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Immune Responses to Adeno-Associated Virus (AAV) Vectors

NIH Recombinant DNA Advisory Committee Meeting

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SUMMARY

Symposium Moderators:

*Dr. Hildegund C.J. Ertl
Dr. Nicholas Muzyczka*

BACKGROUND AND OPENING REMARKS

Dr. Howard Federoff, Chair of the NIH Recombinant DNA Advisory Committee (RAC), stated the goal of this symposium: to increase awareness in the scientific community and the public of recent data on the possible immunogenicity of AAV vectors, and to update RAC members in order to inform future RAC reviews of trials using AAV vectors. The presentations will focus on the results of clinical trials in which immune studies were conducted, two of which suggested a T cell response to AAV capsid and elimination of transduced cells. Also discussed will be preclinical studies that could help clarify the immunogenic potential of AAV, possible contributory factors from current AAV vector system design and production, and available assays for detection of immune responses. He explained that a structured discussion period would follow the presentations. In addition to the moderators, Drs. Ertl and Muzyczka, Dr. Federoff thanked Drs. Dewhurst, Nemerow, Somia, and Vile for their help in organizing this symposium.

AAV Vectors for Gene Transfer

Presenter: Dr. Marina O'Reilly

Dr. O'Reilly provided an overview of AAV protocols registered with the OBA to provide context for the use of AAV vectors in the gene transfer field. Of the 856 gene transfer protocols reviewed by the RAC, 36 (4 percent) use an AAV vector. The first AAV protocol was submitted in 1994 and recently, about four to five AAV protocols per year have been submitted in years in which approximately 65 protocols are reviewed. Of the 36 AAV trials, 16 (45 percent) are closed, 13 (36 percent) are open and enrolling, and the status of 7 (19 percent) is pending. Approximately 400 research participants have been dosed. Regarding clinical indications, AAV trials have been less focused on cancer (which makes up 67 percent of total protocols submitted to the OBA) and include more single-gene disorders (18, or 50 percent, of all AAV protocols) such as cystic fibrosis, hemophilia B and Canavan's disease. The administration route for AAV vectors reported in the 36 protocols is mostly respiratory and intracerebral, but other routes are also represented. The majority (31, or 86 percent) of AAV protocols are in Phase I, whereas three (eight percent) are Phase II and two (six percent) of the AAV protocols are in Phase III.

The Genetic Modification Clinical Research Information System (GeMCRIS) was used to identify 29 initial reports (from 12 trials) of serious adverse events (SAEs) that were both unexpected and possibly related to gene transfer with an AAV vector. The Gene Transfer Safety Assessment Board (GTSAB) has routinely reviewed all of these events. A review of these events collectively did not identify any patterns across AAV trials, and the SAEs reported from AAV trials do not seem to differ from those reported in other gene transfer trials.

SESSION I: AAV VIROLOGY AND IMMUNOLOGY

Biology of AAVs

RAC Presenter: Dr. Muzyczka

Dr. Muzyczka provided background information on AAV to assist participants and the public in evaluating the other presentations at the symposium. AAV is extremely prevalent with approximately 80% of the human population infected. AAV infection does not appear to cause pathology of any kind. Approximately 80 percent of humans have antibodies to AAV serotype 2 (AAV-2), 18 percent to 35 percent have neutralizing antibodies, and 5 percent can generate a proliferating lymphocyte cytotoxic T-lymphocyte (CTL) response.

AAVs are parvoviruses, which are characterized by linear, single-stranded DNA with hairpinned ends and two open reading frames (a capsid gene [*cap*] and a replication gene [*rep*]). Parvoviruses that need a helper virus, in the form of a herpesvirus (HSV) or an adenovirus (Ad), are called dependoviruses or AAVs. Without helper virus, AAV infects cells, DNA is transported to the nucleus, but the virus can not replicate. There is limited expression of *rep* and sometimes integration into a specific site on human chromosome 19. Co-infection with a helper virus is required for productive infection or for integrated copies of AAV to pop out and proceed to a productive infection. The cellular genes required for viral replication are highly conserved, basic replication proteins. The adenovirus helper genes have roles in inducing S phase, preventing apoptosis, mRNA transport to the cytoplasm, transcriptional control, and the preventing the formation of AAV DNA concatamers. Less is known about the mechanisms of the helper genes from HSV or baculovirus.

