REVIEW

Immune responses to adeno-associated virus and its recombinant vectors

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Recombinant adeno-associated virus (rAAV) vectors have emerged as highly promising for use in gene transfer for a variety of reasons, including lack of pathogenicity and wide host range. In addition, all virus-encoded genes have been removed from standard rAAV vectors, resulting in their comparatively low intrinsic immunogenicity. For gene replacement strategies, transgenes encoded by rAAV vectors may induce less robust host immune responses than other vectors *in vivo*. However, under appropriate conditions, host

immune responses can be generated against rAAV-encoded transgenes, raising the potential for their use in vaccine development. In this review, we summarize current understanding of the generation of both undesirable and beneficial host immune responses directed against rAAV and encoded transgenes, and how they might be exploited for optimal use of this promising vector system.

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Introduction

Gene therapy has opened many exciting possibilities for treating diseases that are otherwise recalcitrant to current 'standard' treatments. However, a recognized obstacle to gene replacement therapy is the generation of host immune responses directed against vector-encoded transgene products or the vectors themselves. Vectors derived from recombinant adeno-associated virus (rAAV) have emerged as highly promising for use in gene transfer. rAAV vectors are not pathogenic, transduce nonproliferating cells, including cells of the immune system, and are capable of stable genomic integration and long-term transgene expression.^{1–4} All virus-encoded genes have been removed from currently employed rAAV vectors, resulting in their comparatively low intrinsic immunogenicity. Microarray analyses of cells infected with AAV demonstrated few effects upon overall gene expression even at high multiplicities. The primary effect was reduced expression from genes associated with cell proliferation. In contrast, transduction with an adenovirus E1 and E3 deleted vector resulted in a 'stress response' with augmented expression from cytokine and chemokine genes.⁵

Despite these theoretical advantages, host immune responses can be generated against rAAV vector-encoded transgenes, the type and magnitude of the response being somewhat dependent upon the transgene and the route of vector administration.⁶ In certain settings, as in the expression of a possible therapeutic gene, these immune responses are undesirable. In contrast, others have attempted to capitalize upon the

ability of rAAV transduction to induce host immune responses to generate antigen-specific vaccines. rAAV vector-based genetic vaccines can successfully induce primary humoral and cellular immune responses directed against specific antigens in animal models^{7–9} and *in vitro* cultures with human lymphocytes.^{10–12} Thus, as in most biological systems, there is a delicate balance between the generation of desirable and unwanted effects.

To successfully exploit rAAV vectors for the development of gene therapy it is critical to understand the principles underlining the generation of host immune responses both to the encoded transgene and to the vector itself. In this review, we will summarize the current progress in immune responses to AAV-based vectors, and address additional relevant questions that will need to be resolved before bringing this vector to the clinic. A more thorough discussion of the development of rAAV vectors for vaccine development is presented elsewhere.¹³

Basic AAV biology

AAV are helper-dependent parvoviruses assigned to the *Dependovirus* genus of the subfamily *Parvovirinae*.^{14,15} To date, of the eight recognized AAV serotypes, the biology of AAV2 has been most extensively studied, and the following discussion of AAV biology will focus primarily upon this serotype unless otherwise noted. Details of the other AAV serotypes are provided below. The requirement of helper functions from another virus for AAV productive infection is generally accepted, although an as-yet unconfirmed study reported that AAV2 could replicate autonomously in normal skin keratinocytes.¹⁶ The AAV genome is comprised of a 4.7 kb, single-

stranded DNA flanked by GC-rich inverted terminal repeats (ITRs) (Figure 1). The ITRs serve as *cis*-active elements that are required for encapsidation and serve as elements for self-primed DNA replication. AAV2 is unique among viruses because of its capacity to integrate site specifically into primate DNA into a region termed 'AAVS1', which is located on chromosome 19 in humans.¹⁷ Molecular analyses have demonstrated two primary open reading frames (ORFs) in the AAV2 genome, the left under control of promoters at map positions 5 and 19 (p5 and p19, respectively) termed 'rep' encodes proteins necessary for AAV2 ori-dependent DNA replication and site-specific integration. The right under control of a promoter at map position 40 (p40) termed 'cap' encodes three colinear capsid proteins, VP1-3, that share common carboxy termini, but different amino terminal ends that are generated by alternative splicing or use of alternative translational initiation sites. The AAV virion is a naked, icosahedral particle about 25 nm in size, comprised of VP1 (87 kDa), VP2 (73 kDa) and VP3 (62 kDa) at ratios of approximately 1:1:10. Utilization of overlapping reading frames for capsid proteins maximizes the coding capacity of a small genome, and potentially reduces the number of epitopes against which immune responses might be generated. The primary receptor for AAV2 is believed to be heparin sulfate proteoglycan, with coreceptors including α V β 5 integrin and basic fibroblast growth factor receptor.^{18–20}

Recombinant AAV vectors (rAAV) have been constructed by removal of AAV-encoded ORFs, and replacement with transgenes of interest between flanking ITRs (Figure 2). rAAV are usually encapsidated by transfection of the vectors, as plasmids, into cells expressing both AAV rep and cap functions, and 'helper' virus functions, typically adenovirus E1, E2, E4, and VA RNAs. Both

rAAV2 VECTOR PRODUCTION

Figure 2 General Scheme of rAAV2 vector production. rAAV vectors have been constructed by removal of endogenous viral genes, and insertion of an expression cassette(s) between flanking ITRs. rAAV are generally encapsidated by transfection of a plasmid containing the rAAV vector into cells with provision of AAV rep and cap (as either plasmids or by using cells expressing them) and 'helper' virus functions such as Adenovirus E1, E2, E4, and VA RNAs (either as a plasmid or by viral infection). The resulting vector is then purified for use using density gradient centrifugation (CsCl₂ or Iodixanol) and/or column purification. See text for details.

minimum ($\geq 75\%$ of wild type genome size) and maximum size (≤ 110 – 119%) limitations for optimal rAAV encapsidation have been described.²¹ After a suitable incubation period, the cells are harvested, and the vector purified, classically by isopycnic cesium chloride gradient centrifugation. Recently, other purification strategies, including heparin column chromatography for rAAV2 vectors,²² and iodixanol gradient centrifugation²³ have been employed to simplify vector purification.

