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MINIREVIEW

Building a Better Vector: The Manipulation of AAV Virions

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This review will focus on research directed at manipulating the virion of adeno-associated virus (AAV) with the goals of circumventing the immune response of the virion, as well as retargeting the virus to specific cell types of interest. The use of five AAV serotypes for addressing questions of Ab neutralization, novel tropism, as well as providing natural templates for targeting by virion modification will be discussed.

AAV BIOLOGY

Adeno-associated virus type 2 (AAV2) is a small (20–25 nm), single-stranded DNA (4680 nucleotide), nonenveloped, parvovirus that has emerged as a major player in the viral gene delivery arena. The only *cis* components required to generate AAV2 vectors are the two 145-nucleotide inverted terminal repeats [for review, see (Samulski *et al.,* 1999)]. These terminal repeats are required for the replication and packaging of the recombinant genome into the newly formed AAV virions (McLaughlin *et al.,* 1988; Samulski *et al.,* 1989). The virion is composed of three structural proteins, Vp1, Vp2, and Vp3, that form the 60-subunit viral particle in a ratio of 1:1:20, respectively. The structural proteins are translated from three overlapping transcripts of a single structural gene that differ due to alternative splicing and start codon usage. Therefore, alterations made in the Vp3 coding domain will be present in all 60 subunits while alterations in Vp1 will be present in approximately 3 subunits.

Properties of the wildtype (wt)AAV type 2 as well as advances in the production of rAAV type 2, including the elimination of helper virus contamination (Ferrari *et al.,* 1997) and purification by column chromatography (Zolotukhin *et al.,* 1999) have helped advanced recombinant (r)AAV type 2 use in gene therapy applications. Like nonpathogenic wtAAV type 2, rAAV infects both dividing and nondividing cells and establishes latency for the life of the cell. Although the natural route of infection for wtAAV is upper respiratory, rAAV has demonstrated infection and long-term expression of transgenes in the brain, liver, muscle, retina, and vasculature of experimental animals (Samulski *et al.,* 1999).

Ironically, the broad tissue tropism once considered an advantage of AAV type 2 might be viewed as a liability. The ideal clinical situation for a viral vector would be the delivery of a gene therapy agent by the least invasive manner in the lowest dose required. This could be accomplished if the delivery agent were able to specifically target the organ or tissue most affected by the condition, thereby lowering the effective dose required. The appeal of targeting vectors is the potential for a simple injection of the transfer vehicle to exclusively localize to and transduce the target tissue. For this ideal to become a reality, a number of steps must be accomplished. The foremost obstacle is the host immune response against the virus.

IMMUNE RESPONSE AGAINST AAV

Two aspects of the immune response against AAV type 2-based vectors in gene therapy applications, preexisting immunity and the potential need for readministration, have generated the interest for research using other serotypes and manipulating the virion to alter antigenic determinants.

One of the challenges facing the successful use of AAV type 2 as a gene therapy tool is the impact of natural immunity to the virion. To understand the scope of this challenge, studies have determined the prevalence of serum antibodies in the population. These studies have defined the subset of the population with seropositive antibodies against AAV type 2 between 50 and 96%, with 18 to 67.5% being neutralizing (Blacklow *et al.,* 1968; Chirmule *et al.,* 1999; Erles *et al.,* 1999; Moskalenko *et al.,*