The AAV genome has two terminal repeats (ITR), three promoters, and two open reading frames expressed as alternatively spliced transcripts encoding Rep and Cap proteins. The terminal repeats contain the *cis* elements, rep binding site (RBE) and terminal resolution site (*trs*), required for Rep recognition and binding. Rep has helicase activity necessary for viral replication. Rep also functions in transcriptional control by repressing the p5 promoter, thus, AAV gene expression. The Ad E1a protein relieves this repression.

An AAV vector is generated by deleting *rep* and *cap*, and inserting the transgene cassette between the ITRs. The *rep* and *cap* genes are supplied on a plasmid. The helper virus genes are also supplied on a helper plasmid or by co-infection. Virus containing *rep/cap* sequences and no terminal repeats can be a significant contamination in recombinant AAV stocks if an intact p5 promoter is present in the helper plasmid. Removal of the p5 promoter solves this problem. Regarding AAV integration, vectors persist as episomes, and the mechanism for that persistence is unknown. Risk of tumor formation from insertional mutagenesis should be lower because no integration has been found in muscle, and integration has been demonstrated to be approximately 1 percent in liver.

AAV vectors take a long time to express a gene, typically four to six weeks until maximum transgene expression is reached. This is due to slow trafficking of 12-20 hours for DNA to reach the nucleus. The delay is also caused by the second strand synthesis required before transgene transcription. Viral uncoating is also slow which may mean that capsid is available for an extended period as a cytotoxic T cell target.

Overview of Immune Responses to AAV Capsid

RAC Presenter: Dr. Ertl

Dr. Ertl discussed studies in a mouse model to investigate the immune responses to AAV vectors observed in a human clinical trial for hemophilia B. Because Dr. High would be describing this study in-depth in the following presentation, Dr. Ertl briefly summarized the results in which a research participant developed therapeutic levels of Factor IX (F.IX). However, four weeks after AAV vector administration, the F.IX levels decreased concomitant with the development of transaminitis, which resolved after F.IX levels returned to baseline. The results suggested immune-mediated destruction of AAV-transduced hepatocytes. This result had not been observed in any of the animal models. However, unlike the naïve animals, humans would have been previously infected with AAV2 along with a helper virus, such as adenovirus, and would have developed memory T cells to AAV.

To test the effect of pre-existing AAV-specific CD8+ T cells on AAV mediated gene transfer in a mouse model, mice were administered either an adenoviral vector expressing AAV capsid antigen or AAV vectors expressing an immunogenic transgene product. Nine to 21 days later, the mice were infused with an AAV vector of a different serotype expressing F.IX. The pre-existing CD8+ T cell did not eliminate the transduced hepatocytes although they cross-reacted with the AAV vector used for gene transfer. The studies were modified to allow three to four months before gene transfer. Hepatic AAV gene transfer into mice with a memory immune response to AAV capsid resulted in reduced hF.IX expression, and results obtained with AAV-2-F.IX and AAV-8-hF.IX were similar.

The differences between mouse models and the human hemophilia research participants included that kinetics are accelerated in mice, there is no indication of a booster effect of AAV transfer on AAV capsid-specific CD8+ T cells in mice, and transaminitis in mice is modest compared with that seen in humans. Possible explanations for why results differed between the studies of effector and memory T cells include differences in proliferative capacity, susceptibility to apoptosis, levels of cytokine production, and T cell receptor affinity. A question remains as to why AAV gene transfer results in sustained transgene product expression in nonhuman primates; the hypothesis is that most experiments use young rhesus macaques that may not yet have been infected with AAV or that may have mainly effector—rather than memory—CD8+ T cells.

Mouse models should provide the opportunity to prove or disprove that AAV capsid-specific CD8+ T cells affect AAV-mediated gene transfer and will allow preclinical assessment of modified AAV vectors and preclinical testing of immunomodulatory regimens.

SESSION II: IMMUNE RESPONSES IN CLINICAL STUDIES USING AAV VECTORS

CD8+ T-Cell Responses to AAV Capsid in Normal and AAV-Infused Human Subjects

Presenter: Dr. Katherine A. High

Dr. High described the hemophilia B trial using an AAV2 vector expressing F.IX. She provided a description of research participants in the AAV-2-F.IX trial and discussed studies of lymphocytes from human subjects, *in vitro* studies of peptide-major histocompatibility complexes (MHCs) and cell lysis studies on transduced human cells, and mapping of epitopes.

