AAV: An Overview of Unanswered Questions

Kenneth I. Berns and Nicholas Muzyczka*

Guest Co-Editors, Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, Gainesville, Florida.

AAV has been studied for 55 years and has been developed as a vector for about 35 years. By now, there is a fairly good idea of the dimensions of what would be useful to know to employ AAV optimally as a vector, but there are still many unanswered questions within the system. As with all biological systems, each good experiment raises further questions to answer. This article provides an overview of those areas in which unknown information can be identified and of those questions that have not yet been recognized. Some of these are touched on in the six review articles in this issue of *Human Gene Therapy*.

Keywords: AAV toxicity, AAV persistence, AAV immunity, AAV cell interactions

INTRODUCTION

TODAY, ADENO-ASSOCIATED VIRUS (AAV) is one of the most promising and successful gene-therapy vectors. AAV vectors have been successful in the treatment of several monogenic diseases in early clinical trials.¹ Although work in the past has focused primarily on gene replacement, many investigators are now adapting the vector system to new clinical modalities, including RNAi and gene-modifying strategies such as Crispr/cas9.¹ Moreover, Glybera² has been licensed for clinical use in the European Union for the treatment of a lysosomal storage disease. To reach this level of success has taken >30 years from the initial isolation of an infectious AAV clone and the development of AAV vectors for long-term expression.^{3–5} This has included the development of so-called scalable production systems that promise to deliver large quantities of vector for both clinical trials and, eventually, patient therapies.⁶ Although AAV gene therapy appears on the threshold of success, there are still many gaps in our knowledge about this unusual virus. Here we review some of these areas.

AAV IMMUNITY

Even though it is known that wild-type adeno-associated virus (wtAAV) infection is common (>80% for AAV2),⁷ after 50 years the details of infection remain largely unknown. There is evidence that in an infection with a respiratory adenovirus,

co-infecting wtAAV can be found in tissues of the upper respiratory tract.⁸ It is not known whether all infections require respiratory droplet transmission or whether there are alternative routes of infection, for example the gastrointestinal tract. Many adenoviruses and herpesviruses have been identified as helper viruses in cell culture. However, there is no knowledge of whether any or all of these may be associated with the wtAAV infection of people. Although it is known that recombinant AAV vectors (rAAV) can be used to transfer genes to humans, it is not known if people are infected by wtAAV in the absence of a helper virus co-infection under natural conditions or, if so, under what circumstances and how high a titer is required. More fundamentally, it is unknown how high a titer is required for a successful infection (*i.e.*, one that induces an antibody response). Relatively few studies have been done to determine antibody levels to specific AAV serotypes, usually to see whether subjects in a clinical trial have antibodies to the AAV serotype used as a vector. In fact, one of the contributions to AAV biology by gene therapy has been the determination of the prevalence of AAV in the human population. Because of the apparent lack of AAV toxicity, this was previously of no interest to virologists and clinicians.

Two of the review papers in this issue address the issue of AAV immunity.^{9,10} Two key questions

*Correspondence: Dr. Nicholas Muzyczka, Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, 2033 Mowry Rd, Rm CG208, Gainesville, FL 32610-3610. E-mail: nmuzyczka@ufl.edu

emerge. First, is the AAV inverted terminal repeat (ITR) primarily responsible for the TLR9 response to AAV DNA that subsequently leads to an adaptive immune response? In addition, can this be avoided by modifying the AAV ITR, perhaps by altering the GC content of the terminal repeat? It is now known which sequences within the ITR are essential for viral propagation and could modify some of the GC-rich regions.¹¹ The fact that the AAV2 Rep proteins can package AAV2 ITRs into several of the other capsid serotypes and that only the AAV5 ITR requires its own unique Rep protein for DNA replication¹² suggests that there may be some flexibility in the ITR sequences that can function for both replication and packaging.