For the sake of clarity, we will separate host immune responses against the vector and the encoded transgene(s) in the discussion that follows.

Humoral immune responses predominate following AAV infection, and magnitudes are route and viral dose dependent

Pre-existing immunity to wild-type AAV in humans is predominantly humoral, with a minority of subjects demonstrating marginal lymphocyte proliferation and IL-10 secretion in response to AAV2 proteins.²⁴ Similar results were obtained in immunocompetent mice and rhesus monkeys,^{25,26} indicating activation of Th2 subsets and B cells to viral capsid proteins. AAV2-specific IgM and IgG2 responses were observed in the above experiments, suggesting that host immune responses against AAV were dependent upon both T-cell-dependent and -independent mechanisms. Studies of a wild-type AAV infectious primate model using rhesus macaques demonstrated that primary and memory immune responses were dependent upon both the route of infection and the presence of helper virus. Intranasal infection with AAV2 alone did not elicit neutralizing antibodies (NABs), while intravenous and intramuscular infection did. In contrast, intranasal coinfection with wild-type AAV2 and adenovirus elicited NAB, lymphocyte proliferative responses

AAV2 BIOLOGY

Figure 1 General scheme of adeno-associated virus type 2 (AAV2) biology. AAV is a member of the Parvovirus family. It has a 4.7 kb single-stranded DNA genome flanked by GC-rich inverted terminal repeats (ITRs) that are necessary for viral replication, and encapsidation. The genome is organized into 2 major open reading frames (ORFs), the left under control of p5 and p19 promoters, collectively termed 'rep', and the right under control of the p40 promoter termed 'cap.' Generally, AAV require 'helper' functions from another virus for productive infection. In the absence of helper virus coinfection, AAV integrate into the cellular genome, or may persist episomally. The AAV2 rep gene product has been shown to mediate site-specific integration of the AAV2 genome into a region of primate DNA termed 'AAVS1' which resides on chromosome 19 in humans. See text for details.

to AAV2, and cellular infiltration in local tissue.²⁷ Mechanistically, these differences could result from multiple factors including a possible immune adjuvant effect of adenovirus coinfection, host exposure to higher doses of AAV2 because of virus replication, and systemic AAV2 spread due to disruption of the mucosal barrier by adenovirus infection.

Murine models demonstrate that the magnitude of the host immune response to rAAV2 transduction is dependent upon the virus dose and route of administration. The threshold for induction of significant NABs in a C57BL/6 mouse lung model was 10^6 intrapulmonary rAAV2 particles per mouse. However, administration of vector at doses below this threshold dose also led to low-level transgene expression.²⁸ In contrast, the threshold for the generation of antivector immune responses was higher (10^8 per mouse) for rAAV2 given intramuscularly to C57BL/6 mice.²⁹ Interestingly, lower rAAV doses could transduce some cell types without induction of host antivector immune responses, suggesting that vector readministration might be successful following a lower primary dose. Neutralizing activities against intramuscularly²⁶ or intravenously³⁰ administered rAAV2 in a murine model were totally T-cell dependent, as mice lacking functional T cells (BALB/c or C57BL/6 nude mice, CD40L knockout mice and CD4+ T-cell-depleted mice) did not generate NAB, although they generated similar levels of IgM after immunization. These findings were consistent with those obtained in MHC class I and II knockout mice.²⁹ However, rAAV2 delivered to the liver of mice or rhesus monkeys induced transient neutralizing humoral immune responses that were T cell independent.³⁰

Immunologically privileged sites such as the brain, testis, and the eye exhibit partial or complete loss of antigen-specific immunity as reflected by extended graft survival after transplantation,³¹ and, thus, may be useful targets for gene delivery and sustained transgene expression. The eye (anterior chamber as well as posterior retina) is rendered immune privileged by several factors including the blood–ocular barrier, sequestration of retinal antigens, and expression of local immunomodulators like MIP2 α , TGF- β , and IL10 in the aqueous humor. An interplay of several effector cells like ocular APCs, NKT cells, and activated T-regulatory cells³² mediate the immune deviant properties associated with the eye such as impaired delayed type hypersensitivity (DTH) and an impaired ability to produce complement fixing antibodies. Anand *et al*³³ demonstrated stable and long-term transduction of photoreceptors following subretinal delivery of rAAV2 encoding green fluorescent protein (rAAV-GFP; 1×10^{10} particles/eye). Although a significant antivector capsid humoral response was observed, no anti-GFP specific response was noted, in contrast to Ad-GFP where significant anti-GFP antibodies were demonstrated. These findings suggest that immune privilege might not be complete, and may vary with the nature of the introduced antigen and/or vector. Furthermore, antigen presentation is possible even in an immunologically secure site like the retina. These findings demonstrate that the route of AAV administration, in addition to the major target organ(s) for transduction, play major roles in determining the type and extent of the host immune response that is generated.

Pre-existing immunity to rAAV and its impact on rAAV-mediated gene transfer

In humans, the presence of serum antibodies directed against AAV1, 2, 3, and 5 is very common. Early seroepidemiological studies of AAV1, 2, 3, and 4 demonstrated that the prevalence of AAV1, 2 and 3 antibodies rose steeply between the ages of 1 and 10, and reached a peak of 60% by the age of 10 years. In contrast, antibody against AAV4, originally isolated from non-human primates, was detected much less frequently, with a peak incidence of about 10% between 2 and 5 years of age.^{34–35} The seroepidemiology of AAV5, which was originally isolated from a human genital lesion, may be significantly different: average peak titers occurred between 15 and 20 years of age, and about 50% of adults in the former West Germany tested positive.³⁶ Reports for seroprevalence in adults vary from 35 to 80%, probably due, in part, to the varying sensitivities of the serologic tests and the age of the tested subjects. A recent investigation using ELISA analyses of samples from 572 individuals from diverse areas (Germany, Brazil, and Japan) demonstrated that AAV2 seropositivity was distributed worldwide, and increased with age. About 30% of those less than 10 years were AAV2 seropositive, while this increased to 60% for those between 10 and 19, dropped to 49% of those between 20 and 29, increased again after the age of 30 and peaked at 73% in the 50–59 year age group.³⁷