The Phase I/II trial of AAV-mediated liver-directed gene transfer for hemophilia B was based on data that demonstrated long-term expression of the F.IX gene in the livers of experimental animals. The study had an open-label, dose escalation design with hepatic artery infusion of vector. Observations from one research participant at the highest dose infused in humans identified the therapeutic dose of vector,

indicated excellent agreement between antigen and activity levels, and that the F.IX being synthesized had normal specific activity. It was observed that preexisting neutralizing antibodies blocked transduction by this route of administration. In subject E, the participant that received the highest vector dose, F.IX levels were observed to increase from a baseline of 1 percent to 12 percent, but at four weeks post-administration began to decline back to baseline. At the same time, subject E developed asymptomatic transaminitis with liver function tests peaking at five weeks and then returning to normal. Well-timed laboratory tests detected similar results in Subject G, who received a lower dose, did not develop detectable levels of F.IX and did not experience a Grade I toxicity. The loss of transgene expression and increased liver function test results had not been observed in animal models.

Dr. High discussed subsequent studies to investigate whether the transaminitis and loss of vector expression were due to immune-mediated destruction of the transduced hepatocytes. The vector expresses only two antigens, F.IX and AAV2 capsid proteins which, while initially present in the transduced hepatocytes, would not be synthesized and would be gradually degraded. Interferon- γ ELISPOT assays indicated a T-cell response to AAV capsid but not F.IX. Pentamer staining was used to identify CD8+ T cells specific to capsid peptides. The expansion and contraction of the T cell population paralleled the rise and fall of transaminases following gene transfer. The CD8+ cells are functional, can specifically lyse HLA matched peptide-loaded target cells and cross-react with other AAV serotypes.

While human peripheral blood mononuclear cells exposed to empty capsid process and present capsid antigen in a manner appropriate to stimulate a functional response from capsid specific CD8+ T cells, mice do not generate such a T cell expansion following vector infusion. This difference may be due to the fact that humans are natural hosts for AAV2, and the infection occurs in the context of a helper virus so that CD8+ T cells to both helper and AAV are primed. The requirements for reactivation of memory T cells are less stringent than those for induction of primary responses.

Dr. High summarized that the clinical findings are subtle and would be missed with incorrectly timed or less sensitive laboratory studies. Hemophilia provides an extremely clear and rapidly available end point, so that loss of expression is immediately evident and unequivocally established. Loss of F.IX expression was accompanied by an asymptomatic and reversible rise in serum transaminases and expansion of a population of capsid-specific CD8+ T cells; there was no evidence of a T-cell response to F.IX. Normal subjects carry capsid-specific CD8+ T cells that cross-react functionally with other AAV serotypes. Infusion of AAV vector into naive animals results in long-term expression and does not result in expansion of a population of capsid-specific CD8+ T cells. The working hypothesis is that prior exposure to AAV-2 in the context of infection with helper virus, with resultant formation of a pool of memory CD8+ T cells, underlies this difference between humans and animals. Cloning of T-cell receptors (TCRs) and preparation of soluble murine TCRs allow direct analysis of peptide-MHC complexes on cell surfaces. *In vitro* data with AAV-transduced cell lines indicate peptide-MHC complexes on cell surfaces. Cell lysis assays using a human hepatocyte cell line and human leukocyte antigen (HLA)-matched human peripheral blood mononuclear cells (PBMCs) suggest that human PBMCs can lyse AAV-transduced hepatocytes.

Immune Responses in a Lipoprotein Lipase Deficiency Study Using AAV-1-LPL^{S447X}

Presenter: Dr. Meulenberg

Dr. Meulenberg discussed a gene transfer clinical trial for lipoprotein lipase (LPL) deficiency. LPL is an enzyme normally synthesized in muscle and adipose tissue that breaks down triglycerides in chylomicron and very low density lipoprotein (VLDL) particles. The released fatty acids are either used in the muscle as energy or stored in adipose tissue. LPL deficiency is a rare autosomal recessive disorder caused by mutations in the LPL gene and results in type I hypertriglyceridemia. The clinical phenotype includes hypertriglyceridemia, abdominal pain, and acute pancreatitis that is potentially lethal. Current treatment for LPL deficiency is ineffective.

The investigational agent (AMT-010) was an AAV1 vector expressing the LPLS447X transgene. LPLS447X is a naturally occurring variant truncated at the C terminus by two amino acids which is associated with a beneficial lipid profile and decreased risk of coronary artery disease. The vector with the AAV1 capsid was used because AAV1 more efficiently transduces muscle cells compared to AAV2. Preclinical studies included a study of efficacy of AMT-010 in LPL-deficient $-/-$ mice. The follow-on study was conducted in LPL-deficient cats, with sustained effects seen with immune suppression.

