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Generation and characterization of anti-Adeno-associated virus serotype 8 (AAV8) and anti-AAV9 monoclonal antibodies

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A B S T R A C T

Adeno-associated viruses (AAVs) are promising viral vectors for therapeutic gene delivery, and the approval of an AAV1 vector for the treatment of lipoprotein lipase deficiency has heralded a new and exciting era for this system. However, preclinical and clinical studies show that neutralization from pre-existing antibodies is detrimental for medical application and this hurdle must be overcome before full clinical realization can be achieved. Thus the binding sites for capsid antibodies must be identified and eliminated through capsid engineering. Towards this goal and to recapitulate patient polyclonal responses, a panel of six new mouse monoclonal antibodies (MAbs) has been generated against AAV8 and AAV9 capsids, two vectors being developed for therapeutic application. Native (capsid) dot blot assays confirmed the specificity of these antibodies for their parental serotypes, with the exception of one MAb, HL2372, selected to cross-react against both capsids. Furthermore, *in vitro* assays showed that these MAbs are capable of neutralizing virus infection. These MAbs will be utilized for structural mapping of antigenic footprints on their respective capsids to inform development of the next generation of rAAV vectors capable of evading antibody neutralization while retaining parental tropism.

1. Introduction

Major advances have occurred in the development of Adeno-associated viruses (AAVs) as gene delivery vectors over the last two decades, including improvements in large scale vector production to support clinical trials (Chahal et al., 2014; Martin et al., 2013; Mietzsch et al., 2014). Significantly, recent successes in clinical trials worldwide have resulted in the approval of the use of the first AAV gene therapy product in Europe for the treatment of lipoprotein lipase deficiency (Pollack, 2012), and numerous clinical trials are in progress for many other disease targets (Bainbridge and Ali, 2008; Brantly et al., 2009; Daniel Gaudet and Kastelein 2012; Ginn et al., 2013; Maguire et al., 2008, 2009; Mendell et al., 2009; Smith et al., 2013; Wierzbicki and Viljoen, 2013). However, AAV elicits both a cellular and humoral immune response which must be overcome for improved vector efficacy. In the general population, ~40–70% of individuals have been exposed to AAVs (Blacklow et al., 1968; Boutin et al., 2010; Calcedo et al., 2009, 2011; Liu et al., 2013), and a significant number of potential patients already harbor pre-existing antibodies to AAVs (Ferreira et al., 2014; Halbert et al., 2006; Li et al., 2012; van der Marel et al., 2011). These pre-existing antibodies have been shown, even at low levels, to prevent successful gene delivery (Hurlbut et al., 2010; Manno et al., 2006; Scallan et al., 2006; Wang et al., 2011). In addition, the antibody response is likely to interfere with any re-administration of an AAV vector in the event that therapeutic levels are not maintained for the lifetime of the patient.

To understand AAV-antibody interactions and identify potential epitopes, the first step is to produce and have at hand a panel of anti-AAV antibodies. Here, we generated a panel of anti-AAV8 and anti-AAV9 mouse monoclonal antibodies (MAbs) to aid characterization of their capsid-antibody interactions. AAV8 is known for its enhanced hepatic cell transduction (Sands, 2011) and has been used in numerous preclinical and clinical trials to target the

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liver (Bell et al., 2011; Nathwani et al., 2006, 2007, 2011). AAV9 has been reported to cross the blood-brain barrier (Bevan et al., 2011; Federici et al., 2012; Gray et al., 2011; Schuster et al., 2014; Zhang et al., 2011), and has become the vector of choice for treating genetic disease involving the central nervous system (CNS) (Cearley and Wolfe, 2007; Fu et al., 2011; Spampanato et al., 2011; Xue et al., 2010). However, despite the progress in AAV vector development, detailed antigenic footprint information is lacking for both AAV8 and AAV9. Until now, there has only been one MAb developed against AAV8 and AAV9, namely ADK8 and ADK9, respectively, and one cross-reactive MAb, ADK8/9 (Sonntag et al., 2011). The binding site of ADK8 on the AAV8 capsid surface has been identified, through cryo-electron microscopy (cryo-EM) reconstruction methods and confirmed by mutagenesis, to be located on the top of the protrusions surrounding the icosahedral 3-fold axes of the capsid (Gurda et al., 2012). However, the epitope for ADK9 on the AAV9 capsid surface remains unknown. The only available AAV9 antigenic information based on an *in vivo* library screening showed that residues 453–457, which are also located on the 3-fold protrusion, are important for antigenicity (Adachi et al., 2014). Since the antibody response in humans is polyclonal, the antigenic information from one monoclonal anti-AAV antibody is not sufficient to mimic patient responses. Therefore, in an effort to better understand the region(s) of the AAV8 and AAV9 capsids that are immunogenic/immunodominant, we have generated a panel of new antibodies to AAV8 and AAV9 capsids in mice using the hybridoma method. These antibodies will facilitate further studies, through molecular and structural biology, that will provide a better understanding of the antigenic regions of their respective capsids. This information can then be utilized to develop AAV8 and AAV9 variants, through rational site-directed mutagenesis or structure guided directed evolution, with the ability to evade antibody neutralization while retaining parental tropism.

