cytokines through NF-kB against AAV is documented but is also known to be operational in other viruses such as herpes simplex virus [24-26]. However, in case of viruses used for gene delivery there are fundamental differences associated in the gene transfer protocol, which could contribute to variability in this response. These include the route and mode of vector administration (eg., peripheral vein for AAV and *ex vivo* transfer for lentivirus), the vector/protein load [eg., 10^12 vgs of AAV and 1.9x10^7-10x10^7 TU (Transducing Units) for lenti-virus], the inherent immunogenicity of the outer capsid defined by the variable epitopic regions. Thus while lessons learnt from AAV gene therapy could be applied in principle to other vectors systems and vice versa, it will be necessary to minutely dissect the host-virus interactions to tailor specific strategies.

GENERATION OF IMMUNOLOGICALLY INERT AAV VECTORS

Approaches

AAV serotypes (AAV1-11) and additional capsid variants (>100 variants) have been categorized into six different phylogenetic clades. Among them nine serotypes (AAV1 to AAV9) have shown significant conservation in capsid sequence (62-99%) [27]. The three major capsid proteins [(VP1-736aa), (VP2-599aa), (VP3-534aa)] are splice products, present at a ratio of 1:1:8 on capsid surface and share the C- terminus [28]. In recombinant AAV vectors, the cap and rep genes are deleted from coding sequence and replaced with a therapeutic gene of interest [29]. The structure of adeno-associated virus (AAV2) has been determined by X-ray crystallography [4]. AAV virus structure (T=1 icosahedral structure) is well characterized and consists of exposed structural regions referred to as a cylinder structure protruding from each five-fold axis, the cylinder is encircled by a canyon, each three fold axis has a protruding spike formed by four loops and each two-fold axis contains a depression termed a dimple [30]. The three fold symmetry region of AAV2 capsid has an important role in capsid/receptor binding, for example, two arginine residues located at positions 585 and 588 are responsible for binding heparan sulfate proteoglycan (HSPG) [4, 31-33] and act as the primary receptor binding site [34]. Similarly, another domain located at position 511-513 takes part in capsid binding to α5β1 integrins [35]. AAV capsid structure can thus be used for functional alteration(s) and render them less immunogenic as described below.

Targeted Mutagenesis

Epitope mapping has revealed linear and complex conformational epitopes on AAV capsid surface [36] (Fig. **2**, Table **2**). Techniques such as site-specific mutagenesis strategies are helpful in the stepwise introduction of point mutations within the target epitopes to generate vectors with immune evasion properties [30, 37-40]. Li *et al.* [37] have recently reported an AAV2 mutant (AAV2.5) by replacing 5 amino acids at two fold axis of symmetry of the capsid with residues from AAV1. This AAV2.5 mutant exhibited efficient skeletal muscle transduction, a distinct neutralizing antibody (NAb) profile and higher transgene expression levels in mice pre-immunized against AAV2. Further insertion of 20 different amino acids at a single site (Thr 265) has been shown to alter binding affinity of anti-AAV2 antibody (A20), muscle transduction efficiency and humoral immune profile. In addition, class switching from IgM to IgG during the initial humoral response was noticed post AAV vector immunization in mice.

There are comparatively fewer reports for validation of immune escape phenotypes with their *in vitro* and *in vivo* vector transduction efficiency. A recent study by Martino *et al.* [41] has tested various AAV capsid candidates and their ability to evade capsid specific CD8+T cells *in vivo*. This group has previously demonstrated that modifications in surface-exposed tyrosine residues to phenyl-alanine (Y-F) residues improve the transduction of AAV2 vectors by 10-fold [42, 43]. These Y-F mutant capsids were less efficiently presented by hepatocytes to the MHCI complex and also demonstrated reduced killing of human or murine hepatocytes in comparison to AAV2 wild type vectors. In a similar study, Van den Driessche *et al.* [44] have developed stealth AAV vectors wherein AAV capsid recognition by the patient's immune system was reduced by eliminating antigen presentation *in cis*. They have validated this concept *in vitro* and demonstrated that these vectors decrease T cell activation and recognition of AAV transduced liver cells by ~100 fold when compared to conventional AAV2 vectors. The immune stealth AAV vectors also had comparable vector titre and high F.IX transgene expression. Gabriel *et al.* [39] and Sen *et al.* [40] have shown that site specific mutagenesis of serine, threonine and lysine residues in AAV2 and AAV8 capsids, respectively, results in high transgene expression in hepatocytes with corresponding low NAb titres *in vivo*. These results show that rational manipulation of AAV serotype(s) for generation of novel mutants can result in virion escape from pre-existing antibodies. Peptide insertion has also been proposed as an alternate method to modest point mutations generated. The targeted gene delivery and decreased reactivity