However, some populations are less likely to be AAV seropositive. Interestingly, early reports suggested that infection with wild-type AAV2 was oncoprotective. Oncoprotection may result from the inhibition of transforming genes by the AAV2 rep gene product, or the actual cytolytic activity of AAV2 in p53 mutant cancer cells.^{38,39} The antioncogenic activity of AAV2 infection was supported by seroepidemiological data showing that patients with cervical cancer were less likely to have been infected with AAV2 and were seronegative.⁴⁰ A recent seroepidemiological investigation of cervical cancer patients confirmed the above findings.⁴¹ In addition, 29% of patients with adult T-cell leukemia lymphoma ($n=31$) were AAV2 seropositive compared to 84.6% ($n=39$) of healthy carriers with human T-cell leukemia virus type 1.⁴² In contrast, no difference in the prevalence of serum antibodies against AAV2 helper viruses such as adenovirus and herpes simplex virus was demonstrable between cancer patients and healthy controls in the above investigation. The low prevalence of anti-AAV2 antibodies in certain cancer populations may provide an intrinsic advantage for the use of rAAV2 vectors for cancer gene therapy.

Not all anti-AAV antibodies detected by ELISA are NABs. It was reported that although AAV2-specific antibodies were detected by ELISA in 96% of subjects (74 samples), only 32% had neutralizing activities.²⁴ These findings were somewhat contradictory with the study of Erles *et al*.³⁷ In that study, 40 samples were chosen at random from a larger group of 572. Of these, 32 (80%) were positive for antibodies against AAV2, and 27 (67%) were positive for AAV2 NABs. It is possible that the low concordance of ELISA and NABs results from the first study reflected a sampling bias due to analysis of samples primarily from one specific community.⁴³ However, similar variabilities were observed by Moskalenko

et al,⁴⁴ in which 80% of 50 human serum samples were positive for anti-AAV2 antibodies by ELISA, but only 18% were positive for NABs. This variability could be the result of different assay methods, and emphasizes the importance of specifically monitoring for NABs during clinical trials.

The above findings are potentially important because the presence of NABs could impede *in vivo* rAAV transduction following systemic administration. Several studies have demonstrated that AAV2 immune animals are resistant to subsequent rAAV2 transduction, and that resistance correlated with the appearance of NAB to AAV2 capsid proteins, not to transgene products.^{28,29,44,45} For example, pre-existing neutralizing activity to rAAV2 in C57BL/6 mice diminished the effectiveness of rAAV2 readministration 20-fold.⁴⁶ Similarly, readministration of rAAV2 by the intranasal route to preimmune rabbits was unsuccessful.²⁸ In contrast, Beck *et al*⁴⁷ observed that rAAV2-based vectors could successfully transduce rabbit lung even after repeated administrations and despite the presence of high titer anti-rAAV2 NAB in the serum. These results may not be entirely contradictory, as neutralization of vector on the luminal surface of the lung may be dependent upon the levels of neutralizing secretory IgA, levels of which may not correlate with total serum neutralizing activity. The target organ chosen for transduction may also influence anti-vector immune responses. Anand *et al*⁴⁵ demonstrated that rAAV transduction (1×10^{10} particles/eye) of cells in an immune privileged site like the eye was possible even in the presence of significant anticapsid systemic NABs. Murine strain-specific differences in immune responses have also been described; neutralizing activities in BALB/c mice had much less impact on vector readministration than in C57BL/6 mice.^{26,49}

Perhaps the most pertinent data about the effects of pre-existing neutralizing immunity upon rAAV transduction arose from a recently reported hemophilia B/rAAV-human-factor-IX (hu-FIX) clinical trial.⁵⁰ In this preliminary report, all three subjects had FIX gene point mutations, and the level of neutralizing anti-AAV antibodies ranged from 1:10 to 1:1000. Nevertheless, transduction and local muscular expression of FIX was documented even in the individual with the highest pretreatment neutralizing anti-AAV titer. However, it should be emphasized that this was a preliminary report, that the sample size was very small, and that vector doses were intentionally low to address potential safety issues. Additional studies will be necessary to determine whether these findings are generally applicable.

Approaches to overcome pre-existing AAV immunity for *in vivo* gene therapy

Several approaches have been evaluated for overcoming the possible block of pre-existing immunity to *in vivo* gene therapy.

Development of rAAV vectors from different serotypes

To date, eight different serotypes of AAV have been identified. DNA sequence analyses of AAV1-5 have

shown differences in their capsid genes, with 80% homology at the amino-acid sequence level. AAV2, 3, and 5 were isolated from human clinical specimens, whereas AAV1 and 4 were of simian origin.¹⁵ AAV6 was isolated as a contaminant in a laboratory adenovirus stock, and appears to represent a recombinant consisting of the 5' portion of AAV2 fused to the 3' portion, including the ORFs, of AAV1.^{43,51} Recently, PCR amplification with primers complementary to conserved regions of AAV1-6 was used to screen rhesus monkey tissues for latent AAV infection, and two novel serotypes, AAV 7 and 8, were identified. The capsid proteins from AAV7 and 8 were divergent from those in AAV 1-5. Vectors derived from AAV7 were as efficient for murine muscle transduction as AAV1, previously the most efficient serotype for this purpose. Transduction of hepatic cells with rAAV8 was more efficient than other rAAV serotypes. Furthermore, AAV7 and 8 were not neutralized by heterologous antisera raised to other serotypes, and NABs to AAV7 and 8 were rare in human serum, supporting potential application(s) for AAV7 and 8 as vectors for human gene therapy.⁵²

In humans, pre-existing immunity to AAV2 has been identified at a higher frequency than that directed against AAV1, 3, or 5. Data from 77 healthy human subjects demonstrated that 27% exhibited NAB to AAV2, while 20% had NAB against AAV1. In addition, antibody titers against AAV2 were higher than corresponding antibodies against AAV1. Among these subjects only one had NAB to AAV1 and not to AAV2, whereas six had the antibodies to AAV2 and not to AAV1.⁴³ More recently, the same group analyzed a collection of 85 human sera for neutralizing activities to AAV5 and found none of them positive.⁴⁶ Using serotype-specific PCR, AAV3 and 5 sequences were identified at much lower frequencies than AAV2 in human tissues,⁵³ implying that infection with these strains is less common. Eight human sera were tested by ELISA for anti-AAV2 antibodies, and for NABs to AAV2, 3, and 5. Six of eight samples were positive by ELISA; of these six samples only one exhibited neutralizing activity to all three serotypes, one to AAV2 and 3 but not to AAV5, one to AAV2 and 5 but not to AAV3, two samples to AAV3 only, and one to AAV5 alone. Of the two ELISA-AAV2-negative samples one had NAB to AAV3 and 5 and the other did not.³⁷ Seven serum samples from healthy donors were analyzed for their neutralizing activities to AAV2, 3, and 6 in parallel; four samples were positive to AAV2, AAV3, and AAV6 but the neutralizing titers were lower for AAV3 and much lower for AAV6.⁴⁵

The above data suggest that neutralizing immunity generally does not cross serotypic lines, raising the possibility of using either rAAV vectors based upon different serotypes or vectors pseudotyped with capsids from different serotypes to circumvent problems with pre-existing immunity, and facilitate vector readministration.

