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There Must Be a Way Out of Here: Identifying a Safe and Efficient Combination of Promoter, Transgene, and Vector Backbone for Gene Therapy of Neurological Disease

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I don't know where I am,
I'll never know,
within the silence I cannot know,
I just have to move forward

—Samuel Beckett

The long-term therapeutic success of gene therapy for the treatment of neurological diseases will rely in large part on the vector used.[1] Clinical success will hinge upon a balance between efficient cell transduction to reverse pathological phenotypes, and the extent of adverse inflammatory and immunological neurotoxicity.[2] Ciesielska *et al.* have recently shown that transduction of glial cells in rodents by an adeno-associated virus serotype 9 (AAV9)–based vector expressing green fluorescent protein (GFP) increased immunological neurotoxicity when compared to similar vectors expressing endogenous proteins.[3] In this issue of *Molecular Therapy*, Samaranch *et al.*[4] dissect further and compare in detail the neuropathology triggered by expression of a non-self or self-protein delivered by AAV9- or AAV2-based vectors in adult nonhuman primates (NHPs). The study highlights the continued challenge of optimizing the combination of promoter, transgene, and vector backbone, and how a simple change in the latter can alter both transduction patterns and toxicity.

There has been much excitement about the use of AAV vectors for the treatment of neurodegenerative diseases such as Parkinson's disease,[5–7] and AAV2 has long been considered a safe and efficient serotype to take forward into clinical trials. A few years ago, however, AAV9 was shown to cross the blood–brain barrier and to transduce wider brain areas and more cell types than AAV2 following intravenous delivery. These findings redirected clinical development toward AAV9-based vectors. However, increased transduction of different brain cell types, such as astrocytes, neurons, oligodendrocytes, microglia, and vascular endothelium might potentially enhance the toxicity of AAV9, particularly in the case of systemic administration.

Samaranch *et al.* compared the neuropathology resulting from infusion of AAV9 or AAV2 vectors expressing GFP into the right and left basal ganglia, respectively, of adult cynomolgus macaques. As has been observed in rodents, AAV9 transduced a wider area than AAV2. However, AAV9-GFP also caused greater inflammation and immune activation based on increased immunostaining for microglia/macrophages, major histocompatibility complex class II, lymphocytes, and $CD8^+$ cells. The authors also observed increased neurotoxicity in the side injected with AAV9-GFP. Interestingly, an animal that received AAV9 expressing the human aromatic L-amino acid decarboxylase (hAADC), a surrogate self-protein (based on 97% homology with the NHP protein), showed no adverse behavioral effects, no calbindin-D28k depletion (i.e., potential toxicity to Purkinje neurons in the cerebellum), nor evidence of activated astrocytes or microglia despite levels of transduction similar to that observed for the AAV9-GFP vector. As neurotoxicity was not seen in animals injected with AAV9-hAADC, the authors conclude that the combination of vector-specific transduction patterns and exogenous transgene were responsible for the increased toxicity observed with AAV9-GFP.

The authors also performed intrathecal injections of AAV9-GFP in NHPs, which resulted in a serious imbalance in motor coordination and ataxia due to cerebellar damage, and forced the euthanasia of two animals after 3 weeks. Intrathecal administration yielded a much broader expression of the transgene; by about 3 weeks GFP distribution was detected beyond the cerebellum and cortex. Severe ataxia was attributed to the pronounced depletion of calbindin-D28k (a calcium-binding protein found on Purkinje neurons), potentially leading to aberrant Ca^+ homeostasis and Ca^+-mediated signaling for neuronal function.