Fig. (2). Schematic of neutralizing antibody epitopes on AAV2 capsid. AAV2 capsid linear sequence (714 amino acids) (red) and epitope sequences are denoted by different colors; overlapping regions are also marked (yellow). Linear and conformational epitope sequences reported by Moskalenko *et al.* 2000 and Wobus *et al.* 2000 are highlighted by green and blue respectively.

of AAV2 capsid to NAbs by Huttner *et al.* [45] is a proof of efficient peptide mediated capsid alterations. In this study, two mutants, I-534 and I-573, carrying peptide insertions in surface exposed loop regions showed up to 70% reduced affinity for AAV antibodies. Taken together, these studies demonstrate that targeted modifications on the vector capsid are able to circumvent capsid specific CD8+ T cells or neutralizing effects of AAV specific antibodies.

Directed Evolution

Directed evolution strategies are based on high throughput generation and selection of AAV variant genetic libraries. Maheshri *et al.* [46] have reported combination of DNA shuffling and error-prone PCR mutagenesis strategy for directed evolution of AAV capsid. DNA shuffling uses DNase I digestion to create small fragments of DNA that are then reassembled into new genes through repeated annealing cycles in presence of DNA polymerase. The high-throughput approach helps to generate a large size (>10^6) of AAV2 mutant capsid library with randomly distributed mutations. This protocol resulted in engineering of AAV variants (r2.4 and r2.15) with altered receptor binding and high transduction efficiency in presence of anti-AAV2 NAbs. Affinity chromatography selection was used for yield of mutants with altered heparin affinity as shown for VP3 mutants, N382D on the inner shoulder of the threefold spike and N596D on the outer shoulder of the threefold spike. This work further demonstrated that the mutant r2.15 can evade antibody neutralization and enhance gene delivery 96 fold higher than wild type AAV2 virus *in vitro*.

Perabo *et al.* [47] have used error-prone mutagenesis for randomization of AAV2 capsid and to select vectors with neutralization evasion properties. Mutant clones were selected in the presence of human sera on a highly permissive cell line, HeLa. The viral library possessed 0.9 mutations per clone on an average. The clones contained, most frequently, mutations at capsid amino acid positions 459 and 551 which conferred these vectors with NAb escape characteristics. In another study, Maersch *et al.* [48] performed randomization of immunogenic sites followed by high-throughput selections for infectivity in presence of neutralizing sera. Five immunogenic sites at position 449, 459, 493, 551 and 558 were selected and two vectors (A11 and H6) were significantly less cross neutralized by human sera (3.9 fold and 2.7 fold) or with pooled intravenous immunoglobulin (IVIG) (2.1 fold and 5.1 fold) compared to unmodified AAV2 vectors. However, screening each amino acid substitution for their immune escape properties requires a diversity of 3.2x10^17 clones, hence AAV variant library construction even with a diversity of 10^7-10^8 clones makes error-prone PCR mutagenesis a labor intensive technique [48].

Shuffled Vectors

An *in vitro* evolution strategy based on DNA shuffling through fragment reassembly has been used to randomly combine eight AAV serotype capsid sequences (AAV2, 4, 5,8 and 9, caprine AAV, avian AAV and bovine AAV) and to generate a library of chimeric capsids [49]. Upon selection in the presence of IVIG, a single mutant AAV-DJ (chimera of AAV2, 8 and 9) was isolated. The AAV-DJ isolate was found to be efficient in transgene expression (100,000 fold better), in groups of passively immunized mice at lower IVIG concentrations (4mg or 20mg) and in comparison to wild type AAV8 or AAV9 vectors. In a similar study Koerber *et al.* [50] analyzed seven chimeric AAV vectors (AAV1, 2, 4, 5, 6, 8 and 9) and a mutant with >90% similarity to AAV1/6 had the most efficient immune evasion property. This mutant, cB4, was 400 fold more resistant to neutralization by IVIG than AAV2 and is as of yet one of the most promising stealth AAV vectors described. These studies demonstrate the importance of chimeric virions, where the immune evasion phenotype is incorporated due to the new combination of capsid proteins.