This strategy has been successful in animal models. An rAAV1 vector encoding human α 1-antitrypsin (hu-AAT) was successfully administered to mice with pre-existing immunity to AAV2, and *vice versa*.⁴³ It was reported that NABs to AAV2 cross react with AAV3 but not to AAV6.⁴⁵ Lung transduction of mice with pre-existing immunity to AAV2 with an rAAV6-based vector encoding placental alkaline phosphatase was almost equivalent to that of

naïve mice. In contrast to AAV2 vectors, prior transduction with an rAAV6 vector only partially inhibited transduction with an AAV6 pseudotyped vector, suggesting that the capsid of AAV 6 was less immunogenic.⁴⁵ As noted above, the capsid proteins of AAV1 and AAV6 should be essentially identical, although a direct comparison of these two vectors has not been reported. Vectors derived from AAV5 also demonstrated promise for readministration to AAV2 immune mice.⁴⁶ Vectors based on AAV4 have also been studied, but thorough analyses of immunogenicity have not yet been done.⁵⁴ Overall, this strategy may be promising as a single rAAV2 vector could be packaged (pseudotyped) within capsids derived from different AAV serotypes.⁵⁵ This strategy could also be used to exploit the recognized differences in tissue tropisms of different AAV serotypes to optimize gene transfer to specific tissues.⁵⁴ For example, it has been reported that AAV1 and AAV5 transduce muscle cells more efficiently than AAV2, resulting in higher systemic transgene expression.^{43,56}

Application of AAV vector plasmids for gene therapy

AAV vector-based plasmid DNA, particularly when complexed with specific liposomes, have been shown to deliver transgenes efficiently. When compared to conventional plasmids, AAV-based plasmids displayed 2- to >10-fold higher transgene expression that also appeared more stable.^{57,58} *In situ* hybridization and Southern analyses demonstrated both integrated and episomal forms following AAV-based plasmid transfection.⁵⁹ As this strategy does not depend on specific host cell receptors, it may extend host cell range. Furthermore, encapsidation size limitations are circumvented. This could be another approach to evade problems with pre-existing immunity to AAV capsid proteins. Liposome complexed or naked AAV-plasmid DNA have been administered intravenously in rodent models, resulting in transgene expression in multiple organs.^{59,60} However, additional experiments are needed to confirm the reproducibility of this strategy.

Ex vivo manipulation of target cells for gene therapy

One strategy to circumvent pre-existing antivector immunity is to transduce target cells *ex vivo*, in effect isolating them from the immune system. Transduced cells can then be reimplanted. In fact, the earliest gene therapy clinical trials utilized *ex vivo* manipulation of hematopoietic stem cells (HSCs), appealing targets for genetic manipulation, as HSCs are easily obtained, and many inherited diseases affect the blood. One important property of rAAV vectors is the ability to transduce quiescent or nonproliferating cell populations, an important characteristic of true stem cells.^{61–63} Thus, we reasoned that rAAV vectors might be promising for HSC transduction. Although some investigators have noted difficulty with long-term transduction of HSCs with rAAV vectors, we and others have demonstrated stable rAAV transduction and integration in human HSCs associated with long-term transgene expression, both *in*

vitro and in the NOD/SCID xenograft model *in vivo*.^{64–68} S. Chatterjee, personal communication. It is highly likely that, as described in other tissues *in vivo* including muscle, optimal transgene expression from single-stranded rAAV genomes may take weeks to develop in quiescent HSCs, accounting for some of the discrepancies in transduction. Vector modifications are in progress to potentially further improve the transduction efficiency of rAAV for HSCs.⁶⁹ While this strategy is useful for hematopoietic cells, the obvious problems with this approach are that not all tissues are amenable to *ex vivo* culture and manipulation, and that it is technically labor intensive and costly.

Identification of neutralizing AAV capsid epitopes

As animal experiments demonstrated that NAB to AAV capsid protein(s) play important roles in blocking vector readministration, the identification of neutralizing epitopes could aid in the development of less immunogenic vectors, or haptens designed to block NABs. Several mutational analyses of AAV2 capsid proteins have been done to both analyze areas of cell receptor binding and to identify points for insertion of peptides to modify vector tropisms.^{70,71} Six peptides that blocked human serum neutralizing activities were identified by screening human sera against a peptide library.⁴⁴ The epitopes for three AAV2-specific murine neutralizing monoclonal antibodies were mapped, and found to differ from those defined above by human polyclonal NABs.⁷² The consensus between the above two studies is that no single linear epitope was responsible for AAV2 neutralization, even for a single monoclonal antibody. This suggests that NAB might recognize conformational epitopes, making this strategy more difficult to bring to fruition. Recently, the crystal structure of the AAV2 virion has been delineated, and will guide rational engineering of rAAV vector capsids to tailor cellular targeting and to circumvent host antivector immune responses.⁷³

Immune modulation to reduce pre-existing immunity

Humoral immunity appears to be the predominant host immune response directed against AAV2, and neutralizing activities appear to be T cell dependent. The monoclonal anti-CD40 ligand (anti-CD40L) antibody, MR1, combined with soluble CTLA4Ig block both CD40 and B7 costimulatory pathways and have been successful in reducing primary host immune responses against recombinant adenovirus vectors, permitting vector readministration. However, a single dose of this regimen resulted in only transient immune suppression.⁷⁴ Identical regimens were successfully used to circumvent generation of antivector immune responses to rAAV2 vectors administered intranasally.²⁸ Another approach to prevent antivector immune responses included *in vivo* administration of anti-CD4 antibody to deplete CD4+ T cells at the time of rAAV2 vector administration, which reduced NAB formation and allowed vector readministration.²⁹