Two sets of data suggest the likelihood of peripheral systemic exposure to the AAV9-GFP vector, and the priming of a systemic immune response against AAV9-GFP. In the case of the intrathecal injections, distribution of GFP was widespread, cerebellar toxicity was significant, and antibodies specific to GFP were detected. Given the administration of vector into the cisterna magna, a peripheral activation of the immune system cannot be discounted. Equally, in experiments in which AAV9-GFP was injected into one hemisphere, and AAV2-GFP was injected into the contralateral hemisphere, inflammation surrounding the AAV2-GFP injection side also suggests priming of a systemic immune response by AAV9-GFP that then recognizes GFP expressed from AAV2, as suggested by the authors. As innate immune responses have not been seen to diffuse beyond the local inflammatory stimulus, systemic activation of antibodies specific to AAV9 and/or to GFP is thus highly plausible.

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To explain their findings, the authors focus on the transduction of astrocytes, which can upregulate major histocompatibility complex class II, a crucial step in the activation of a systemic immune response. This would suggest local priming of the immune response in the brain itself, a phenomenon that has not yet been proven beyond reasonable doubt. However, the greater level of transgene expression from AAV9 could also contribute to the greater neurotoxic response. Alternatively, the capacity of AAV9 vectors to diffuse through the blood–brain barrier could potentially allow AAV9 vectors to escape to peripheral lymph nodes, where they could initiate a full-blown immune response. Long-term expression (up to 12 months) of the non-self protein β-galactosidase from adenoviral vectors in the brain—in the absence of neurotoxicity and inflammation—has previously been shown to depend strictly upon avoiding exposure of the systemic immune system to either the vector or transgene.[8] It is thus likely that, in addition to the differential capacity of AAV9 and AAV2 to transduce brain cells, the intrinsic propensity of the vectors to diffuse out of the brain may contribute to systemic immune responses. It will be important to determine whether AAV9 escape has indeed occurred, as it will help distinguish between competing hypotheses to explain how the immune system recognizes viral vector-encoded antigenic proteins expressed in the brain.

Although the induction of the immune response appeared to require the expression of a foreign marker protein, expression of endogenous proteins may not necessarily obviate potential toxicity. The level of expression of transgenes is determined by the combination of the particular gene, the promoter used, and the viral vector backbone.[9] For example, expression of an endogenous protein in heterogeneous sites, or at levels well beyond normal expression, has been shown to be sufficient to prime systemic immune responses.[10,11] As noted by the authors, an endogenous human protein to be used in a clinical trial will certainly act as a foreign protein in experimental animals, seriously compromising the interpretation of preclinical toxicity studies.

The safety and efficacy of any combination of promoter, transgene, and vector backbone in humans will ultimately be determined only through careful evaluation in large randomized controlled double-blinded clinical trials.[12,13] The human immune system—including that of the brain—differs substantially from that of other species. Data produced in experimental animals may therefore not predict all the challenges that will be encountered in the clinic. Furthermore, it will be possible to clone and express an endogenous gene encoded by a patient's genome, a potential advantage vis-à-vis adverse immune reactions. The human brain itself may also provide certain advantages as a therapeutic target. For example, its large size is likely to retain injected viral vectors without leakage to the systemic compartment, a technical challenge in smaller rodent brains. However, if AAV9 vectors are able to escape the brain more easily than AAV2, these dynamics will have to be determined in experimental models before moving to clinical trials. Although it is unlikely that marker proteins such as GFP will be used clinically in humans, other elements used to visualize injected vectors could act as exogenous immune-stimulatory proteins. Therefore, the continued emphasis of the authors in working toward optimizing a safe and efficient combination of vector, promoter, and transgene remains the only, yet very difficult and labor-intensive way, to move toward the safe and efficient implementation of clinical trials for the treatment of human neurodegenerative diseases.

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Development of Preclinical Models for Immunogene Therapy of Brain Cancer: It's Not Monkey Business!

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The choice of an appropriate animal model for toxicology studies is one of the most important decisions in the US Food and Drug Administration (FDA)–directed investigational new drug process for gene therapies. A model should, as closely as possible, recapitulate the complexity of the human body, so that preclinical toxicity and safety can be established before any human clinical trials. In a study appearing in *Molecular Therapy—Methods & Clinical Development*, VanDerVeen *et al.*

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