The second question is whether we can modify the capsid to remove the epitopes that are commonly recognized as antigenic sites. Many of the epitopes that are recognized for an increasing number of circulating monoclonal antibodies are now known.¹³ It may be possible to engineer viral vectors that are much less antigenic or at least escape recognition by the most prevalent memory cells in the human population. Work toward testing capsids in which antigenic sites have been ablated is actively being pursued, but modification of the ITR has not yet been tried. Of course, it is known that AAV is extremely old in an evolutionary sense. AAV-like viruses have been isolated from a variety of vertebrates, for example snakes.¹⁴ Such isolates are presumably less likely to contain epitopes to which humans have been exposed and would be good candidates for testing as vectors. Unfortunately, it is also now clear that throughout the AAV family, there are some regions, for example the so-called pH quartet region, that are highly conserved. This region, which contains a protease site¹⁵ and a transcription regulatory site,¹⁶ is believed to serve some critical function that has not yet been defined. It may account for some of the cross-reactivity seen for some monoclonal antibodies among AAV serotypes.

AAV PERSISTENCE

What, if any, are the consequences of the propensity of AAV to establish persistent infections? In animal models, AAV vectors persist in tissues primarily as extrachromosomal elements.^{17,18} These elements persist predominantly as circles in which the AAV genomes are lined up in a tandem (head-to-tail) array in which the ITRs occur as so-called double-D elements between expression cassettes.¹⁹ Integration in animal tissues is a rare event and is most often detected in tissues that are dividing at some rate (*e.g.*, liver hepatocytes). It is

now clear that wtAAV also persists primarily as free episomes.²⁰ WtAAV genomes isolated primarily from human tonsil and adenoid tissues were found to exist as circular tandem head-to-tail concatemers separated by double-D elements, similar to vector DNA. The structure of the episomes points to a simple homologous recombination event between the ITRs to form the circular intermediate, which occurs after synthesis of the second strand of DNA. This mechanism also provides a substrate for rescue and replication of the wt genome from latency using the known biochemical activities of the viral Rep protein. This does not preclude the possibility of wt or vector DNA integration, but integration for both wtAAV and rAAV appears to be a rare event. In contrast, in dividing cells in culture, rAAV persists primarily as an integrated copy and shows a propensity, at least in some cases, for integration into a region of chromosome 19q13.4.²¹ This region has an almost identical Rep binding element to that in the AAV ITR, and the recognition of the chromosome site is involved in the initiation of nonhomologous recombination with the chromosome.²² Surprisingly, there is only one report of wtAAV integrating into human tissue.²³ In addition, wtAAV has also been recovered from other human tissues, including muscle, the spleen, and blood.^{20,24,25} Recovery from blood is surprising, since this is tissue that is either actively dividing or has recently divided, raising the question of whether reinfection with wtAAV is common in human populations, or whether integration is the preferred mechanism of persistence in blood.

Although the high frequency of AAV-specific antibody in the human population is known, there are no data on the frequency of AAV persistence following natural infection. As noted above, wtAAV DNA can be detected in epithelial cells in the upper airway a short time after an adenovirus infection, and episomal copies of wtAAV have been detected in tonsils and adenoids, consistent with airway infection. Recently, AAV was found latent in human leukocytes at a high frequency (34%), suggesting that this may be a natural reservoir.²⁴ In another study, very high levels of wtAAV sequences were found in the urogenital tract tissue²⁶ of women suspected of having herpes infection. Neither of these findings has been independently confirmed. If AAV DNA persists, can the virus be rescued, either by superinfection with a helper virus or by exposure to genotoxic conditions, as has been reported in studies of latently infected cells in culture? The wt copies of AAV isolated from adenoid tissues and leukocytes were shown to be intact and capable of producing infectious virus particles.

Thus, they could potentially be rescued *in vivo*.^{20,24} The question has been considered because of a report linking wtAAV activation to fetal miscarriage,²⁷ a finding that was not confirmed by another group.²⁶ However, recombinant genomes, which lack the viral *rep* gene essential for replication, are not likely to cause fetal toxicity.

The most curious aspect of AAV vectors is that it is still not known why they work. This question emerged when Kessler *et al.*²⁸ and Xiao *et al.*²⁹ showed that an AAV vector would continue to express its transgene for 6–12 months *in vivo*. Subsequently, expression from an AAV vector in a canine eye persisted unabated for up to 12 years (William Hauswirth, unpublished), and similar results have been reported for muscle and brain transductions. Many laboratories have transfected circular expression plasmids into animal models and achieved only short-lived persistence and expression. Yet, AAV vector DNA, which also persists as a circular plasmid, not only persists for long periods of time but continues to express its transgenes from a variety of promoter cassettes. Not only are these episomes not cleared from cells, but they are also apparently not targets for epigenetic silencing in a wide variety of tissues. Herpes DNA also persists as an episome in neural tissues, but its expression is severely attenuated. WtAAV expression is similarly completely shut off by Rep protein expression, but this is not the case with AAV vectors where Rep is not present.