Although these strategies were successful in obviating a primary antivevector immune response, they do not address potential problems of pre-existing antivevector immunity, a situation that would likely exist in human clinical trials. Neither treatment with the immunosuppressant cyclosporin A nor CD4 depletion facilitated transgene expression in mice with pre-existing anti-AAV2 immunity following rAAV2 transduction.^{29,44} Cyclophosphamide, another immunosuppressant, also did not block the formation of NABs against rAAV2 capsid proteins, although, as described in further detail below, it effectively blocked formation of antibodies directed against an rAAV-encoded hu-FIX transgene in another study.⁷⁵ More studies are needed to optimize the dose and schedule of administration of immunosuppressives to make this approach viable.

Host immune responses to transgene products delivered by rAAV in replacement gene therapy

The original concept of gene therapy simply focused upon replacing defective gene(s) responsible for inherited diseases. It is now clear that the situation is more complex, and that host factors, particularly the host's immune response to the 'replaced' gene, are important obstacles to gene replacement therapy. Several studies have shown that host responses to inserted 'replacement' transgenes are clearly dependent upon the type of gene mutation.⁷⁵ Diseases may result from a spectrum of genetic mutations that vary from total absence of gene expression (null mutant) to a single point mutation that affects a critical site in protein function. In the former instance, 'replacement' of the missing gene would result in expression of an entirely new protein which could be considered as 'foreign' by the host's immune system. In the latter case, replacement of the gene would result in expression of a protein that was virtually identical to a pre-existing host protein and potentially less immunogenic. In retrospect, these types of responses could have been anticipated given clinical information about the development of 'inhibitors' following protein replacement therapy as, for example, in hemophilia B (see below).

A variety of animal models engineered to mimic human genetic disease have been developed to assess gene replacement strategies, and further characterize antitransgene immune responses. For example, several small and large animal models of hemophilia B, an inherited deficiency of clotting factor IX (FIX) that is associated with a bleeding tendency, have been developed. Several studies focusing upon defective FIX gene replacement using rAAV vectors, with resulting host anti-FIX immune responses, are summarized in Table 1.^{75–83} The first four examples closely mimic two types of genetic defects in human hemophilia B, either point or null mutations. Therapeutic expression of canine FIX (cFIX) following intraportal administration of rAAV/cFIX in both murine and canine animal models was described.⁸⁰ In the canine model a therapeutic effect was demonstrable for more than 7 months without development of FIX inhibitors. Similar results were reported by others in murine and canine models.⁷⁹ Sustained (>17 month) expression of cFIX was obtained following

intramuscular administration of rAAV/cFIX, to levels that could be therapeutic in a human clinical trial.⁷⁷ In this study, the animals had a point mutation in the FIX gene, and FIX inhibitors were either absent or transient. In a later study employing a FIX null mutant canine model, FIX inhibitors were detected, and their formation blocked by cyclophosphamide treatment,⁸² findings similar to those of Fields *et al.*⁷⁵ and Mount *et al.*⁸³ These studies underscore the importance of the mutational background in the generation of host anti-transgene immune responses and immunomodulation to prevent inhibitor formation, issues discussed in greater detail below. Ultimately, they provided sufficient safety and efficacy data to permit transition to an ongoing human clinical trial described above.

Immune responses to transgene products depend on several factors including intrinsic host differences, the type of vector employed, the route and dose of vector administration, the immunogenicity of the transgene itself, the promoter controlling transgene expression, and the subcellular localization of the transgene product within the target cells. These factors are not mutually exclusive, but often interdependent. For example, one important factor determining generation of an immune response may be overall transgene expression. This, in turn, is related to vector type, the target tissue for transduction, the transcriptional promoter used, and the dose of the vector used. Major factors that have been shown to influence host immune responses following systemic vector administration are discussed below.

Intrinsic differences in host immune responses

The genetic background of the host itself can significantly influence immune responses against specific transgenes. This principle has been most avidly demonstrated in murine models, in which an identical vector encoding a specific transgene can be successful in one genetically homogeneous mouse strain, but unsuccessful in another. For example, intramuscular administration of an rAAV2 vector encoding hu-AAT to C57BL/6 mice resulted in long-term transgene expression at therapeutic levels. In contrast, an identical vector administered intramuscularly to BALB/c mice generated potent anti-hu-AAT responses, with rapid elimination of transgene expressing cells.⁸⁴ The magnitude of immune responses to readministered rAAV vectors may also be strain dependent, as discussed below. In contrast to most animal models, inbred murine systems provide identical genetically and immunologically defined populations so that immune responses can be dissected and intensively studied. These advantages are lost when moving to larger, more heterogeneous animal systems, including humans. These differences undoubtedly account for a portion of the variabilities in immune responses reported in clinical trials. Furthermore, as described above, some of the apparent disparities of generation of immune responses against 'endogenous' gene products may reflect differences in the genetic mutational background of the study population, as well as differences in the ability of specific HLA alleles to present antigens and generate immune responses (eg null *versus* point mutation, see above and Table 1).^{77,82}

Table 1 Host immune responses to rAAV-encoded clotting factor IX (FIX) in different experimental settings