Chemical Modifications

It is possible to generate neutralization resistant AAV vectors by shielding the immunogenic sites in vectors by polymer coating or encapsulation. Polymeric materials such as Poly-ethylene glycol (PEG) and Poly-HPMA [(Poly-N-(2-hydroxypropyl) methacrylamide] that have been used for coating adenoviral and AAV vectors [51]. PEG is non-toxic and resists protein-binding by steric hindrance and blocking surface charge characteristics. PEG has been used extensively to conjugate proteins to extend their half-life in circulation and to reduce immune response directed against them [52]. The process of PEG conjugation, also called as PEGylation has been widely used to protect viral vectors from neutralizing antibodies [53], for vector retargeting [54] and to increase vector stability [55]. These compounds are covalently conjugated through active groups on the polymer termini to nucleophilic amino acid side-chains present on the viral surface, such as lysine or cysteine residues [56]. A drawback of PEGylation on AAV is the reported loss of viral infectivity at critical threshold conjugation ratio (PEG: Lysine ratio of 1:1000 to 2500) due to possible loss of key residues and the steric hindrance of viral surface regions critical for receptor binding [57]. In another report, hydrolysis of the PEG/AAV linkages gradually revealed antigen binding sites and made the conjugation process less effective [58].

Co-administration Strategies

One major hurdle with the use of genetically modified stealth vectors is their inherent variability or loss of transduction efficiency when targeted to multiple tissues or organs. Thus, alternate approaches like co-administration of antibodies against select ligands and pharmacological interventions have been proposed [59]. These include co-administration of anti-B cell ligands, pharmacological inhibitors and plasmapheresis to reduce the frequency of adverse immunological events [60-62]. Pre-existing antibodies impede successful gene transfer. Therefore some studies have emphasized the development of B cell targeting therapy for NAb titer reduction [63]. Pharmacological modulators such as Bortezomib have been reported to be effective in reducing IgG producing plasma cells in secondary lymphoid organs as well as minimizing cytotoxic T cell response due to characteristic proteasomal inhibition [64, 65]. However, Bortezomib does not completely deplete memory B cells which results in expansion of antibody producing B cell population overtime and subsequently an increase in anti-AAV circulating antibodies [66].

Several immuno-suppressive agents have been used to counter the effect of NAbs in the host. Halbert *et al.* [67] demonstrated persistent gene expression in alveolar tissue, upon co-administration of anti-CD40 ligand antibody (MR1) and a soluble CTLA4-immunoglobulin fusion protein (CTLA4Ig) *in vivo*. Zhang *et al.* [68] used adoptive transfer of AAV infected dendritic cells (DC)s into normal C57BL/6 and CD40 ligand deficient CD40L(-/-) mice to demonstrate the role of CD40 ligand in antigen presentation and CTL activation. These data were further confirmed by use of MR1, the anti CD40 ligand antibody to block the T and B cell cross talk in dystrophic *mdx* mouse model of muscular dystrophy [69]. In a recent landmark trial in patients with hemophilia B, the importance of short-term immunosuppression intervention, the gluco-corticoid therapy by Prednisolone, have been recognized to overcome AAV specific CD8 T cell response [70]. Further examples of immuno-suppressive regimens include Mycophenolate Mofetil and Cyclosporine A which reduce T cell response during AAV vector administration have been proposed [71]. Nayak *et al.* [72] used a combination of Rapamycin and IL-10 to induce tolerance *via* T-regulatory cells against an AAV vector encoding F.IX. Recently, Mingozzi *et al.* [62] have shown the effect of immuno-suppressants on non-human primates (Rhesus macaques) by using anti-B cell monoclonal antibody, Rituximab and Cyclosporine A for overcoming NAbs directed against the h.FIX transgene. These animals were injected intravenously with AAV8 vectors containing human F.IX transgene and the immuno-suppression regimen did not interfere either with vector transduction or with Treg (CD4+CD25+FoxP3+) induction.

The effectiveness of plasmapheresis is attributed to reduction in levels of antibodies, immune complexes and pro-inflammatory molecules. It is a therapeutic plasma exchange technique for removal of immuno-globulins (IgG) and toxic proteins from the blood plasma. This technique is practiced during organ transplant and helps to keep neutralizing factors at an undetectable level; it has been reported to provide vector stability in patients with low NAb titres [73]. However, in cases of patients with high NAb titres, plasmapheresis in combination with immunosuppressive treatments will prove more useful as this technique alone fails to completely abrogate the neutralizing antibody levels even after frequent procedures [6].

