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Research Article

Cost-Effective Approach to AAV Viral Vector Manufacturing

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Abstract: Due to its outstanding safety profile and effective transduction to diverse target tissues, adeno-associated virus, often known as AAV, has emerged as a major platform for the delivery of therapeutic genes for the treatment of a wide range of disorders. In contrast, the manufacturing of viral vectors at large scales and their long-term storage are inefficient processes, which leads to lower yields, middling purity, and a shorter shelf life as compared to those of recombinant protein treatments.
Keywords: Adeno-associated virus, tissues, viral vectors.

Introduction

Over the course of several decades, significant technical improvements have been achieved, enabling options for the treatment and control of life-threatening disorders. Gene therapy is one of the most ground-breaking medical breakthroughs that have occurred in this modern era. The transport of the gene of interest into the cells of a patient as a therapy to cure a life-threatening condition is an example of gene therapy, which is also known as human gene transfer. The most fundamental definition of gene therapy describes the process. The transport of genetic material to the target cells in an effective and safe manner is a critical factor in determining whether or not gene therapy will be successful in clinical trials. For the purpose of gene delivery, several viral-based vectors and non-viral delivery methods, including as nanoparticles, liposomes, lentivirus, adenovirus, and adeno-associated virus, have been put through their paces and found wanting (AAV). When compared to other delivery systems for gene therapy[1,2], AAV has emerged as the predominant vector due to its many desirable attributes. These desirable attributes include a lack of pathogenicity, efficient infection of dividing and non-dividing cells, and sustained maintenance of the viral genome. Additionally, AAV has emerged as the predominant vector due to its ability to maintain the viral genome. There are now two gene therapy medicines available on the market that have been granted FDA approval and are based on AAV delivery methods.

Adeno-associated virus

The genome of the adeno-associated viral (AAV) vector is roughly 4.7 kilobases in size, and it is protected by a protein coating that surrounds it (kb). The viral vectors used in AAV infections are members of the parvovirus family. Co-infection with a second helper virus, such as adenovirus or herpesvirus, is required in order for the AAV genome to replicate and express its viral genes in an effective manner. The structure of an AAV is comprised of an eight-stranded antiparallel b-barrel as well as an a-helix. The ssDNA that has been packed is encased within the capsid, which is formed by the b-sheet creating the inner surface of the capsid and the b-barrel forming the core of the capsid[2].

Rep (Replication), Cap (Capsid), and aap are the three genes that are included inside the genome's single strand (Assembly). These coding sequences are surrounded by inverted terminal repeats, also known as ITRs, which are important for the packaging and replication of the genome. Rep78, Rep68, Rep52, and Rep40 are the four proteins that are encoded by the Rep gene. These proteins are necessary for the replication and packaging of the viral genome. The Cap gene is responsible for encoding three structural viral capsid proteins that overlap one another. These proteins, known as VP1, VP2, and VP3, create the outer capsid shell that shields the viral genome. Additionally, the Cap gene is actively involved in cell interaction and internalization.

The viral capsid coat is made up of 60 different proteins that are organised into an icosahedral shape, and the molar ratio of the viral coat proteins to the capsid proteins is around 1:1:10 (VP1:VP2:VP3). Among the proteins that make up the capsid, VP1 has the greatest molecular weight (87 kDa), followed by VP2 (72 kDa) and VP3 (62 kDa). The three capsid proteins have a b-barrel domain, but their N-terminal extensions are distinct from one another. Variable loops provide unique surface topologies for each AAV variation, and these topologies mediate the molecular interactions that are crucial for cell attachment, entrance, and immunological characteristics. Variable loops also contribute to the diversity of AAV. An alternative reading frame of the aap gene, which overlaps with the reading frame of the cap gene, encodes the assembly activating protein (AAP). This nuclear protein functions as a scaffolding component during the capsid building process[1,2].