Since the only common element to all AAV vectors is the terminal repeat sequence, it seems likely that there is some kind of signal(s) in the ITR that promotes both plasmid persistence and prevents epigenetic shut off. This area of research has not received much attention. The ITR is the primer for second-strand synthesis when single-stranded vectors are used. It consists of an overall palindrome in which there are short palindromic sequences on either side of the midpoint of the ITR sequence. The simplest secondary structure that is envisioned for the ITR after it folds on itself is a T- or Y-shaped structure in two dimensions.³⁰ The ITR is a GC-rich sequence, and several alternative hairpins are possible within the so-called A-segment (nucleotides 1–41 and 86–125). Early studies with double-stranded forms of the ITR produced after renaturation suggested the presence of multiple forms near the termini, and more recent studies have supported this notion.³¹ However, the overall spectrum of alternative secondary structures is not known, and there is little, if any, information on tertiary or higher-order structures. For example, there is indirect evidence

that the ITR can adopt a non-B, non-Z, triple-stranded structure seen in telomeres.³² Detailed structural studies of the ITR and its possible role in the persistence of AAV vector DNA have not yet been done and may point to a mechanism that explains the persistence of AAV vector genomes. One possibility is that the extraordinary secondary structures possible for the ITR may inhibit methylation of the chromatin so that shut down of expression is averted. It has been reported that the ITR can function as a promoter for AAV vectors.³³ Earlier results suggested that the ITR could also function as an enhancer that is responsive to the Rep protein.³⁴ Studies on the structure of the ITR and their role in the persistence of AAV episomes are feasible and should be informative. This is particularly true with respect to structures of DNA protein complexes. Multiple transcription element binding sites have been identified in the AAV ITR, including the binding site for the histone acetylase YY1, as well as p53, Sp1, and others. Although it is known that the ITR has low-level promoter activity that does not use a TATA box,³⁵ with few exceptions,^{36,37} relatively little work has been done on the role of specific ITR sequences in gene expression or episomal persistence. Detailed studies of AAV chromatin, including histone association and modification, and DNA methylation have not been done on AAV expression cassettes in animal models and are overdue.

AAV TOXICITY

There have been several reports that suggest that AAV integration may be oncogenic (see the review by Chandler *et al.*³⁸). AAV infection of newborn mice from a mutant line has been reported to lead to an increased incidence of liver cancer. This work has been reproduced but seems to be specific to a particular strain. The overwhelming majority of AAV infections of mice (with either wtAAV or AAV vectors) have not shown an increased incidence of tumors. Similarly, negative results have been found following injection of AAV vectors in other animal models. A recent report from France has suggested a link between primary hepatic cell carcinoma and integrated AAV sequences (see the review by Srivastava and Carter³⁹). The frequency of cases associated with AAV sequences was low, but the AAV sequences were found in cell sequences implicated as potential tumor inducers. Several groups of scientists wrote responses that argued that the data presented did not support the authors' conclusion that AAV was linked to the

tumors under study. The AAV sequences were not associated with tumor tissue, and only a small stretch of sequence from the right end of the AAV genome were found integrated.^{40,41} Similar results were observed following co-transfection.³⁹

In contrast to the suggestion that AAV may be oncogenic, there have been several reports that AAV may inhibit oncogenesis.³⁹ Women with cervical carcinoma were much less likely to be seropositive for AAV2 than the general population. This result was obtained independently by several groups at different times but was not given much credence. After human papillomavirus (HPV) was recognized as the etiologic agent in cervical carcinoma, the ability of AAV to inhibit papilloma virus and the ability of HPV to act as a helper virus for AAV were both demonstrated.^{42,43} Thus, at least at the experimental level, the notion that AAV can inhibit cervical carcinoma has some support. Furthermore, it is supported by cell culture data that both the *rep* gene and the ITR by itself are capable of inducing repair pathways that can lead to apoptosis in tumor cell lines.^{44,45} These data are congruent with the finding that AAV is often found in female urogenital tissue that also contain HPV.⁴⁶ The clinical significance of these observations is uncertain, but the concept that AAV can inhibit viral oncogenesis conceptually nicely complements the notion that AAV is fundamentally nonpathogenic. The data and the concept are intriguing, but further studies of the question are clearly needed to help resolve the issue. Of course, the notion that AAV may be both oncogenic and anti-oncogenic constitutes an amusing biological conundrum. Neither or both may prove to be true.