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TABLE 1

Pairwise Comparison of Amino Acid Sequences in AAV Serotypes

Serotype[a]	Total (%)[b]	Vp1 (%)	Vp2 (%)	Vp3 (%)	Variable (% of Vp3)[c]	% difference in variable[d]
1vs2	122 (16.6)	21 (15.3)	13 (20.0)	88 (16.5)	66 (75)	41.50
1vs3	102 (13.9)	11 (8.0)	13 (20.0)	78 (14.6)	49 (63)	30.80
1vs4	289 (39.4)	16 (11.8)	34 (56.7)	239 (44.8)	128 (54)	80.50
1vs5	323 (44.6)	39 (28.7)	55 (98.2)	229 (43.0)	123 (54)	77.40
2vs3	94 (12.8)	20 (14.6)	14 (21.5)	60 (11.3)	47 (78)	29.60
2vs4	296 (40.3)	28 (20.6)	38 (63.3)	230 (43.2)	119 (52)	74.80
2vs5	316 (43.6)	44 (32.3)	50 (89.3)	222 (41.7)	119 (54)	74.80
3vs4	290 (39.5)	14 (10.3)	41 (68.3)	235 (44.1)	125 (53)	78.60
3vs5	310 (42.8)	35 (25.7)	53 (94.6)	222 (41.7)	116 (52)	73.00
4vs5	385 (53.2)	40 (29.4)	65 (116.1)[b]	280 (52.6)	143 (51)	89.90

[a] Pairwise alignments were based on AAV5 capsid alignment (Chiorini *et al.,* 1999).

[b] Total number and percentage differences of amino acids including insertions and deletions.

[c] Variable domain extends from tyrosine 443 to lysine 601 (AAV2 numbers). Total number and percentage of differences in variable domain over differences in Vp3 between paired serotypes.

[d] Percentage of different amino acids in variable domain between paired serotypes.

2000). In experimental animals, neutralizing antibodies have been shown to eliminate (Fisher *et al.,* 1997; Kessler *et al.,* 1996; Xiao *et al.,* 1996) or greatly reduce (Chirmule *et al.,* 2000; Xiao *et al.,* 2000) the levels of transgene expression of the readministered vector. Two studies report conflicting results upon readministration to the rabbit lung in the presence of neutralizing antibodies. One study demonstrates transgene expression after the third administration, while the other shows no expression upon readministration (Halbert *et al.,* 1997; Hernandez *et al.,* 1999). Some of these discrepancies may be related to the purity of the vector and method for administration. However, with the exception of lung, most studies have determined the block to readministration to be a humoral response associated with T-cell-dependent B cell activation against the virion and not the transgene product as seen with other viral vectors (Chirmule *et al.,* 2000; Hernandez *et al.,* 1999). These results are supported by studies of transient immunosuppression during primary administration which allows successful readministration of the same vector (Halbert *et al.,* 1998; Manning *et al.,* 1998). Although these observations support humoral response to the AAV virion as the major immune response in rAAV animal studies, two exceptions are noted. A cell-mediated immune response to transgenes expressing a foreign cell surface viral glycoprotein, and a foreign secreted protein were observed after rAAV infection (Brockstedt *et al.,* 1999; Manning *et al.,* 1998). A cell-mediated immune response to the transgene expressed from AAV is rare, and one explanation may relate to inefficient transduction of mature dendritic cells and the presentation of neoantigen (Jooss *et al.,* 1998; Zhang *et al.,* 2000). The virus unsuccessful infection of mature dendritic cells may allow for the intracellular expression in target cells and immunologic tolerance of foreign antigens. These studies not only illustrate the need to better understand the immune response against AAV type 2 but also a need to develop new strategies to overcome or circumvent neutralizing antibodies existing in the population. Two obvious approaches are available: one strategy would utilize the other AAV serotypes as alternative vectors, the second strategy would alter domains of AAV type 2 that are immunogenic so that preexisting neutralizing antibodies will not hinder readministration.

SEROTYPES

The five serotypes of AAV represent a group of replication defective viruses that are a rare resource for the gene therapy community. Questions related to their potential utility are (1) How similar are they to the type 2 vector? (2) Will neutralizing antibodies cross-react with these virions? And (3) Do the serotypes have biologically relevant differences such as tissue tropism?