The clinical program for AMT-010 was an observation study of baseline data on triglyceride levels of LPL-deficient patients on a low-fat diet (N=14) and a pivotal proof-of-concept study to demonstrate safety and efficacy in LPL-deficient subjects (N=8) after intramuscular (IM) administration of AMT-010. This gene transfer study was an open-label, dose-escalation study of IM administration—at multiple sites on the body—of a single dose of AMT-010. Evaluation occurred at 12 weeks, with a 5-year followup. Four research participants were injected with 1×10^{11} gc/kg, and four were injected with 3×10^{11} gc/kg. The primary objectives were to assess the safety profile of AMT-010 and the efficacy of AMT-010, defined as a reduction of median triglycerides to less than or equal to 10 mmol/L or 40-percent reduction of median triglycerides. The secondary objectives were to determine LPL^{S447X} activity and expression, evaluate immune responses, and assess shedding of vector.

In the clinical trial, no SAEs and no dose-limiting toxicity were encountered, and no clinically relevant abnormalities have been observed in laboratory measurements, physical findings, or other measurements. A reduction in median triglyceride level was seen in all subjects. Analysis of immune responses showed no antibodies observed against the LPL transgene product. However, antibodies were observed against the AAV-1 capsid in all eight research participants. In addition, capsid-specific T-cell responses were detectable in four of eight research participants 12 weeks after gene transfer. A pattern similar to that seen in the hemophilia B trial was observed in one subject in the higher dose group. Following an initial decrease in triglycerides, there was a rebound in the level coinciding with an increase in creatinine phosphokinase (CPK) levels (a two fold increase not considered an adverse event) and the T cell response.

Conclusions from this gene transfer clinical trial are that no humoral and cellular immune responses were detected against the LPL transgene, humoral responses against the AAV-1 capsid were detected in all eight research participants, and cellular immune responses against AAV-1 capsid were detected in four out of eight participants. In at least one participant, T-cell activation was associated with CPK elevation and loss of therapeutic effect. The kinetics of CPK elevation overlaps that of T-cell responses. One possible solution is to administer a short course of an anti-T-cell regimen at the time of gene transfer, which may allow long-term persistence of the transgene by reducing T-cell responses to capsid. This follow-on study will be conducted in Canada using AMT-011.

Immunological Response to Alpha-1-Antitrypsin in a Phase I Trial Using AAV-2 Vector Delivered Intramuscularly

RAC Presenter: Dr. Muzyczka

Dr. Muzyczka presented these data at the request of principal investigators Terence R. Flotte, M.D., and Mark Brantly, M.D., Powell Gene Therapy Center, University of Florida. Alpha-1-antitrypsin (AAT) is a protease inhibitor which inhibits neutrophil elastase, a remodeling protein in the lung. Patients with AAT deficiency acquire an emphysema-like disease at an early age.

A Phase I, recombinant AAV-2-human alpha-1 antitrypsin (rAAV-2-hAAT) study was conducted at a single site (University of Florida) as an open-label, single-dose study, with dose escalation between cohorts and intramuscular administration with ultrasound guidance to avoid vascular structures. There were four cohorts of three research participants each. Safety findings for all four cohorts showed that all 12 research participants were treated safely, with doses up to 6.9×10^{13} vector genomes per person. No serious vector-related adverse effects occurred, antibody levels and responses were variable, and there was no evidence of cell-mediated immunity. The conclusions of this study were that rAAV-2-hAAT did not

result in acute toxicity with the doses tested, vector DNA was present in blood but not semen at all doses within the first 3 days, and humoral responses to AAV-2 capsid were observed but no humoral response to AAT was observed.

A second trial using a different vector based on AAV-1 has just begun. To date no antigen-specific lymphocyte reaction to AAV or capsid has been observed. ELISPOT assays for T lymphocyte responses will be performed in this trial.

SESSION III: ANIMAL MODELS OF AAV IMMUNE RESPONSES

AAV Gene Transfer and Vaccine Studies of T-Cell Responses in Mice

RAC Presenter: Dr. Ertl

Dr. Ertl discussed rAAV vectors as vaccine carriers, which are currently being used in HIV trials. AAV2 vectors expressing *gag* of HIV-1 were pseudotyped with capsid from AAV1, 2, 5, 7, 8, and 9. Mice were immunized intramuscularly and CD8+ T cell responses were measured at different time points. AAV vectored vaccines induced transgene product-specific CD8+ T cells (with AAV1, 7, and 8 inducing the highest levels). A prime-boost strategy was investigated using AAV2/7 vaccine followed by a chimpanzee adenoviral vector; however, the AAV-induced CD8+ T cells do not expand upon a booster immunization.