Another source of potential toxicity that appears to be emerging occurs at high-input doses of AAV, or when AAV is used for transduction of embryonic stem cells. There have occasionally been anecdotal reports of acute toxicity, presumably due to apoptosis following rAAV transduction in adult animal models. This has been seen in brain experiments where rAAV transduction was associated with what appeared to be apoptosis at the site of injection and not simply inflammation. Perhaps the clearest example of this was reported by Ulsoy *et al.*⁴⁷ This group was investigating the possible toxicity of RNAi constructs as a function of input dose and used a GFP-expressing virus as a control, as well as viruses that contained scrambled genes. Strikingly, toxicity was observed with all of their constructs, including the GFP-containing construct, when the same input dose was reached, suggesting that some aspect of the vector and not the transgene might be responsible for toxicity. It is

now clear that use of rAAV for transduction of embryonic stem cells is toxic; see for example Hirsch *et al.*⁴⁸ Again, the ITR and its interaction with repair pathways is likely to be the culprit, but, as yet, a clear explanation is not available. It suggests, however, that the long-held view that AAV and rAAV are never toxic does not necessarily apply to the specialized conditions present in gene-therapy experiments, where very high localized doses of vector are sometimes used in specialized cells.

MISSING ACTORS AND VIRUS-CELL INTERACTIONS

AAV was considered initially to be an interesting model virus because the small size of the genome suggested a simple strategy for the viral life cycle. This is now recognized to have been an enormously naïve notion. What has become clear is that in order to perpetuate its genome, AAV must perform all the functions of much larger viruses. The fact that one can use insect cells and baculovirus as a helper virus to produce the same titers that are obtained from mammalian cells using herpes or adenovirus shows how remarkably adaptable the virus can be. The small size of the genome means that AAV is much more clever (or devious) in the strategy to replicate and perpetuate its DNA.

Originally, the AAV genome was considered to encode four nonstructural proteins in an ORF in the left half of the genome and three structural capsid proteins in an ORF in the right half. Early studies on RNA translation showed that the VP2 capsid protein is initiated from an unusual ACG codon.⁴⁹ Then, after many years, an additional protein was discovered that functions during virion assembly.⁵⁰ More recently, an additional coding region embedded in the AAV capsid gene has been reported to be involved in the viral life cycle.⁵¹ Both lipase and proteinase functions have been described for the structural proteins.^{15,52} It is now clear that antisense transcription and translation may play a role cellular gene expression,⁵³ and this could also be a part of the virus repertoire. Indeed, a recent study has detected antisense transcription from the AAV genome.⁵⁴ In addition, in recent years, the significance of non-coding RNAs has been appreciated in virus biology and a recent study suggests that non-coding RNAs are transcribed from AAV as well.⁵⁵

Finally, to date, most of the work on AAV-cell interaction has focused on identifying the proteins involved in AAV DNA replication and transcription factors that bind to AAV promoter regions. However, we still know very little about AAV interac-

tions with cellular proteins. Recently, thanks to more sophisticated proteomics techniques, a number of proteins have been identified that are involved in attenuating AAV viral yield⁵⁶ and potentially participating in AAV assembly.⁵⁷ This work will be very important for generating improved vector production systems as well as improved transduction.

AAV is turning out to be a remarkably versatile tool for both molecular biology and clinical therapy. However, it is thought that we are only at the beginning.

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AUTHOR DISCLOSURE

K.I.B. has no conflicts of interest to declare. N.M. is a founder of Applied Genetic Technologies Corporation (AGTC) and Lacerta Therapeutics. Both companies are involved in commercializing AAV vectors for various clinical applications.

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