AAV Natural Infection Induces Broad Cross-Neutralizing Antibody Responses to Multiple AAV Serotypes in Chimpanzees

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Cross-sectional studies of primates have revealed that natural neutralizing antibody (NAb) responses to adeno-associated viruses (AAV) span multiple serotypes. This differs from the phenotype of the NAb response to an AAV vector delivered to seronegative nonhuman primates that is typically restricted to the administered AAV serotype. To better understand the mechanism by which natural AAV infections result in broad NAb responses, we conducted a longitudinal study spanning 10 years in which we evaluated serum-circulating AAV NAb levels in captive-housed chimpanzees. In a cohort of 25 chimpanzees we identified 3 distinct groups of animals: those that never seroconverted to AAV (naïve), those that were persistently seropositive (chronic), and those that seroconverted during the 10-year period (acute). For the chronic group we found a broad seroresponse characterized by NAb reacting to multiple AAV serotypes. A similar cross-neutralization pattern of NAb was observed in the acute group. These data support our hypothesis that a single natural infection with AAV induces a broadly cross-reactive NAb response to multiple AAV serotypes.

INTRODUCTION

ADENO-ASSOCIATED VIRUS (AAV) is a replication-defective member of the Parvoviridae family. AAVs have been developed as gene therapy vectors given their favorable safety profile, ability to transduce both dividing and nondividing cells, and transduction efficacy in small and large animal models of disease as well as in clinical trials.¹ Interestingly, although AAV genomes have been found latent in many different human tissues,^{2,3} AAV infection has not been associated with disease in humans.

Many species, including dogs, sheep, pigs, macaques, and humans, have serum-circulating neutralizing antibodies (NAb) to AAV at various levels,^{4–11} with a strong correlation between AAV NAb and the AAV serotype recovered from tissues. For example, the most prevalent AAV serotype in humans, AAV2, is also associated with a higher prevalence of AAV2 NAb in the population,^{10,12,13} whereas, on the contrary, AAV8, a nonhuman AAV, has higher prevalence among rhesus and cynomolgus macaques.¹⁴ Despite the strong correlation between serotypes and epidemiology, we and others have

reported that, in humans, presence of naturally occurring serum-circulating AAV-specific NAb almost always correlated with presence of serum-circulating NAb to various diverse AAVs.^{12,15,16} These findings differ from observations in gene therapy trials, in which a replication-deficient serotype-specific AAV vector is injected into subjects with no preexisting AAV NAb, in which the ensuing NAb response is limited to the AAV serotype that was injected.^{17,18}

To further understand the phenomenon of naturally occurring broadly cross-neutralizing NAb responses observed in nonhuman primates, we conducted a longitudinal study designed to analyze serum samples collected from captive housed chimpanzees over 10 years for AAV NAb. Chimpanzees were chosen given their genetic similarity to humans and the availability of consecutive, long-term serum samples in a contained population. AAV8 was the principal AAV serotype screened for two reasons: first, as it is known to be highly prevalent in several nonhuman primates species and, second, as AAV8 has emerged as the vector of choice for several ongoing clinical trials.^{19–22}

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MATERIALS AND METHODS

Serum samples

Sera were obtained from 25 captive chimpanzees housed at the UT MD Anderson Cancer Center. The samples were collected sequentially from 2002 to 2011 and were analyzed for the presence of AAV-specific NAb. Samples were stored frozen until the time of analysis.

Neutralizing antibody assay

Chimpanzee serum samples were thawed, heat-inactivated at 56°C for 30 min, and analyzed for NAb to AAV1, AAV5, AAV8, AAV9, or AAVrh10 by an *in vitro* transduction inhibition assay on Huh7 cells, as previously described.¹² Briefly, AAV vectors expressing LacZ were premixed with twofold serum dilutions of serum before transducing Huh7 cells. Cells were lysed and LacZ expression was quantified the following day using a luminescent-based assay. A serum dilution of 1:5 was used to define the assay detection limit. Reproducibility of AAV NAb titer of a test sample is ± 1 twofold serial dilution. AAV vectors carrying the CMV.LacZ cassettes were produced by the Vector Core at the University of Pennsylvania. All AAV vectors were produced, as previously described,²³ by transfection of HEK293 cells and iodixanol gradient centrifugation of concentrated supernatants. AAV vector genomes were

quantified by three repeated real-time PCR. AAV vectors were also analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis to verify vector purity.

RESULTS AND DISCUSSION

The AAV8 NAb analysis of 25 chimpanzees for NAb identified 12 animals with no detectable levels of AAV8 NAb (titers <5) throughout the 10-year period (Table 1; referred to as naïve). We identified five animals that were characterized by persistent and relatively stable levels of AAV8 NAb for 10 years (Table 1; referred to as chronic). The remaining eight animals seroconverted from a negative status to AAV8 NAb positive status during the period of observation and remained AAV8 NAb positive (Table 1; referred to as acute). NAb levels in this group waxed and waned throughout the years that samples were evaluated. It is unclear whether the seroconverting animals did so after infection with AAVs acquired from other animals in the facility or from caretakers. We have noted a similar seroconversion of previously naïve rhesus and cynomolgus macaques housed in our facility (results not published).