All of the AAV serotypes have been sequenced and five of the six have significantly divergent amino acid sequences. Serotypes 1 and 6 share >99% amino acid homology in their capsid proteins, and sequence analysis supports a recombination event between types 1 and 2 resulting in isolation of this laboratory strain type 6 (Rutledge *et al.,* 1998; Xiao *et al.,* 1999). For simplicity, we will refer to AAV type 1 in this review. The degree of difference between the five serotypes is shown in Table 1. Pair-wise comparisons of amino acid sequences between each serotype were made. The total number and percentage difference between each serotype for the entire capsid, Vp1, Vp2, and Vp3 and the variable domain within Vp3 are reported (Table 1). From these comparisons, three groups of serotypes emerge based on their

total percentage difference. Original evidence suggests that AAV types 1, 2, and 3 were serologically distinct based on complement fixation assays derived from guinea pig sera, an assay that requires 10^9 particles for detection (Hoggan *et al.,* 1966; Parks, 1967). Sera epidemiological studies of AAV types 1, 2, and 3 in pediatric patients demonstrated near identical neutralizing antibody profiles suggesting simultaneous infection by all three viruses (Blacklow *et al.,* 1968). However contrary to these observations, AAV type 1 has never been seen in clinical isolates (Blacklow *et al.,* 1971). Amino acid sequence identity as shown in Table 1 demonstrates that AAV types 1, 2, and 3 may be more similar antigenetically. Therefore, defining of the AAV serotypes by complement fixation assays may not reflect their role in nature. We have used a compilation of sequence data, serological and biological features to help define the 5 serotypes of AAV.

The first group consisting of AAV types 1, 2, and 3 share homology across the three capsids with 8 to 21.5% differences in Vp1, Vp2, and Vp3. The most divergent region of the capsid is located in Vp3 and referred to as the variable domain (see Table 1). Interestingly, members of this group all bind heparan sulfate (Summerford and Samulski, 1998); unpublished data) and antibodies can cross-react to varying degrees (Blacklow *et al.,* 1968; Blacklow, 1971). AAV types 4 and 5 do not share homology across the three capsids. Compared to types 1, 2, and 3, with the exception of the AAV4 Vp1 domain, these viruses appear distinct (see Table 1). The uncommon similarity of the Vp1 domain shared between AAV serotypes 1, 2, 3, and 4 is suggestive of a recombination event. The amino acid sequences of Vp 2 and 3 of serotypes 4 and 5 are as different from each other as from those in group 1. This attribute is most likely why serotype 4 and 5 are also immunologically distinct. There is no serological evidence that AAV types 4 and 5 have cross-neutralizing antibodies, and neither of these viruses are competed by soluble heparan sulfate (Chiorini *et al.,* 1999) suggesting that they may use distinct and unique receptors. Comparing the degree of capsid homology between the AAV serotypes and some autonomous parvoviruses reveals just how potentially different the AAV serotypes can be.

Some autonomous parvoviruses contain minor changes in the capsid amino acids that result in differences in host ranges and tissue tropism. To illustrate this point, less than a dozen amino acid differences exist between feline panleukopenia and canine parvoviruses capsids (Chang *et al.,* 1992). Furthermore, only two amino acid substitutions are required to change the immunosuppressive T cell tropism of MVM (MVMi) to a nonlethal fibrotropic strain (MVMp) (Ball-Goodrich and Tattersall, 1992). By comparing these relatively subtle changes which result in alterations in host range and tissue tropism to the degree of capsid homology between AAV serotypes one might conclude that the AAV serotypes would have nothing in common. However, it should be noted that these divergent amino acids of the autonomous parvoviruses are all surface localized based on crystal structure analysis, an interpretation not available for the serotypes of AAV. Therefore, experimental analysis has been used to establish that the differences in the amino acid sequences between AAV serotypes are biologically significant. As mentioned above, evidence regarding cross-neutralizing antibodies between the different serotypes in animals and human was one of the first points established when studying AAV (Hoggan *et al.,* 1966).