Transgene product	Promoter	Experimental host	Route	Immune responses	References
Canine FIX	CMV-IE ^a	Dog with a null mutation	IM ^b	Inhibitory antibody (<i>n</i> =3)	Herzog <i>et al</i> (2001) ⁸²
Canine FIX	ApoE/hAAT ^c	Dog with a null mutation	Portal vein	Inhibitory antibody only in 1/3 (<i>n</i> =3)	Mount <i>et al</i> (2002) ⁸³
Canine FIX	CMV-IE ^a	Dog with a point mutation	IM ^b	Only one in five showed transient inhibitory antibody	Herzog <i>et al</i> (1999) ⁷⁷
Canine FIX	MFG ^d	Dog with a point mutation	Portal vein	No inhibitory antibody (<i>n</i> =2)	Snyder <i>et al</i> (1999) ⁷⁹
Canine FIX	LSP ^e	Dog with a point mutation	Portal vein	No antibody (transgene expression over 7 months)	Wang <i>et al</i> (2000) ⁸⁰
Murine FIX	CMV-IE ^a	C57BL/6 with a null mutation	IM ^b	Inhibitory antibody (<i>n</i> =6)	Fields <i>et al</i> (2001) ⁷⁵
Human FIX	MFG ^d	C57BL/6 with a null mutation	Portal vein	Noninhibitory antibody (<i>n</i> =3, functional FIX persist)	Snyder <i>et al</i> (1999) ⁷⁹
Human FIX	CMV-IE ^a	C57BL/6	IM ^b	Inhibitory antibody (<i>n</i> =4, no FIX in plasma)	Herzog <i>et al</i> (1997) ⁷⁶
Human FIX	MFG/CMV ^f	C57BL/6	IM ^b	Antibody (<i>n</i> =15, no FIX in plasma)	Ge <i>et al</i> (2001) ⁸²
Human FIX	MFG/CMV ^f	C57BL/6	Portal vein	Antibody (<i>n</i> =12+15, FIX persist in plasma)	Snyder <i>et al</i> (1997) ⁷⁶ ; Ge <i>et al</i> (2001) ⁸²

Note: ^aCMV-IE, cytomegalovirus IE enhancer/promoter; ^bIM, intramuscular delivery; ^cthe promoter is comprised of the human 1-antitrypsin promoter and 4 copies of the ApoE enhancer; ^dMFG, murine Moloney virus long terminal repeat (MLV LTR) promoter; ^eLSP, liver specific promoter; thyroid hormone-binding globulin promoter; ^fInternal CMV promoter in MFG retroviral vector.

Role of delivery vectors in host immune responses to transgene products

Gene transfer vectors themselves are important factors in host immune responses to encoded transgenes, which may be perceived by the host as neoantigens. Strongly immunogenic vectors such as recombinant first-generation adenoviruses can provide 'danger signals,' induce cellular production of inflammatory cytokines, helper epitopes to transferred neoantigens, and may act as immune adjuvants by mobilizing and activating the cells necessary for T-cell activation. The reasons for this are multifactorial and generally beyond the scope of this discussion. Briefly, however, while rAAV vectors have been engineered to express only the transgenes of interest, standard larger vectors, including adenovirus, express a wide variety of endogenous viral genes in addition to the inserted transgene. Furthermore, larger vector virions consist of multiple components, some of which may exhibit intrinsic cellular toxicity, are inherently more complex, and potentially more immunostimulatory than AAV capsids.⁸⁵ Finally, several available viral vectors are based upon common pathogenic viruses, and may elicit potent memory immune responses if the host has already been infected with a serologically related virus.

We will cite several examples to illustrate these issues. In early studies immunocompetent C57BL/6 mice were transduced intramuscularly with either rAAV2 or adenovirus vectors encoding the *lacZ* reporter gene under control of the cytomegalovirus immediate early (CMV-IE) promoter. Mice transduced with the rAAV2 vector exhibited long-term *lacZ* expression without the development of significant anti-*lacZ* cellular or humoral immune responses. In contrast, anti-*lacZ* host immunity was elicited when delivered by adenovirus vectors.^{25,49,86,87} Potentially stronger immunogenic antigens including hu-FIX and influenza virus hemagglutinin delivered by rAAV similarly induced less inflammation and host immune responses than that delivered by

corresponding adenovirus or even plasmid vectors.^{88,89} Even in immune privileged sites such as the subretinal space, immune responses in C57BL/6 mice to rAAV and its encoded transgene, green fluorescent protein (GFP), were weaker than those to GFP encoding adenovirus vectors.³³ Differences in vector transduction of antigen-presenting cells (APC) were initially invoked to account for the differences in immune responses. It was reported that rAAV2-transduced mouse spleen-derived mature DC failed to express transgenes that were necessary for the induction of cellular immune responses.^{25,89} Other groups observed that rAAV2-encoded transgene products induced Th2 subset activation and secretion of IL-10 that could modulate host cellular immune responses.^{26,88} Subsequently, it was demonstrated that rAAV2 encoding the *lacZ* gene could transduce murine bone marrow-derived immature DC *in vitro*. Adoptive transfer of the transduced DC markedly diminished transgene expression, and was associated with an antigen-specific cytotoxic T lymphocyte (CTL) response. Interestingly, adoptive transfer of the transduced immature DC into CD40L-knockout mice failed to elicit a CTL response, suggesting that CD40L-CD40 interactions played a critical role in regulating immature DC-mediated immune responses following rAAV2 gene transfer.⁹⁰ Blocking the CD40/CD40L pathway could induce host tolerance specific to the rAAV2 transferred neoantigen influenza virus hemagglutinin, but not to the same neoantigen delivered by an adenovirus vector.⁹¹ It is important to emphasize that cell-mediated immunity was less intensively studied than humoral immunity in the above experiments.

Immunogenicity of the transgene

It is clear that the strength of the host immune response is also transgene dependent. Thus, expression of a protein that is virtually identical to an endogenously expressed gene product is unlikely to be identified as 'foreign' and less likely to generate an immune response,

while expression of a highly divergent gene product will. This general rule has been supported by a variety of animal studies, in addition to human clinical trials. For example, human immunodeficiency virus (HIV)-1 infected individuals eliminated autologous T cells genetically marked with a retroviral vector encoding a herpes simplex thymidine kinase-bacterial hygromycin phosphotransferase (TKHygro) fusion protein. Host immune responses were elicited against the TKHygro fusion protein that was perceived as 'foreign' even in a population that would be considered immunosuppressed.⁹²

Recombinant AAV2 vectors have been successfully used to transfer several neoantigens including *LacZ*,²⁵ luciferase,²⁹ human placental phosphatase,²⁸ hu-AAT,⁹³ and α -galactosidase A,⁹⁴ in animal models, without the generation of host immune responses against encoded transgenes. In contrast, rAAV-mediated gene transfer of other transgenes, including herpes simplex virus glycoprotein B,⁷ ovalbumin,⁶ *N*-methyl-D-aspartate receptor,⁹⁵ influenza virus hemagglutinin,⁸⁹ HIV proteins,⁹ and hu-FIX,⁸² induced strong host immune responses. The subcellular location of the transgene product may also influence immune responses. Retargeting rAAV2-encoded *LacZ* from the cytoplasm to the cell membrane of muscle cells elicited murine CTL responses to the protein that were not observed in analogous experiments using the cytoplasmic form of the transgene.⁹¹ Presumably, the augmented immune response was the result of improved antigen presentation at the cell surface.