We analyzed the breadth of the NAb response as a surrogate for infection with other serotypes from the naïve animal group by conducting NAb analysis

Table 1. Time course of naturally occurring AAV8 neutralizing antibody titers in chimpanzees

Group	No.	Chimpanzee ID	Year									
			2002	2003	2004	2005	2006	2007	2008	2009	2010	2011
Naïve	1	2830	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
	2	5432	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
	3	5062	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
	4	5823	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
	5	5658	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
	6	5063	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
	7	5061	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
	8	5430	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
	9	1844	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
	10	2242	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
	11	3915	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
	12	5315	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Chronic	1	2429	40	40	40	40	80	80	40	80	40	80
	2	1719	5	5	5	5	5	5	5	10	5	5
	3	5957	5	5	5	40	20	10	10	10	20	20
	4	1841	20	40	40	40	40	80	40	40	20	40
	5	1929	160	80	40	40	40	40	40	20	40	20
Acute	1	4510	5	<5	<5	5	5	5	5	<5	<5	<5
	2	6427	<5	<5	<5	<5	5	5	<5	<5	<5	<5
	3	5073	<5	<5	40	10	5	5	5	5	5	5
	4	5491	<5	<5	5	<5	5	5	<5	<5	<5	5
	5	5442	<5	<5	<5	<5	5	5	<5	<5	<5	<5
	6	2200	<5	<5	<5	<5	<5	<5	<5	20	<5	5
	7	5618	<5	<5	<5	<5	40	20	20	20	20	10
	8	5687	10	<5	<5	<5	10	5	10	5	<5	5

AAV, adeno-associated virus.

Table 2. Cross-reactive AAV neutralizing antibody response in chimpanzees

	Naïve 2004* (2830)	Chronic		Acute	
		2006* (2429)	2007* (1841)	2004* (5073)	2006* (5618)
AAV8	<5	80	80	40	40
AAV1	<5	160	40	160	20
AAVrh10	<5	640	80	40	40
AAV5	5	640	80	80	40
AAV9	<5	80	160	40	80

*Year (animal ID).

to four other AAV serotypes (Table 2). The four AAV serotypes we screened for belong to clades A, E, and F, allowing us to explore the breadth of the NAb response to AAV.¹² In addition, we included AAV5, an ancestral AAV from which the different clades emerged.³ Although the analysis did not include members from all clades, it included AAV serotypes isolated from human and nonhuman primates that are currently in clinical trials. An animal from the naïve group (#2830) in 2004 was NAb negative to AAV8, AAV1, AAVrh10, and AAV9. However, this animal did have NAb to AAV5, although the response was weak. A similar AAV NAb analysis was performed in two chimpanzees from the chronic infected group, #2429 (serum sample isolated in 2006) and #1841 (serum sample isolated in 2007). In these two animals we found high levels of NAb not only to AAV8 but also to AAV1, AAVrh10, AAV5, and AAV9. Although the data obtained in this study were generated using animals from a single colony, they support previous findings in humans worldwide,^{4,12} where subjects positive for one AAV serotype were also found positive for other AAV serotypes.

What is difficult to reconcile is the substantial breadth of the NAb response in these chimpanzees compared with the sero-specific response that is observed after AAV vector injection into seronegative nonhuman primates. It is possible that the chronically seropositive chimpanzees have been subjected to multiple episodes of sequential natural infections by a diverse group of AAVs. An alternative explanation is that primates become colonized with AAVs in the absence of a humoral response, and an infection (e.g., an adenoviral infection) may trigger a global activation of an endogenous reservoir of latent AAVs. Fluctuations in the AAV NAb titers over time in the chronic and acute groups could be because of activation of endogenous AAV by adenovirus and/or other unknown helper viruses. In fact, we have shown that despite the absence of an active adenovirus infection, chimpanzees are colo-

nized with adenoviruses.^{24–26} In this scenario, the initial NAb reaction may be weak and below the limits of detection of our *in vitro* assay when samples were evaluated before an “activation” event. However, postactivation, the increased “mobilization” of multiple AAVs may have induced a higher polyclonal recall (memory) response. This is supported by the observations that acutely infected animals did appear to show a response that fell below limits of detection at various times after NAb development.

One way to differentiate between the two explanations is to characterize the serologic response in naïve animals during an episode that leads to seroconversion. Another way would be the isolation of AAVs from tissue samples from animals undergoing seroconversion—a difficult endeavor when using endangered animals such as chimpanzees.

In conclusion, we showed that seroconversion in a previously naïve animal was associated with a broad and persistent NAb response that (1) argues against multiple sequential infections as the reason for the breadth of humoral immunity in primates and (2) is consistent with activation of endogenous AAVs during seroconversion activation event. Our data also highlight the difference between the humoral immune response induced by natural AAV infection and by AAV vector administration. In clinical trials, local administration of AAV vectors does not seem to be affected by low levels of preexisting NAb. The injection of AAV into patients not naïve to AAV will induce a strong broad cross-reactive immune response that will preclude them from vector re-administration, if required. Improved understanding of the humoral immunity to natural AAV infection in nonhuman primates will be instrumental in designing strategies to prevent its occurrence or circumventing it in gene transfer trials.

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AUTHOR DISCLOSURE

J.M.W. is an advisor to Regenxbio, Dimension Therapeutics, and Solid Gene Therapy and is a founder of, holds equity in, and has a sponsored research agreement with Regenxbio and Dimension Therapeutics; in addition, he is a consultant to several biopharmaceutical companies and is an inventor on patents licensed to various biopharmaceutical companies. R.C. has no conflict of interest to disclose.

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