Literature review

One of the gene therapy vehicles that is being researched the most extensively is called adeno-associated virus (AAV). As a result of its discovery in the beginning as a contamination of adenovirus preparations[1,2], it was given this designation. To put it more simply, the AAV genome is protected by a protein shell that encircles and shields a rather modest single-stranded DNA sequence that is roughly 4.8 kilobases (kb). AAV is a member of the parvovirus family and in order for it to proliferate; it has to be co-infected with other viruses, most often adenoviruses. After being identified through serology at first, hundreds of distinct AAV strains in a wide variety of animals have been found thanks to the molecular cloning of AAV genes. It has three genes: rep (which stands for replication), cap (which stands for capsid), and aap. Its genome is single-stranded (Assembly).

Through the utilization of three separate promoters, alternate translation start sites, and differential splicing, these three genes are responsible for the production of at least nine different gene products. These coding sequences are surrounded by inverted terminal repeats, also known as ITRs, which are important for the packaging and replication of the genome. Rep expression gives rise to the viral capsid proteins (VP; VP1/VP2/VP3), which form the outer capsid shell that protects the viral genome, as well as being actively involved in cell binding and internalization[3]. The Rep gene encodes four proteins (Rep78, Rep68, Rep52, and Rep40), which are required for viral genome replication and packaging. Cap expression gives rise to the viral capsid proteins (VP; VP1/VP2/VP3). It is believed that the viral coat is made up of sixty different proteins that are grouped in an icosahedral form, and the capsid proteins are in a molar ratio of one to ten, one to two, and three (VP1:VP2:VP3)[4]. An alternative reading frame for the aap gene, which overlaps with the reading frame for the cap gene, encodes the assembly-activating protein (AAP). It is believed that this nuclear protein performs a scaffolding function necessary for capsid assembly[5]. Although AAP is required for the nucleolar localization of VP proteins and the construction of the capsid in AAV2, the subnuclear localization of AAP differs across the 11 other serotypes that have been recently investigated, and AAP is not required for the replication of AAV4, AAV5, or AAV11[6].

Although there is a great deal more to the biology of wild-type AAV, a great deal of which is not completely known, the form that is utilised to make gene therapies is not the form that is employed. Because it does not contain viral DNA, recombinant adeno-associated virus, also known as rAAV, is essentially a protein-based nanoparticle that has been designed to pass through the cell membrane. Once there, it may ultimately transport its DNA payload into the nucleus of a cell. ITR-flanked

transgenes that are expressed within rAAV may form circular concatemers even in the absence of Rep proteins[7,8]. These concatemers continue to exist as episomes in the nucleus of cells that have been transduced. As a result of the inability of recombinant episomal DNA to integrate into the genomes of host organisms, its copy number will gradually decrease over the course of time as the cell goes through successive cycles of replication. This will, in time, lead to the loss of both the transgene and the expression of the transgene; the pace at which the transgene is lost is dependent on the turnover rate of the cells that have been transduced. Because of these properties, rAAV is an excellent candidate for use in many gene therapy applications. Based on our existing knowledge of viral biology and the present status of the platform[9,10], the following provides an outline of the practical factors that need to be taken into account prior to using rAAV as a gene therapy agent.

Adeno-associated virus biology

Structure of the genome, replication of DNA, and assembly of viruses

In 1965, the human adeno-associated virus, also known as AAV, was found to be a contaminant that was present in adenovirus (Ad) preparations. AAV is one of the tiniest viruses, with a non-enveloped icosahedral capsid that measures roughly 22 nm. Its crystal structure has just recently been resolved to a resolution of three angstroms, making it one of the most precise examples of its kind. Dependovirus is the name of a distinct genus within the Parvoviridae family that contains AAV serotypes. This is due to the fact that a productive infection would typically not take place without the presence of a co-infecting helper virus. Despite the great seroprevalence of AAV in the human population (about 80% of individuals are seropositive for AAV2), the virus has not been connected to any human ailment that has been documented.[6,7]