The AAV induced CD8+ cells had limited proliferative capacity. The loss of the CD8+ T cells' proliferative capacity was specific for the transgene product, but not for the Ad boost. It was independent of the timing between the two immunizations but is dependent on the dose of the rAAV vector used for priming. AAV-induced T cells are not as efficient as Ad vectors at killing transduced muscle cells or protecting as assayed by clearance of vaccinia infection. After AAV*gag* booster immunization, the Ad-induced CD8+ T-cell response cannot be increased by a second boost. AAV impairs antigen-driven differentiation of naive CD8+ T cells, but B-cell responses are not impaired.

The mechanism by which this occurs is currently unknown but possibilities include inappropriate antigen presentation, lack of T helper cells, induction of T regulatory cells (Tregs), or exhaustion. AAVs do not fully drive maturation of dendritic cells. An adjuvant was found to have little effect on rescuing CD8+ T-cell responses. AAV induces Tregs; however, the Tregs do not affect the rAAV-induced CD8+ T-cell response, and the effect cannot be transferred by cells or sera. Some exhaustion markers, such as PD1 and CD244, are up regulated in some populations of AAV induced *gag*-specific T cells. Dr. Ertl theorized that antigen may not be cleared after AAV transduction resulting in a partially exhausted T cell population that is incapable of proliferation. Although AAV-induced CD8+ T cells are impaired in mice, questions remain as to whether human CD8+ T cells are similarly infected and whether this infection would accelerate the vaccine recipient's progression to AIDS after HIV-1 infection.

Impact of AAV Capsid Structure on T-Cell Responses in Mice and Monkeys

Presenter: Dr. James M. Wilson

Dr. Wilson described studies to investigate the activation of capsid-specific T cells and the killing of transduced target cells. Potential sources of capsid antigen are input vector capsid protein, contaminants in vector preparation, and activation of endogenous AAV.

Prior to 2002, AAV had been categorized into six serotypes based on immunologic properties. Using PCR strategies, endogenous (possibly latent) viruses (~ 120 AAVs) were detected in humans and nonhuman primates (NHP). There exists evidence for low level *cap* messenger RNA expression from endogenous virus. Immunologic evidence of remote infections includes neutralizing antibody and memory T cells in humans and monkey populations. The impact of endogenous flora on AAV gene transfer should be considered, and nonhuman primates are good models for studying host-AAV interactions in humans.

Vectors derived from the novel AAV viruses have been studied for efficacy of gene transfer in different target cells and activation of capsid specific T cells. In mouse studies, T cell activation was seen with AAV1, 5, 7, 8, and 9 capsid but at lower levels than for AAV2. These differences may be due to antigen processing and presentation. AAVs, such as AAV2, that bind heparin, also bind dendritic cells effectively which may contribute to T cell activation. Having memory T cells did not generate a more robust priming of heparin-deficient AAV.

Dr. Wilson described NHP studies of different AAV vectors expressing self and non-self transgenes that measured transgene expression, capsid T cells and endogenous AAV. He described the results of the AAV studies in NHP muscle: levels of capsid T cells were higher with AAV-2 than with AAV-7 or AAV-8; transgene T cells were detected with all serotypes; endogenous AAV was detected in all animals, and evidence for vector-derived *cap* in tissues was observed in lower quantities in fewer animals. Two primate studies were conducted using AAV expressing self-transgenes in NHP liver, with injections into the portal vein. Results of these AAV liver studies were that transgene expression is biphasic but stable, liver function tests and pathology were within normal limits, but erythropoietin showed a twofold to threefold increase in week 1.

The capsid T-cell hypothesis has not been validated in animal models so questions remain as to how the transduced cells are killed. One alternative hypothesis is that endogenous production of Cap, either from rcAAV, AAV-cap contaminants or activation of endogenous AAV, renders transduced cells as targets for CTLs; however, this is not consistent with the stoichiometry and may require a paracrine effect. A second hypothesis is that capsid T cells are not relevant to vector performance.

Future research should assume that capsid T cells are relevant until proven otherwise. Key animal studies have yet to be conducted, for example, using nonhuman primates with preexisting memory T cells and high doses of immunogenic AAV. To minimize potential immune responses, efforts should be made to minimize vector dose by making capsid modifications to enhance transduction, improving transcription, and using self-complementary vector genomes when possible. Capsid immunogenicity could be minimized by decreasing dose, using less immunogenic capsids, eliminating or reducing empty capsids in preparations, and reevaluate manufacturing and product characterization methods to avoid AAV-*cap* and replication-competent AAV (rcAAV).