The understanding of vector-host cell biology has pinpointed the role of previously unknown factors. Zhu *et al.* [68] and Faust *et al.* [74] have reported correlation between vector intra-cellular trafficking events and recognition of CpG motifs by endosomal receptor TLR9 as a pathogenic stimuli and activation of innate immune response against AAV. These reports suggest that it may be possible to block

AAV capsid specific T cell response by co-administration of an oligonucleotide decoy with AAV vector to inhibit TLR9 activity. AAV infection in host cells and subsequent intracellular trafficking are known to cause activation of TLR-9Myd88 pathway; this further leads to production of interferon(s) and activation of CD8+ T cells and/or B cells [26, 75]. AAV infection can also cause inflammatory response and result in activation of transcription factors like NF-kB, as observed in case of scAAV2 mediated hepatic gene transfer [76]. Unfolded protein response (UPR), which recruits chaperone proteins for efflux of mis-folded proteins also causes NF-kB activation [77]. However, co-administration of Bay-11 or metformin attenuated the cellular NF-kB or UPR pathways, *in vivo* [77].

Recently, an elegant study has demonstrated the utility of AAV capsid decoys to overcome pre-existing humoral immunity against AAV [78]. Passively immunized C57BL/6 mice neutralized gene expression from an AAV8 vector expressing coagulation factor IX (5x10^9 vg/mouse) at anti-AAV NAb titres ranging from 1:3 to 1:100, but not when increasing amounts of AAV8 empty capsid were co-administered. Interestingly, the use of empty decoys from AAV 5, 6 and 8 vectors along with non-infective AAV2 mutants also rescued gene expression and prevented CD8+T cell mediated clearance of vector transduced cells. This strategy suggests that it is possible to overcome the barrier of pre-existing immunity against AAV in humans.

CHALLENGES WITH IMMUNOLOGICALLY INERT AAV VECTORS

Variable Vector Efficiency

Targeted mutations in the immunogenic domains of AAV can also lead to pitfalls like loss of infectivity or gene expression and undesirable tissue tropism. For e.g., mutations introduced to redirect the AAV2 tropism have shown decreased binding and neutralization by pre-existing antibodies [45]. Lochrie *et al.* [30] have reported an exhaustive list of AAV2 mutants with variable level of transduction and neutralization levels. Certain mutants such as the D529V in AAV2 had only 0.0005 fold infectivity *in vitro*, in contrast to another plateau mutant R471A with 318 fold infectivity and 42 fold reduced neutralization in human serum. Thus choice of target amino acids for substitution that results in the best combination of immune-escape phenotype, a high infectivity and desired tissue tropism, is very crucial. In addition, it is important to incorporate only a limited number of modifications in the vector [79]. Perabo *et al.* [47] and Maersch *et al.* [48] have independently reported the increase in relative amounts of small hydrophilic amino acids and a simultaneous decrease in the fraction of large, hydrophobic amino acids at mutation sites and demonstrated their effect on capsid architecture. This study underscores the importance of minimizing major biochemical changes to the capsid.

Different methods have been used to define AAV capsid immunogenic sites such as pepscan, peptide competition, peptide insertion, site directed and evolution directed mutagenesis [56]. Each technique has some limitations and thus identification of specific "immune signatures" in capsid surface is very crucial. Moreover, available data from cryo-electron microscopy on the Mab-20 and AAV2 complex interactions [80] to recognize antibody binding footprints revealed that the three dimensional domain architecture of the capsid protein rather than simple linear sequence is important. Recently, Gurda *et al.* [81] have also reported the presence of an antigenic region on AAV8 capsid threefold symmetry region using ADK8 MAb and cryo-electron microscopy. These studies emphasize the importance of recognizing target residues precisely by a variety of methods possible.