The genome of the AAV is roughly 4.7 kilobases in length and is linear and single-stranded DNA (kb). Due to the multi-palindromic nature of its terminal 125 bases, the AAV2 DNA termini are composed of an inverted terminal repeat (ITR) that is 145 nucleotides long and may fold on itself via complementary Watson-Crick base pairing to produce a distinctive T-shaped hairpin structure. This secondary structure, in accordance with the AAV DNA replication model, supplies a free 3' hydroxyl group for the commencement of viral DNA replication by a self-priming strand-displacement mechanism that involves leading-strand synthesis and double-stranded replicative intermediates. Since the virus does not include an encoding for a polymerase, it must rely on the activity of cellular polymerases to copy its DNA. The ITRs are located on each side of the viral genes rep (which stands for replication) and cap (which stands for capsid), which code for nonstructural and structural proteins, respectively. The rep gene makes use of two promoters that are located at map positions 5 (p5) and 19 (p19), as well as an internal splice donor and acceptor site, in order to encode four different regulatory proteins. These proteins have been given the names Rep78, Rep68, Rep52, and Rep40 based on the apparent molecular weights of each of them. Through their interactions with the Rep-binding element (RBE) and terminal resolution site (trs) sequences that are found inside the ITRs, the Rep78 and Rep68 proteins take part in the process of DNA replication that is carried out by AAV[8].

Furthermore, in response to environmental signals such as the presence or absence of a helper virus, these proteins either positively or negatively control the expression of the AAV gene, depending on the case. The formation of single-stranded viral genomes from double-stranded replicative intermediates requires the participation of the Rep52 and Rep40 proteins, which both play a role in this process. The single-stranded genomes that arise are bundled with similar effectiveness, regardless of whether they have a positive or negative polarity. The efficiency with which AAV performs its tasks is mind-boggling, and it results not only from the overlapping genetic arrangement of its genome but also from the incorporation of a wide variety of metabolic processes into each of its relatively few gene products. For example, Rep78 and Rep68 are both strand-and site-specific endonucleases in addition to being DNA binding proteins that are site-specific. In addition to that, they possess the helicase and ATPase functions that are likewise possessed by Rep52 and Rep40.

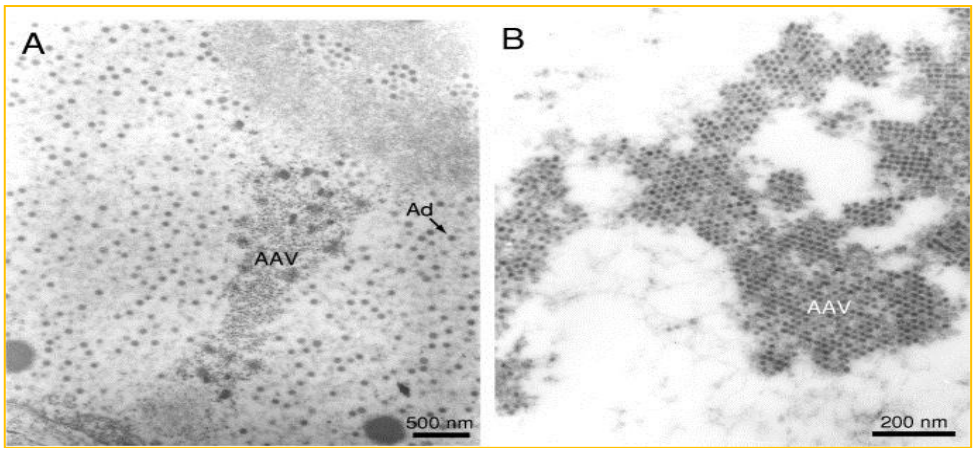

Figure 1. Transmission electron microscopy of AAV2 and Ad5 particles in human cells