New studies analyzing neutralization antibodies in animals has demonstrated that the route of infection is important, with AAV type 2 neutralizing AAV type 1 when both are administered through the spleen, but not if administered through the muscle (Xiao *et al.,* 1999). This may help explain the early results observed by Blacklow and colleagues in humans suggesting that two out of three AAV positive adults had neutralizing antibodies against AAV types 1, 2, and 3 although type 1 has never been isolated from clinical specimens (Blacklow *et al.,* 1971). Therefore, it is important to note that readministration of vector using AAV serotypes 1 versus 2 will depend on the route of administration. This is not the case for serotypes 4 and 5. Neutralizing antibodies against serotypes 4 or 5 developed in mice do not cross-react with any other serotype (Li and Samulski, unpublished data). Another recent study identifying the immunogenic epitopes of AAV type 2 capsid using overlapping peptides that block neutralizing antibody binding has been attempted (Moskalenko *et al.,* 2000). Mapping these peptides onto an alignment of the different serotypes reveals several regions that are conserved. Information obtained from this type of analysis could lead to the genetic alteration of those amino acids in an effort to alter the immune response. Alternatively, using these peptides as Ab blocking reagents may allow for repeated administration. Regardless of the approach, all of these data support further analysis of AAV vectors and host cell immune response when using different AAV serotypes.

SEROTYPE TROPISM

In addition to the different immune responses seen using AAV serotypes, different biological aspects such as tissue tropism have been observed. Xiao and coworkers have compared AAV type 1 and AAV type 2 in both liver and muscle (Xiao *et al.,* 1999). Using equivalent numbers of viral genomes, the levels of transduction in liver were 10- to 50-fold greater for AAV type 2 vectors. In contrast, AAV type 1 vectors produced ~100% more transgene product in muscle (Xiao *et al.,* 1999). This may imply differences in viral entry, trafficking, uncoating, or other unidentified steps when infecting specific cell

types (Bartlett, 2000; Summerford and Samulski, 1998; Chiorini, 1999). In another report comparing AAV serotypes 2, 4, and 5 *in vivo,* investigators observed different regional distribution and transduction efficiency in the central nervous system at three and fifteen weeks after injection (Davidson *et al.,* 2000). The regional transduction in either striatal cells or intraventricularly may partially reflect primary receptor binding differences, however efficiencies are still questionable since the titers used were not equal. Regardless, these results suggest that the differences in capsid amino acids can impact both immune and transduction profiles. For example, analysis of AAV type 2 and 5 for binding and transduction of human airway epithilia cells and in mouse lungs also demonstrated tropism differences. AAV type 5 bound (5- to 10-fold better) and transduced (50-fold) more efficiently than type 2 (Zabner *et al.,* 2000). Although these differences are intriguing and highlight the advantage of having multiple replication defective AAV serotypes to test, it also points to an area of concern when comparing serotype specific vectors, namely determining titer. In all of the above studies, particle numbers were generally used to determine titer. It is apparent that in addition to immune and tropism differences observed for the AAV serotypes, physical to infectious particle numbers may also vary. These differences would greatly influence interpretation of *in vivo* transduction data (Davidson *et al.,* 2000; Xiao *et al.,* 1999; Zabner *et al.,* 2000). The infectious center assay was developed specifically to determine the number of infectious particles within a stock (Haberman *et al.,* 1999). It is apparent that utilization of this assay will be required when comparing transduction efficiencies between different AAV serotype vectors.

The differences in the tropisms of the serotypes have just begun to be studied in animal models. Large-scale biodistribution studies should elucidate tissue tropism differences between the serotypes and help match the appropriate vector with target tissues. For these studies to be of clinical use, it is important that serotype-specific tropism established in animals translates to humans. Regardless, the elucidation of the mechanism of tissue tropism observed for the different AAV serotypes will help in designing AAV-specific targeting vectors.