Presence of High-Titer Neutralizing Antibodies

The presence of pre-existing immunity against AAV especially at high titers is a major challenge that is yet to be surmounted convincingly. NAb mediated elimination of transduced cells due to viral antigens or transgene has not been reported. Ocular gene transfer studies (AAV mediated transfer of RPE65) have also shown that post gene transfer, an increase in neutralizing antibodies in eye does not affect recombinant RPE65 protein levels [82]. In an AAV-mediated phase I study in patients with hemophilia B, one patient with a higher pre-existing NAb titer (1:17) was found to express lower levels of F.IX (3%) in the serum than another patient (11.8%) with lower preexisting NAbs (1:2) against AAV2 at the same vector dose administered [16]. These results suggest that preexisting NAbs in the human population can attenuate vector transduction efficiency and inhibit transgene expression [56, 83]. Available data from various populations show that such anti-AAV NAbs are widely prevalent (Table **1**). In a detailed study, Calcedo *et al.* [84] have shown pre-existing antibodies against AAV in different age groups (<1 year to 18 years) suggesting that infection by AAV not only occurs early in human life but is common as well (60-80% of the population is sero-positive for AAV2) [36, 83,84]. Further analysis of human samples has identified that IgG1 antibodies are predominantly active against AAV [79]. Indeed the most optimum time for AAV vector introduction in humans appears to be between 7-11 months of age before the immune system is primed against AAV exposure [84]. Another drawback is the lack of very sensitive techniques to detect low levels of NAb [85]. Newer techniques such as ELISpot offer higher sensitivity to detect the adaptive immune responses and should be routinely employed in pre-screening protocols.

Absence of Suitable Models to Predict Immune Response

One of the major drawbacks in pre-clinical gene therapy research is the lack of suitable animal models to evaluate and predict immune response against AAV vectors [86, 87]. While the CD8+ cytotoxic T cell response against the AAV vector encoding factor IX was seen in humans administered with high dose (2 x10e12 vgs) of vectors, a similar dose in mice and dogs had no priming of cellular immunity in these models [88]. Mingozzi *et al.* [88] compared the T cell mediated immune response in mice with humans where they analyzed capsid specific CD8+ T cell frequency in immunocompetent mice infused with AAV2 capsid at different doses. The authors found that in contrast to immune response in humans, mice did not exhibit an increase in capsid specific CD8+ T cells. Even among mouse strains there have been reports of variable immune response between different lines of hemophilia B mice [89, 90]. In addition, the transcrip-

Table 1. Prevalence of anti-AAV antibodies across different populations.

AAV Serotype(s)						Geographical Area
AAV1	**AAV2**	**AAV5**	**AAV6**	**AAV7**	**AAV8**	
	100%					France [106]
	60%					Germany/Brazil/Japan [107]
		50%				West Germany [108]
19%	25%	Negative				USA [109]
20%	27%					USA [110]
	30%	10-20%	20-30%			USA [111]
50.5%	59%	3.2%	37%		19%	French [112]
39%	46%			36%	30%	Belgium [83]
30%	36%			29%	22%	Greece
15%	25%			12%	15%	Italy
33%	64%			20%	18%	Entebbe (Uganda) [83]
35%	46%			29%	29%	Kakira (Uganda)
42%	55%			38%	46%	Rwanda
58%	64%			40%	38%	South Africa
50%	63%			29%	29%	Kenya
42%	55%			26%	26%	Zambia
30%	35%			29%	28%	Australia [83]
28%	35%			27%	24%	Europe
45%	57%			32%	32%	Africa
21%	30%			12%	14%	United States

tional and translational response of target tissue/organ may differ [91]. The currently available mice models fall short in the type of disease conditions they can mimic. For eg., hemophilic mice are not spontaneous bleeders into the joint cavity. Due to these variables, the concept of humanized mice for molecular testing has come to fore-front [92]. These mice models are selected on the basis of characteristic traits (e.g., absence of immune cells) and designed to engraft/transplant human cells to "create" sites to be monitored during the course of treatment [93]. These designer mice models can benefit gene therapy trials (pre-clinical and clinical) immensely if real-time effect of gene transfer can be evaluated.