Role of transcriptional promoters controlling transgene expression on host immune responses

The transcriptional promoter regulates the tissue-specific pattern and the extent of transgene expression, both of which may influence host immune responses. In mouse muscular dystrophy models, expression of defective genes, by rAAV2 intramuscular delivery, was efficient if under the control of muscle-specific promoters, but inefficient when placed under the control of the nontissue-specific CMV promoter. Furthermore, decreased expression from a CMV promoter was associated with the generation of cellular and humoral immune responses directed against the neoantigen.^{96,97} Similar findings have been reported using other vectors. The liver-specific, murine albumin promoter, ubiquitously expressed RSV (Rous sarcoma virus LTR) promoter, and murine PgK (phosphoglycerate kinase) promoter were compared in first-generation adenovirus vectors encoding hu-AAT administered by portal vein injection.⁹⁸ Again, use of the tissue-specific promoter resulted in a lower incidence of antitransgene host immune responses. Studies using vaccinia vectors comparing early and late vaccinia virus promoters indicated that the early promoter was more effective for the generation of murine immune responses against bacterial β -galactosidase. This was believed to be the result of differences of promoter strength in APC, particularly DC. *LacZ* expression under control of the early promoter was 10-fold higher in DC than that from the late promoter. In contrast, *LacZ* expression was equivalent from both promoters in other cells including Epstein-Barr virus

transformed B lymphocytes.⁹⁹ The apparent unifying principle in these studies is that expression of transgenes from promiscuous promoters within adventitiously transduced APC, particularly DC, can elicit antitransgene immune responses. The use of tissue-specific promoters to minimize collateral transgene expression in APC may be particularly important when using vectors such as adenovirus, which generate potent host inflammatory/immune responses that may recruit large numbers of APC to the site of transduction. Such issues may not be as important for less immunogenic rAAV vectors.^{93,96} Furthermore, given the results from the previous studies, the induction of antitransgene immune responses following rAAV transduction⁹⁶ provides circumstantial evidence that APC might be transduced with rAAV vectors *in vivo*.

In light of the above studies, it would be interesting to determine whether the lack of transgene expression in rAAV-transduced mouse spleen-derived mature DC was the result of poor vector transduction or poor CMV promoter activity.²⁵ The CMV-IE promoter is rapidly silenced in murine lung⁹³ and liver.¹⁰⁰ Comparisons of CMV-IE and other promoter strength in mouse mature DC would be enlightening. Differential transduction of immature *versus* mature DC might also provide an explanation to these somewhat contradictory observations.

In addition to limiting transgene expression to particular cell types as in the use of 'tissue-specific' promoters, use of different promoters could lead to a wide range of transgene expression that might be outside of the optimal immunogenic dose range.^{101,102} For example, in mouse hepatocytes, the CMV-IE promoter generated low-level hu-FIX expression, and the development of circulating anti-hu-FIX antibody. In contrast, use of the human elongation factor promoter in an analogous setting generated high-level hu-FIX expression without eliciting anti-hu-FIX responses, perhaps via the induction of high-level immune tolerance.¹⁰² High-level expression of hu-FIX using other approaches in murine models also induced tolerance (see below).^{56,103}

Influence of route of and dose of rAAV administration on host immune responses

Evidence is accumulating that the route and dose of rAAV administration influence the extent and type of host immune responses, both to rAAV capsid proteins and encoded transgene products. For example, several studies have described development of humoral responses to hu-FIX in mice given rAAV2/FIX intramuscularly, but not when the same vector was administered hepatically (see Table 1).^{76,79,82,103} The mechanism(s) responsible for this difference is unclear. It is possible that the intramuscular route provides a different immune stimulus/pathway than the hepatic route, the natural site of FIX production.^{82,101} However, several observations contradict this hypothesis. First, inhibitory antibodies did not develop in the same mouse strain transduced intramuscularly with an rAAV1 vector encoding the same transgene.⁵⁶ Secondly, a similar neoantigen human clotting factor VIII (hu-FVIII) expressed intrahepatically via rAAV transduction in C57BL/6 mice did induce inhibitory antibody.¹⁰⁴ An-

other potential explanation for these findings is the generation of high-dose tolerance of C57BL/6 mice to FIX. Systemic expression of FIX is generally much higher after liver than muscle transduction with rAAV/FIX.^{76,80,82,101} Human FIX expressed at high level in C57BL/6 mouse liver after delivery by an adenovirus vector also induced tolerance, even in mice that generated anti-FIX humoral responses after intramuscular transduction with rAAV2/FIX.¹⁰³ The immune system of C57BL/6 mice may be very sensitive to hu-FIX expressed in muscle; C57BL/6 mice with pre-existing immunity to rAAV2 capsid protein developed anti-hu-FIX immune responses after intramuscular rAAV2/FIX transduction without detectable plasma FIX.⁸² Finally, proteins that are normally expressed in specific cell types may be post-transcriptionally processed utilizing tissue-specific routes, including alternative splicing, for example. Such processing may not occur or may aberrantly occur when the protein is expressed ectopically, potentially resulting in the generation of new immunogenic epitopes.

Other experiments using virus antigens or tumor-associated antigens encoded within rAAV2 vectors also demonstrated that the delivery route influenced the type of host immune responses. The *env*, *tat*, and *rev* genes of HIV were inserted into rAAV2 under the control of the CMV-IE promoter. The highest titer of specific serum IgG was observed in BALB/c mice after immunization with these vectors by intramuscular as compared to intranasal, intraperitoneal, or subcutaneous routes. In contrast, the highest secretory IgA titer was induced by intranasal inoculation.⁹ C57BL/6 mice injected with an rAAV2 vector encoding ovalbumin intraperitoneally, intravenously, or subcutaneously developed potent ovalbumin-specific CTL as well as anti-ovalbumin and anti-AAV2 antibodies. In contrast, mice injected with the same vector intramuscularly developed a humoral response to the virus and the transgene with only minimal ovalbumin-specific CTL.⁶ Thus, varying routes of vector administration may expose the same transgene product to differing immune pathways, which, in turn, may result in different immune presentation and responses.