2. Materials and method

2.1. Expression and purification of AAV8 and AAV9 capsids

Recombinant AAV8 and AAV9 virus-like particles (VLPs) were expressed using the Bac-to-Bac baculovirus-Sf9 insect cell expression system (Gibco/Invitrogen,Carlsbad, CA) and purified using a 20% sucrose cushion followed by sucrose gradient (5–40% [wt/vol]) as previously reported (Lane et al., 2005; Mitchell et al., 2009). Purified AAV8 and AAV9 VLPs were concentrated to 1–3 mg/ml and buffer exchanged into 1X PBS, pH 7.4. The concentration of the samples was estimated by optical density measurements (using OD_{280} and $E = 1.7$ for calculation in mg/ml), as well as SDS-PAGE gel electrophoresis with BSA concentration standards. Prior to use, the purity and integrity of the VLPs were also monitored by SDS-PAGE and negative stain EM, respectively (data not shown).

2.2. Generation of AAV capsid specific monoclonal antibodies

The anti-AAV8 and anti-AAV9 hybridoma clones were generated in collaboration with the Interdisciplinary Center for Biotechnology Research (ICBR) Hybridoma Core Lab, University of Florida. Six-week-old female BALB/CByj mice were immunized three times with subcutaneous injections of 5, 10, 25, 50 or 75 μg of AAV capsids at 21-day intervals and one intraperitoneal injection on day 120 as the last boost. The first three subcutaneous injections were accompanied by a Sigma Adjuvant System (Sigma-Aldrich, St. Louis, MO), which contain 0.5 mg monophosphoryl lipid A, 0.5 mg synthetic trehalose dicorynomycolate in 44 μl squalene oil, 0.2% TWEEN 80 and water. Test bleeds from immunized animals were obtained 10–14 days after every booster injection, following animal care protocols. The collected sera were tested for high specific antibody response using ELISA and Dot Blot (against intact capsids) assays as described below. Four days after the final boost injection, the splenocytes of immunized mice were fused with mouse myeloma Sp2/0 cells using 50% PEG 1500 (polyethylene glycol) as the fusing agent. The fused hybrids were cultured in HAT (hypoxanthine-aminopterin-thymidine) (Sigma-Aldrich, St. Louis, MO) supplemented Dulbeccos Modified Eagles Medium (DMEM) to eradicate the unfused myeloma cells. To obtain the positive hybridoma clones, with the highest specific anti-AAV capsid antibody response, the supernatants from the resulting hybridoma cells were collected and screened by a total of 5 rounds of ELISA assays, as described below. Use of Animals in the UF Hybridoma Core Lab at University of Florida is under the guidelines of the Institutional Animal Care and Use Committee.

2.3. Screening of mice serum or hybridoma supernatants using VLP ELISA

The supernatants of hybridomas were screened in the Hybridoma Core Lab, ICBR, University of Florida, using AAV8 and AAV9 VLPs ELISA assays. Briefly, Nunc Maxisorp 96 well plates (Thermo Scientific, Rochester, NY) were coated with AAV VLPs at 4° C O/N prior to each ELISA assay. The plates were then blocked with 1% BSA in PBS at RT for 1 h, and then washed with washing buffer (1× PBS with 0.5% Tween 20). The immunized mouse serum or the hybridoma supernatants were applied to the plate and incubated at RT for 1 h. After washes, the secondary antibody, a rabbit anti-mouse IgG whole molecule AP (alkaline phosphatase), goat anti-mouse IgG gamma chain specific AP, or goat anti-mouse IgM mu chain specific AP (Sigma-Aldrich, St. Louis, MO) were added at 1:1000, 1:4000, and 1:4000 dilution in PBS with 1% BSA, respectively, for 1 h at RT. Finally after several washes, the substrates, *p*-Nitrophenyl Phosphate Disodium (Sigma), was applied to the plate and incubated for 1 h at RT, then optical density readings were taken at 405 nm using a Molecular Devices SpectraMax 384 Plus (Sunnyvale, CA).

2.4. Anti-AAV VLP dot blot analysis

AAV VLPs were allowed to adsorb onto supported nitrocellulose membranes (Bio-Rad, Hercules, CA) in the dot blot manifold (Schleicher and Schuell, Dassel, Germany). Excess fluid was drawn through the membrane by vacuum filtration. The membrane was removed from the manifold and blocked with 10% milk in PBS, pH 7.4 for 1 h at RT. Primary antibody in the form of anti-AAV mouse serum, hybridoma supernatant, or purified MAbs, in different dilutions depending on the sample being tested, was applied to the membrane in PBS with 5% milk and incubated for 1 h at RT. Following this, the membrane was washed with PBS and horse radish peroxidase (HRP)-linked secondary antibody was applied at a dilution of 1:5000 in PBS and incubated for 1 h at RT. The membrane was washed with PBS and then Super Signal West Pico Chemiluminescent Substrate (ThermoFisher, Waltham, WA) was applied to the membrane and the signal detected on X-ray film. The B1 antibody, which binds to the C terminus of the viral capsid proteins in all the AAV serotypes except for AAV4 (Wistuba et al., 1995), was used as a control to confirm the presence of AAV capsid proteins using denatured capsids (boiled and blotted). ADK8 and ADK9 (Sonntag et al., 2011) were used as positive controls for AAV8 and AAV9, respectively, to detect in intact (non-boiled) capsids.

2.5. Determination of the isotypes for the anti-AAV MAbs

The isotypes of the newly generated anti-AAV antibodies were determined in the ICBR Hybridoma Core Lab, University of Florida,