The internal transcription regulator of AAV2 (ITR) functions as the origin of replication and is made up of two arm palindromes (B-B' and C-C') nestled inside of a bigger stem palindrome (A-A'). The ITR is capable of acquiring two different setups (flip and flop). Both the flip (which is shown) and the flop configurations have the B-B' palindrome closer to the 3' end, but the flop configuration has the C-C' palindrome. Because the D sequence is only found once at either end of the genome, the genome is still in its original single-stranded state. The Rep-binding element (RBE) is where the AAV Rep78 and Rep68 proteins bind, and the boxed motif correlates to this element. The RBE is made up of a tetranucleotide repeat that follows the pattern of 5'-GNGC-3'. The ATP-dependent DNA helicase activities of Rep78 and Rep68 remodel the A-A' region, producing a stem-loop that places the terminal resolution site (trs) in a single-stranded state at the summit of the area. A nick is introduced at the trs by the strand-and site-specific endonuclease catalytic domain of Rep78 and Rep68 when they are arranged in this arrangement. The nucleotides that are shaded at the top of the structure that is shaped like a T correspond to an extra RBE that is known as RBE'. This RBE helps to maintain the connection between the two biggest Rep proteins and the ITR[5,6].

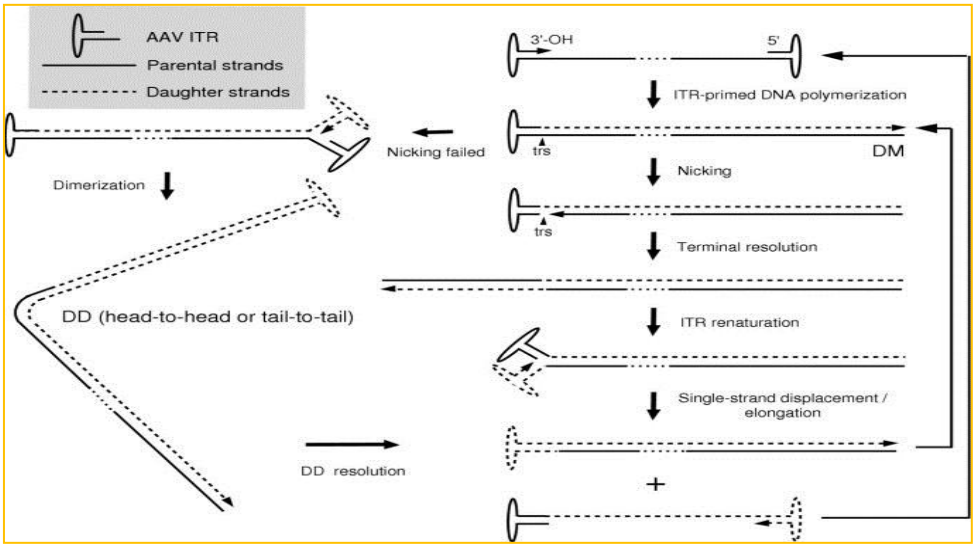

Figure 2. Schematic illustration of the model used to describe how AAV DNA replicates

The self-priming single-strand displacement mechanism that is expected to be involved in AAV DNA replication is thought to be triggered by DNA polymerization at the 3' hairpin primer of input single-stranded genomes. This process is thought to include AAV DNA replication. This results in the creation of linear unit-length double-stranded molecules, also known as duplex monomers (DMs), which have one end that is closed off by a covalent bond. By making site-specific nicks in the parental strand in the direction that is directly opposite the original 3' end location, these constructs can be resolved at the terminal resolution site (trs) (i.e., at nucleotide 125). DNA

polymerases need a substrate in order to unwind and duplicate the inverted terminal repeat, and the newly created free 3' hydroxyl groups offer this substrate (ITR). Last but not least, the palindromic linear duplex termini are able to renaturate form terminal hairpins, which positions the 3' hydroxyl groups in the correct location for single-strand displacement synthesis. Following this step, single-stranded genomes and new DM replicative forms will be created. In the event that nicking does not take place, elongation continues through the covalently closed hairpin structure, which results in the generation of linear double-length double-stranded molecules known as duplex dimers, DDs. These DDs can have either a head-to-head or a tail-to-tail arrangement. By utilizing the AAV ITR sequences that are positioned along the axis of symmetry, the DD replicative intermediates have the potential to be resolved to DMs[7,8].