To be effective, an antigen for vaccination must be administered within an 'optimal' dose range; too much or too little could result in the generation of high- or low-dose immune tolerance. In addition, low dose rAAV2 administration circumvented the generation of neutralizing anti-AAV capsid antibody, and promoted transgene expression^{28,29} experiments, suggesting an increased likelihood of inhibitory anti-FIX antibody development with increased vector doses.¹⁰⁵ A recent report described development of a hu-FVIII inhibitory antibody in C57BL/6 mice that persisted for 9 months after liver transduction with rAAV2/hu-FVIII. Unexpectedly, hu-FVIII was detected at a level similar to immunodeficient mice at 10 months after transduction, and hu-FVIII inhibitory antibody disappeared simultaneously, presumably signaling the development of hu-FVIII tolerance.¹⁰⁶

Immunomodulation and persistent transgene expression

As with antivector immunity, modulation of the immune system has been attempted to prevent and/or overcome

host immune responses to transgene products. *In vivo* administration of anti-CD4 antibody to delete CD4⁺ T cells was successful in reducing immune responses in BALB/c mice to influenza hemagglutinin, a model antigen, delivered intramuscularly by rAAV vectors. In contrast, the anti-CD40L antibody, MR1 (100 µg), administered intravenously on days -2, 0 (day of vector administration), +2, +4, and +12, only elicited transient and partial immunosuppression in an identical model.⁸⁹ To prevent the generation of anti-FIX antibody responses, Fields *et al.*⁷⁶ intraperitoneally injected hemophilia B mice with 100 µg MR1 and CTLA4Ig individually on days -3, 0, +3, +6, and +9, but observed only transient (60 days) and partial suppression of FIX inhibitor development. As discussed previously, MR1 or CTLA4Ig have been partially effective in eliminating antivector immune responses.^{28,74} Although CD40L is important both in the costimulatory pathway of antigen recognition and DC maturation,⁹⁰ the immune system may contain redundant signaling pathways. Thus, combination therapy with these two agents, and optimization of dose and schedule of administration need to be evaluated.

Cyclosporin A, cyclophosphamide, and Tacrolimus (FK506) were evaluated for their ability to prevent the generation of FIX inhibitors in hemophilia B mice injected intramuscularly with rAAV-FIX.⁷⁵ In this study, cyclosporin was administered multiple times intraperitoneally at 50 mg/kg during the first month of vector injection, but exhibited considerable renal toxicity without improving transgene expression. In contrast, FK506 injected subcutaneously at 125 µg per mouse every other day starting on the day of vector administration was effective in blocking inhibitor antibody formation, but required continuous drug administration. Cyclophosphamide was administered intraperitoneally at doses of 20 or 50 mg/kg starting at the day of vector injection and biweekly thereafter up to week +6. The 50 mg/kg dose suppressed inhibitory antibody formation for up to 12 months, the length of the study, without toxicity, but was ineffective in eliminating pre-existing inhibitors.⁷⁵ Successful circumvention of FIX inhibitors in the canine model of hemophilia B was also obtained with cyclophosphamide at the same dose and schedule as described above.⁸² Thus, treatment with cyclophosphamide at the time of vector administration could be an option for the abrogation of antitransgene immune responses in a high-risk population, such as individuals with a null mutation in which gene replacement is planned.

Prenatal gene transfer

Although *in utero* gene therapy is controversial,¹⁰⁷ theoretically transgenes expressed early in fetal development could induce host immune tolerance. Recombinant AAV2 vectors encoding hu-FIX injected intramuscularly into 15-day C57BL/6 fetuses resulted in long-term transgene expression without induction of host immune responses to either hu-FIX or to AAV capsid proteins.¹⁰⁸ These results were in contrast to similar experiments conducted in adult mice.⁸⁸ In a similar experiment, intramuscular transduction of 15-day-old CD-1 fetal mice with an rAAV2 vector encoding luciferase resulted in persistent transgene expression for 18 months without detectable humoral immune responses to luciferase or to rAAV2.¹⁰⁹ In contrast,

luciferase and rAAV2 were immunogenic to adult CD-1 mice.^{6,45,106} In contrast, recently only partial induction of tolerance was noted in 15-day fetuses of a variant murine strain B6/129 F1 injected with AAV/LacZ.¹¹⁰

Conclusions and future directions

It is clear from these studies that there is a fine balance between the generation of untoward and desired (as for possible vaccine development) antitransgene immune responses following rAAV transduction, and that the factors responsible for the shift towards immunity or tolerance have yet to be identified. The latter issue is a problem that is not unique to rAAV transduction *per se*, and permeates the entire field. What conclusions can be drawn from these sometimes conflicting studies? For avoidance of host immune responses to encoded transgenes there are several. First, when compared to comparable vectors, rAAV vectors appear to be less likely to generate anti-transgene host immune responses, although this is clearly relative and not absolute. Secondly, immune responses that are generated following either wild-type infection or rAAV transduction are predominantly humoral. For rAAV vectors, the magnitude and type of immune responses are route, promoter, transgene, and perhaps dose dependent. Thus, humoral immune responses are greater following intramuscular administration of an rAAV vector containing a promiscuous promoter such as CMV or RSV, and less following use of a tissue-specific promoter that is inactive in antigen-presenting cells or after hepatic administration. Pre-existing anti-AAV immunity, particularly with NABs, can obviate systemic rAAV transduction and/or vector readministration in animal models, although the significance in human clinical trials needs further clarification. In contrast, successful transduction and expression of cells residing in immune-privileged sites, such as the eye, may occur even in the presence of pre-existing anti-vector capsid NABs. Immunosuppression might be used to circumvent the development of host antivector or transgene immune responses, and appears to be more effective in obviating primary immune responses than in eliminating pre-existing immunity. As cross immunity to various rAAV serotypes does not necessarily occur, use of rAAV vectors pseudotyped with differing serotypic capsids to circumvent anti-AAV immunity might be a less toxic approach. It is clear that a more thorough understanding of the interplay between rAAV and their encoded transgenes and the host immune system is necessary for the optimal development of this promising vector system.

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