MODIFICATIONS OF AAV TYPE 2 VIRION FOR GENE THERAPY APPLICATIONS

Why modify the virion? The appeal of targeting a vector to a specific cell type has generated the interest in modifying the virion of AAV type 2. Two approaches have been used to modify AAV's virion: chemically cross-linked bifunctional antibodies and genetic manipulation of the capsid gene. Chemical cross-linking is an approach that does not require knowledge of the crystal structure or surface-displayed domains (Fig. 1A). However, the genetic approach relies on identification of positions on the virion surface (Fig. 1B) in order to modify the capsid in an efficient manner. Eliminating primary receptor binding as a way of restricting the natural host range, while adding surface-localized targeting moieties to broaden or specify the virus tropism, is the ultimate goal of both approaches. Early developments in AAV type 2 vector retargeting will be described.

The use of chemically cross-linked monoclonal antibodies to target AAV type 2 to a nonpermissive cell line has recently been demonstrated. A bispecific $F(ab'\gamma)_2$ antibody was constructed (Fig. 1A) with specificity for the virion of AAV type 2 and the surface receptor $\alpha_{IIb}\beta_3$ integrin (Bartlett *et al.,* 1999). After incubation of virus with bispecific antibody, modified virions were able to transduce the nonpermissive human megakaryoblast cells, demonstrating an extension of AAV type 2 host range (Bartlett *et al.,* 1999). While showing the feasibility of this approach, the modified virions also displayed a slight reduction in transduction efficiency in permissive HeLa cells (Bartlett *et al.,* 1999). In these experiments, a monoclonal antibody that neutralizes AAV type 2 was used for the virion binding ligand (Wistuba *et al.,* 1995). The use of a high-avidity neutralizing monoclonal antibody as one component of the bispecific Ab may have decreased the infectivity of this virus to nontargeted cell lines. The potential for this strategy is only limited by the chemistry involved in constructing the reagents, the determination of cell-type-specific receptors that mediate endocytosis, and the efficiency of generating optimum cross-linked virions.

A second approach for altering AAV type 2 tropism is to genetically alter the capsid coding region. For this to be successful, the location of domains on the surface of the virion must be determined. The crystal structure of autonomous parvoviruses has determined surface amino acids and topology accessible for epitope manipulation. In the absence of a crystal structure, three strategies have been employed to determine those amino acids or domains with surface orientation for AAV type 2. In one innovative approach, a collection of overlapping AAV type 2 capsid peptides was generated. These peptides were incubated with AAV type 2 neutralizing serum to determine if any would block binding activity, thereby defining peptides pools that represent immunogenic regions on the surface of the virion (Moskalenko *et al.,* 2000). These regions are now available for genetic manipulation by linker oligo insertion analysis. In two other studies, aligning the capsid amino acid sequence of AAV type 2 with the known crystal structure of CPV has allowed inferences about surface localization. Both investigative groups predicted similar regions of the capsid that would be surface localized (Girod *et al.,* 1999; Wu, 2000). Mutational analysis in these sites has established defined positions on the surface of the virion. The final approach utilized classical random linker insertion mutagenesis (Rabinowitz *et al.,* 1999). All of these studies