CONCLUSIONS

The concept of gene therapy from the bench to the bedside is becoming a reality. Ever since the first clinical trial with AAV vectors was performed in year 1996 for Cystic Fibrosis [94] that demonstrated the safety of the delivery system, recent successes seen in the treatment of diseases such as hemophilia B, Leber's congenital amarousis augurs well for the translation of this mode of therapy to standard of care. Indeed, the launch of the first commercial gene therapy product Glybera, for the treatment of Lipoprotein liapse deficiency is right step in this direction [95]. However, for a treatment modality to be widely embraced, it should be potentially applicable and efficacious in a large number of patients. The presence of naturally occurring antibodies to AAV (Table **1**) and the immunotoxicity seen with these vector systems (Fig. **1**) in some of the clinical trials could potentially limit the universal application of this promising vector system. It thus becomes imperative to identify immunologically naïve AAV either by extensive characterization of naturally occurring isolates or alternatively by generation of rationally designed vectors. While the isolation of serotypes such as AAV5 and AAV8 with a low sero-prevalence [27, 83] in humans is promising, further improvisation of these vector systems by targeted or random disruption of immunogenic sites by a combination of molecular (eg., targeted mutagenesis, capsid shuffling, directed evolution) and chemical (eg., pegylation) methods has been crucial in the generation of stealth AAV vectors. Despite the many advantages seen with these vectors, achieving a fine balance between an AAV isolate with immune escape potential along with a concomitant increase in packaging ability, infectivity

Table 2. Summary of AAV2 and AAV8 capsid T cell epitope sequences. Several immuno-dominant epitopes have been reported to form part of T cell receptor repertoire. The number(s) in brackets correspond to epitope start and end from AAV2 capsid (714 amino acids) and AAV8 capsid (738 amino acids) VP1, VP2 and VP3 domains respectively.

Species/Strain	AAV2 Capsid Sequence	AAV8 Capsid Sequence	Reference
Human	GSGAPMADNNEGADG (206-220)		
Human	SADNNNSEY (492-500)	IPQYGYLTL (375-383)	Mingozzi *et al.*, 2007 [88]
Human	VPQYGYLTL (372-380)		
Human	PADVFMVPQYGYLTL (366-380)	PADVFMIPQYGYLTL (369-383)	Manno *et al.*, 2006 [16]
Human	GNNFTFSYTFEDVPF (406-420)	GNNFQFTYTFEDVPF (409-423)	
Mice (C57BL/6)	NGRDSLVNPGPAMAS (511-525)		
Mice (C57BL/6)	SNYNKSVNV (702-710)	NSLANPGIA (517-525)	
Mice (BALB/c)	FRPKRLNFKLFNIQV (306-320)	LTSEEEIKTTNPVAT (562-576)	Sabatino *et al.*, 2005 [113]
Mice (BALB/c)	HSQSLDRLMNPLIDQ (426-440)	IPQYGYLTL (375-383)	
Mice (BALB/c)	VPQYGYLTL (372-380)		
Mice (MHC I)	QYGSVSTNL (373-381)	KYLGPFNGL (50-58)	
Mice (MHC II)	GVLIFGKQSEKTNV (336-350)	LEPLGLVEEGAKTAP (126-140)	Chen *et al.*, 2006 [114]
Mice (MHC II)	QVSVEIEWELQKENS (475-489)	EYGIVADNL (577-585)	

or stringent tissue tropism has been a challenge. Thus it is cognizable that a combination of approaches is needed to circumvent this phenomenon. This could be potentially driven by synergistic action of a combination of rationally engineered viral structural and transgene elements [41, 96], manipulation of the immunological status of the recipient [59, 62, 73, 78], targeted pharmacological disruption of innate and adaptive immune response signals during and after AAV mediated gene transfer [26, 97]. Alternatively, generation of hybrid AAV vectors by pseudo-typing AAV with beneficial components (structural/non-structural) from other vector systems is another area that could be potentially explored.

Despite some of the lacunae associated with AAV, it is perhaps the safest system available for gene therapy in humans. Therefore overcoming the barriers, which are primarily related to host immunological response should be rewarding to this field. A thorough characterization of the intricate host-virus interactions is likely to offer further clues and help design better strategies to improve the therapeutic efficiency and safety of this promising vector system.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

SH is supported by a senior research fellowship from Council for Scientific and Industrial Research (CSIR), Government of India. GRJ is supported by research grants from Department of Science of Technology, Government of India (Swarnajayanti Fellowship 2011), Department of Biotechnology (DBT), Government of India (Innovative Young Biotechnologist award 2010: BT/03/IYBA/2010; Grant: BT/PR14748/MED/12/491/2010; Grant: BT/01/COE/08/03; Grant BT/PR5021/MED/30/757/2012) and an early career investigator award-2010 from Bayer Hemophilia Awards program, Bayer Inc, USA.

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Received: January 03, 2014 **Revised: March 13, 2014** **Accepted: March 25, 2014**