The production of two transcripts is initiated by alternative splicing at two acceptor sites. The bigger transcript encodes virion protein 1, also known as VP1, which is the capsid protein component with the most amino acids. The shorter mRNA has a downstream conventional initiation codon (AUG) that directs the synthesis of VP3, in addition to a noncanonical start codon (ACG) that is used to make VP2. This combination is employed to generate VP2. The N termini of the VP1, VP2, and VP3 proteins are where they differentiate from one another. Additionally, their apparent molecular weights range from 87, 72, and 62 kDa, respectively. Together, they self-assemble into a nearly spherical protein shell that has T = 1 icosahedral symmetry and consists of a total of 60 subunits. Recent research has showed that small holes at the 12 axes of symmetry of a fivefold symmetry are important for the infectivity of viruses and the packaging of genomes.

In AAV particles, the ratio of VP1 to VP2 to VP3 in terms of molar concentration is 1:1:10. It is believed that the relative quantity of the two cap gene transcripts and the relative efficiency of translation initiation at the three start codons for the structural proteins are reflected in this stoichiometry. A conserved phospholipase A2 (PLA2) motif has been found to have a biological importance in AAV2 infection. This motif was first discovered inside the distinct N-terminal region of the parvoviral VP1 proteins. To be more specific, the VP1-embedded PLA2 activity seems to play a key role at some stage between the translocation of the AAV genome from the endocytic to the nuclear compartment and the initiation of viral gene expression, despite the fact that it is not required for the assembly of the capsid, the packaging of the DNA, or the internalization of the virion. In recent years, mutational analysis of amino acid residues involved in AAV2 capsid pore architecture has indicated that conformational changes of the virion structure that occur during infection cause the VP1 N termini to protrude through the capsid pores, thereby inducing the PLA2 enzymatic activity that is necessary for successful infection. At the level of virion formation, immunofluorescence data shows that the VP1 and VP2 proteins are found primarily in the nuclei of infected cells, whereas VP3 is nearly evenly distributed between the nucleus and the cytoplasm of infected cells. This is due to the fact that the virion formation process takes place in the nucleus of infected cells.

However, when VP1 and/or VP2 are present, VP3 begins to accumulate in the nucleus. This behavior suggests that the main capsid protein is transported to the nucleus via a connection with the proteins VP1 and VP2, which carry nuclear localization signals. Based on the results of the immunofluorescence study, it appears that capsid construction is restricted to the nucleoli of infected cells. The participation of nucleolar chaperones in this process has been proposed as a possible explanation. In a mechanism that is reliant on AAV Rep, fully constructed AAV capsids make their way into the nucleoplasm. Because of this redistribution of the structural proteins, all of the components that are required for the generation of infectious particles, including viral genomes, capsids, and Rep proteins, become co-localized with one another. It is believed that different sections of the nucleoplasm are the sites of the several steps involved in the packaging of AAV DNA. Protein-protein interactions between pre-formed empty capsids and complexes of Rep78 or Rep68 with the viral genome are thought to guide selective AAV DNA encapsidation. This is hypothesized to be the case. Following this, it is postulated that the helicase domains of the capsid-docked Rep52

and Rep40 proteins operate as molecular motors that unwind and transport newly generated single-stranded DNA into empty particles through the holes positioned at the fivefold symmetry axis.