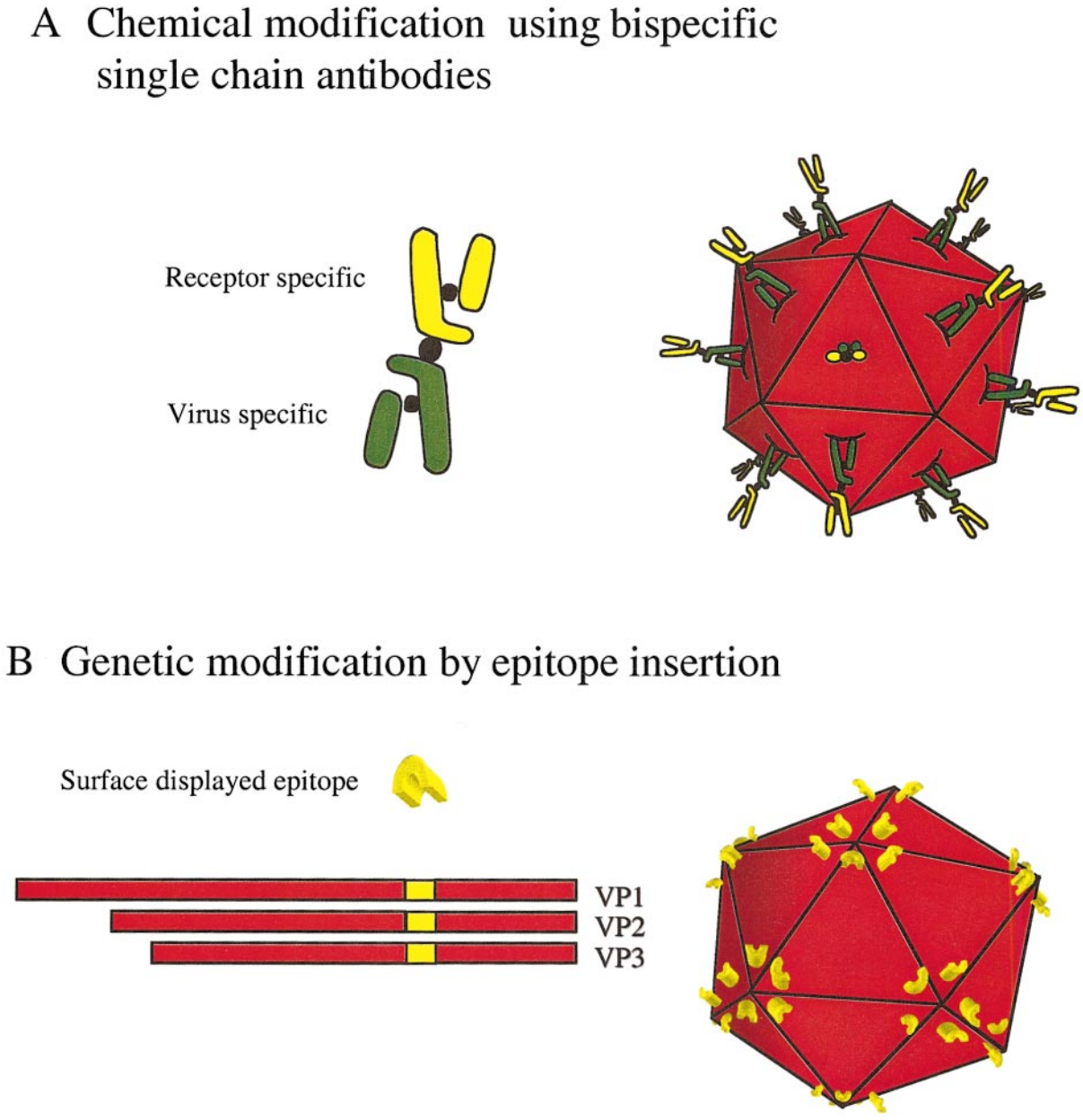

FIG. 1. Methods to manipulate the virion of AAV. (A) The antigen binding fragments (Fab) of two monoclonal antibodies are joined by chemical cross-linking resulting in a F(ab)'2 with two different antigen binding moieties. Here one Fab (green) binds the virion surface (red) and the other Fab (yellow) binds a cell surface receptor that mediates a new tropism. Genetic modification of the capsid (B) introducing the coding sequence for an epitope that mediates binding to a specific receptor. In this illustration the epitope (yellow) is present at the fivefold axis of symmetry of each subunit on the surface of the virion (red).

were accomplished by inserting foreign epitopes either randomly (Rabinowitz *et al.,* 1999; Wu, 2000), at positions predicted to be displayed (Girod *et al.,* 1999; Wu, 2000), or at the amino and carboxy termini (Wu, 2000; Yang *et al.,* 1998).

A major goal in developing a targeted vector is to reduce the level of transduction of nontarget cells. From the random mutagenesis approach, a 4-amino-acid insertion at position 520 resulted in an AAV virion that lost the ability to bind heparin affinity columns and infect HeLa cells while still protecting the vector DNA (Rabinowitz *et al.,* 1999). Additional mutational studies using the CPV alignment approach revealed two clusters of amino acids (509–522 and between 561 and 591) that also reduced or eliminated heparin column binding and *in vitro* transduction (Wu, 2000). These observations begin to define the region of AAV type 2 required for receptor binding. In contrast to the capsid domains identified above, an infectious AAV type 2 mutant containing a 14-amino-acid insertion at position 587 was still inhibited by soluble heparin (Girod *et al.,* 1999). The viable mutant at position 587 and the fact that the cluster domain

TABLE 2

Compilation of Epitope Insertions into AAV2 Capsid

Amino acid position[a]	Titer[b]	A20 (±)[c]	Surface (±)[d]	Infectious[e]	Extend host range[f]	Epitope[g]
261	1×10^{12}	Negative	ND	ND	ND	RGD
266	1×10^{7}	Positive	Positive	6×10^{7}	ND	HA
328	1×10^{7}	Positive	Positive	No	No	HA
381	1×10^{12}	Negative	Unknown	ND	ND	RGD
447	1×10^{9}	Positive	Positive	6×10^{7}	No	HA
447	1×10^{13}	Positive	Positive	1×10^{6}	No (B16F10)	RGD
522	1×10^{9}	Positive	Positive	No	No	HA
534	5×10^{11}	Positive	Negative	ND	No	RGD
553	1×10^{9}	Positive	Positive	No	No	HA
573	1×10^{12}	Positive	Positive	ND	No (B16F10)	RGD
587	4×10^{13}	Positive	Positive	1×10^{7}	Yes (B16F10)	RGD
591	1×10^{9}	Positive	Positive	6×10^{7}	ND	HA
664	1×10^{9}	Positive	Positive	6×10^{7}	ND	HA
1	1×10^{9}	Positive	Positive	6×10^{6}		HA
138	1×10^{10}	Positive	Positive	6×10^{8}	Yes (IB3)	HA/SERP
138	ND	ND	Positive	4.6×10^{4}	Yes/no (KG-1)	sFv anti-CD34
203	Undetectable	Negative	No capsid	No		HA
735	Undetectable	Negative	No capsid	No		HA
28	2.5×10^{11}	Positive	Negative	No	No	Histidine
34	1×10^{10}	Positive	Positive	6×10^{8}	Yes (IB3)	HA/SERP
517	2.4×10^{10}	Positive	Positive	No	No (A9 HABK2)	BRDY
529	1.5×10^{11}	Positive	Positive	No	No (A9 HABK2)	BRDY
586	5×10^{11}	Positive	Positive	No	No (A9 HABK2)	BRDY

[a] The first 12 positions were determined by alignment with CPV. The next 5 are at the amino and carboxy termini of Vp1, 2, and 3. The next 5 were determined by insertional mutagenesis.

[b] Physical titers/ml were determined by dot-blot hybridization or A20 ELISA as described by the authors.

[c] A20 is a mouse monoclonal antibody that recognizes an AAV2 conformational epitope.

[d] Determined by ELISA or immunoprecipitation.

[e] Infectious titers are defined for virions with epitope inserts on AAV2-permissive cells.

[f] Extended host range on nonpermissive cell types (listed) for AAV2 with epitope inserts.

[g] RGD epitope (QAGTFALRGDNPQG), HA (YPYDVPDYA), SERP (serpin FVFLI or KFNKPFVFLI), histidine (ASGGHHHHHHHHGGSA), BRDY (bradykinin AGSGGGSRPPGFSPRSGGA).

mutants do not contain a classical heparin binding motif suggest that loss of heparin binding to these mutants is due to conformational changes. Regardless, these mutant virions represent a starting point for developing a targeting virus without normal host range.