The vectorology of the Adeno-associated virus

Throughout the course of history, the majority of recombinant adeno-associated viral (rAAV) vectors have been based on serotype 2 (AAV2), which serves as the prototype for the genus. Those who are interested in using rAAV for gene therapy applications should take into consideration the faulty nature of the parental virus as well as the virus's purported lack of pathogenicity. Not only did the discovery that a molecularly cloned AAV genome could in Ad-infected cells recapitulate the lytic phase of the AAV life cycle and give rise to infectious virions make it possible to conduct in-depth genetic studies of the virus, but it also made it possible to generate rAAV particles by providing a substrate for their production. The fact that the AAV ITRs contain all cis-acting regions needed in genome rescue, replication, and packing made the latter job much simpler to do. Furthermore, because the AAV ITRs are separated from the viral encoding regions, the design of rAAVs can follow the whole-gene-removal or "gutless" vector rational of, for example, retrovirus-based vectors. This is possible in the sense that the cis-acting elements involved in genome amplification and packaging are in linkage with the heterologous sequences of interest, while the virus encoding sequences necessary for genome replication and virion assembly are provided in rAAV particles are typically produced by transfecting producer cells with a plasmid containing a cloned rAAV genome composed of foreign DNA flanked by the 145 nucleotide-long AAV ITRs and a construct expressing in trans the viral rep and cap genes. This procedure is followed by the transfection of the producer cells. In the presence of Ad helper functions, the rAAV genome is put through the lytic processes of wild-type AAV by being rescued from the plasmid backbone, replicated, and then packaged into pre-formed AAV capsids as single-stranded molecules. This takes place while the rAAV genome is in the process of being subjected to the wild-type AAV lytic processes[8-10].

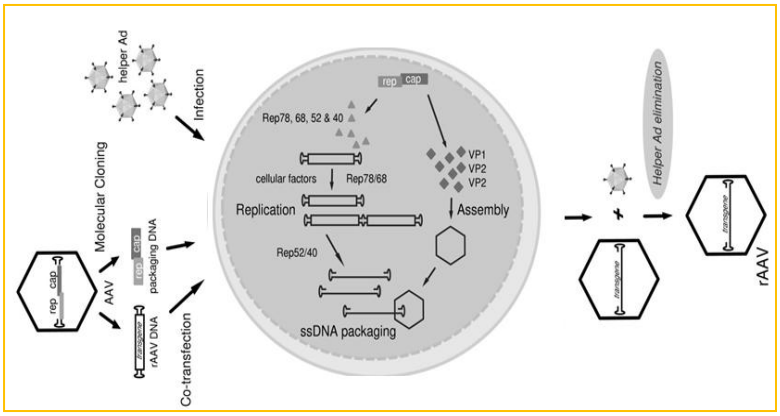

Figure 3. Recombinant AAV production system

The first infectious clones of AAV were able to be generated, which made it possible to conduct functional dissections of the viral genome. Because of this, it was possible to construct plasmids that encode rAAV genomes in which the minimal complement of wild-type sequences required for genome replication and packaging (i.e., the AAV ITRs) frame a gene of interest (a transgene) rather than the AAV rep and cap genes. Producing rAAV particles requires transfecting these constructs into packaging cells together with an expression plasmid for rep and cap. This process results in the generation of rAAV particles. As shown in the illustration, the infection of the packaging cells with wild-type Ad was first responsible for the introduction of the helper activities that were necessary for the activation and support of the productive phase of the AAV life cycle. Instead of using viral infection as the means of production, modern approaches that rely on transfection make use of recombinant DNA that encodes the helper activities. The actions of cellular DNA polymerase, in conjunction with the proteins Rep78 and Rep68, cause a buildup of replicative intermediates in both the duplex monomer (DM) and duplex dimer (DD) forms. The catalytic activities of the Rep52 and

Rep40 proteins are most likely responsible for the incorporation of a portion of the newly synthesised DNA in the form of a single-stranded molecule into previously formed capsids that are otherwise empty. The infectious rAAV virions that are produced by this process are then discharged from the producer cells together with the assistance Ad particles. Using a combination of sequential heat treatment and buoyant density centrifugation, it is possible to selectively remove the helper virus from the final preparation of rAAV[10].

Immunogenicity of the AAV

The fact that rAAV is made up of biomolecules, specifically proteins and nucleic acids, makes it an attractive candidate for use as a vector for the transfer of genetic material. A full-package virus, on the other hand, does not contain any modified lipids or other chemical components. These are the kinds of things that might contribute to undesirable toxicities or immunogenicity's in a way that may not be totally understandable or foreseeable. In comparison to other viruses, the AAV has been found to have a lower immunogenic potential. Although the exact explanation for this is not entirely clear, one theory suggests that it may be related to the discovery that certain AAVs are unable to transduce antigen-presenting cells in an effective manner (APCs). Additionally, in contrast to other methods of viral delivery, rAAV does not include any viral genes; hence, there will be no active viral gene expression to enhance the immune response. This is because rAAV does not contain any viral genes. Although it has been demonstrated that AAV is not as immunogenic as other viruses (such as adenovirus), both the capsid proteins and the nucleic acid sequence that is transmitted can stimulate various components of our immune system. This is made even more complicated by the fact that the vast majority of people have already been subjected to AAV and have already developed an immune response against the specific variants to which they have previously been subjected, which has resulted in a pre-existing adaptive response. This makes the situation even more difficult. This can include non-specific antibodies (NAbs) and T cells, both of which have the potential to reduce the clinical efficacy of subsequent re-infections with AAV and/or the removal of cells that have been transduced. It should come as no surprise that one of the most difficult challenges involves determining how to administer an effective therapeutic dose of rAAV to a patient population that already possesses a sizeable quantity of circulating NAbs and immunological memory against the virus. The virus will be recognised as a foreign protein regardless of whether it is supplied locally or systemically; as a result, the adaptive immune system will make an effort to eradicate it. The humoral response to AAV is mediated by the absorption of the virus by professional APCs, as well as their presentation of AAV capsid peptides to B cells and CD4+ T cells in the setting of class II major histocompatibility proteins (MHCs). This results in the creation of plasma cells and memory cells, both of which are able to produce antibodies that are specific to the AAV capsid. These antibodies can either be neutralizing, which has the ability to prevent further AAV infection, or non-neutralizing. Neutralizing antibodies have the capacity to prevent subsequent AAV infection. It is believed that non-NAbs opsonize the viral particles, making it easier for the spleen to remove them from the body[3,5,10].

Conclusion

During the course of the natural infection process, the virus is internalized by clathrin-mediated absorption into endosomes once it has entered target cells and begun the process of natural infection. After the virus has successfully escaped from the endosome, it is carried to the nucleus, which is the location where the ITR-flanked transgene is decodified from the capsid. The method and mechanism of AAV intracellular trafficking and processing are not completely known, and there are quite a few areas of contention with relation to our present level of expertise. The most widely accepted theory is that capsid breakdown and uncoating take place after endosomal egress and then subsequent nuclear translocation, respectively. On the other hand, it is speculated that the complete virus could undergo ubiquitination in the cytosol as it is being transported to the nucleus. This would be an essential step in the process of directing capsid proteins to the proteasome for proteolytic processing into peptides in order to present them to class I MHC. The fact that proteasome inhibitors or mutations in capsid residues that are sites for ubiquitination can limit class I presentation and T-cell activation provides

support for this hypothesis. T-cell activation in response to various AAV variants that share a significant amount of sequence identity has, however, been observed to behave in a manner that is inconsistently distinct from one another. It is not known at this time whether this is because of subtle differences in the capsid sequence, susceptibility to MHC I presentation, or differential cellular processing that is inherent to the various AAV variants, or whether it is simply because of contaminants in the vector preparations.

Conflicts of interest: The authors declare no conflicts of interest.

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