Once the normal tropism is eliminated, retargeting the virion becomes the next challenge. As described above, insertional mutagenesis strategies identifying positions within the capsid that can tolerate exogenous amino acids have been determined (Fig. 1B) (Girod *et al.,* 1999; Rabinowitz *et al.,* 1999; Wu, 2000). Surface localization was established by engineering targeting ligands into these positions (Table 2) (Girod, 1999; Wu, 2000; Rabinowitz, unpublished data). One insertion mutant at amino acid position 34, containing a serpin epitope, was shown to be infectious and mediate host range extension (Wu, 2000). Using CPV for alignment, 12 predicted positions were examined for surface localization of which 9 were positive. Of the 9 positives, 5 were infectious but with titers at least two logs lower than wild type (Girod *et al.,* 1999; Wu, 2000) (Table 2). Of the 5 infectious mutants, only 1 with a 14-amino-acid RGD insertion at amino acid 587 extended the host range of rAAV type 2 (Girod *et al.,* 1999). These results point to the need for the AAV type 2 crystal structure.

In another set of studies, targeting epitopes were inserted at the amino termini of Vp1, Vp2, and Vp3 as well as the carboxy termini of Vp3 (Wu, 2000; Yang *et al.,* 1998). As described above, insertions at the amino terminus of Vp2 were shown to mediate host range extension in the case of the serpin epitope (Wu, 2000). The use of a CD34 specific single-chain antibody fusion to Vp2 cells yielded mixed results. The mutant capsid was unable to generate transducing complexes unless wild-type helper plasmid was present which yielded titers of 2×10^{2}. Since viable virions were never characterized by EM or Western and evidence exists for AAV pseudo transduction (Alexander *et al.,* 1997), the exact mechanism of this targeting remains unresolved.

These studies demonstrate the difficult task of determining surface localized domains that will display targeting ligands (i.e., three positions have been determined by

the genetic approach and one by chemical cross-linking). Although limited in number, these mutants illustrate the point of extending the host range of AAV type 2 by capsid alteration. A more extensive repertoire of insertion sites that allow for targeting epitopes coupled with mutants that have lost the ability to bind heparin will eventually provide the gene therapy field a new generation of AAV targeting vectors.

CONCLUSIONS

The utility of adeno-associated virus type 2 as a gene therapy vector is just beginning to be exploited. Production and purification protocols have improved the titer and quality of AAV vectors available for clinical trials (Monahan and Samulski, 2000). However, preexisting immunity to natural infections could limit the extended use of this vector. Therefore, it is clear that alternative strategies must be investigated to resolve this problem. At present, the five serotypes of AAV will aid in resolving vector readministration. Preliminary evidence indicates that some of these serotypes do not cross-react and more importantly have different tissue tropism. The serotype-specific tropism provides the unique opportunity for transducing distinct cell types *in vivo* more efficiently (Chao *et al.,* 2000). Ultimately, a molecular understanding of the entry pathways for the various AAV serotypes will be required to fully exploit this attribute (i.e., identification of AAV type 2 primary and secondary receptors). This coupled with the genetic analysis of AAV capsids should advance the field in the development of new parvovirus targeting vectors. At present, it is expected that the various serotypes of AAV (i.e., types 1 and 5) will displace AAV type 2 for certain *in vivo* gene delivery applications. This is based on preliminary studies that demonstrate non AAV type 2 vectors are more suited to transduce certain target cells *in vivo* (i.e., brain, lung, muscle, etc.). However, it is predicted that laboratory generated chimeric viruses composed of preferred portions from these various serotypes (e.g., type 1/2), will eventually displace all serotype specific vectors for use in human gene therapy. These custom designed delivery systems will no doubt prove that building a better vector by manipulating the AAV virion is a feasible and justified endeavor.

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