Immune Liver Injury in Response to AAV-2 in Mice

Presenter: Dr. Nicholas Crispe

Dr. Crispe discussed experiments that examined direct versus indirect (cross-) presentation of AAV-encoded transgene expressed in hepatocytes and the mechanism of CD8+ T-cell-mediated liver injury in response to AAV-encoded antigens.

To study presentation in a mouse model, an AAV-2 vector expressing ovalbumin, a strongly immunogenic protein in mice, was delivered by direct intrahepatic injection. The control vector encoded enhanced Green Fluorescent Protein (eGFP). Three possibilities for presentation of AAV-encoded transgene include direct presentation, local cross-presentation, or transportation to extrahepatic sites such as lymph nodes or the spleen and presentation by bone marrow derived antigen presenting cells (APC). Antigen outside the liver was not detected. The results of bone marrow transplantation studies were not consistent with cross-presentation by bone marrow-derived APC. Therefore, the working hypothesis is direct presentation of antigen by the transduced hepatocytes.

To study the mechanisms by which T cells drive liver injury, mice received a direct intrahepatic injection of AAV vector expressing GFP-SIINFEKL (an immunodominant ovalbumin octapeptide) fusion protein. Upon introduction of OT1 T cells (which are specific for SIINFEKL) into the mouse, a focal hepatitis occurred, including inflammatory lesions in the liver, abundant Kupffer cells and apoptotic nuclei. The Kupffer cells were activated, and interferon-gamma (IFN- γ) and tumor necrosis factor-alpha (TNF- α) were induced. Hepatocellular injury followed.

Studies of the role of IFN- γ suggested two pathways of IFN- γ pathogenesis in CD8+ T-cell-mediated AAV hepatitis. The liver is the site of primary CD8+ T-cell activation, and liver priming results in long-lived T cells that recirculate. The T-cell response causes hepatitis, and T-cell IFN- γ drives liver injury. IFN- γ also causes bone marrow-derived cells to make TNF- α . The studies suggest that both IFN- γ and TNF- α contribute to liver injury; therefore, inflammatory cytokines could be therapeutic targets.

SESSION IV: AAV VECTOR PREPARATION

Recombinant AAV Generation and Purification: Strategies to Reduce Vector Immunogenicity

Presenter: Dr. J. Fraser Wright

Dr. Wright discussed vector generation and production methods; vector purification methods; and vector characterization and quality control with focus on considerations for reducing vector immunogenicity. He also provided an evaluation of the Avigen clinical lot, which was administered to Subject G in Dr. High's Phase I/II trial of AAV-mediated liver-directed gene transfer for hemophilia B.

In considering possible human immune responses to a vector, researchers need to consider potential immune responses caused by the AAV capsid itself, any trace impurities in purified vectors prepared for use in clinical studies (e.g., nonhuman components used in the manufacturing process), or amplified by undesirable physicochemical features of the product (e.g., particle aggregation) caused during administration.

Dr. Wright reviewed the pros and cons of the major cell culture systems for vector generation. The advantages of transient transfection of plasmids into human cell lines include no need for helper viruses, good clinical experience, and flexibility during early phase studies. However, the scalability of this type of system is poor and the plasmid DNA may introduce immunogens. For vector systems using helper virus (e.g., Ad, HSV), the advantages are greater scalability, and good clinical experience, but the helper viruses may be immunogenic. More recently, systems have been developed using baculovirus in insect cell lines. This system has the advantages of greater scalability and that the helper viruses used do not infect humans. There is no clinical experience with this system yet and the insect cell or baculovirus may introduce immunogens.

Regarding cell culture process design to reduce vector immunogenicity, Dr. Wright recommended rigorous qualification testing of raw materials, components, and process intermediates to avoid contamination during product manufacturing. The vector synthesized per cell should be maximized while empty capsid contamination should be minimized. The vector should be designed to optimize transgene expression, minimize non-vector DNA packaging, and the helper/packaging plasmids should be optimized to eliminate the potential for generation of replication competent AAV or species capable of expressing *rep*, *cap* or helper virus genes (e.g., deletion of P5 promoter). Vector purification should remove or reduce to the lowest level possible process-related impurities (residual production cell-derived impurities, production plasmid impurities, and residual process materials such as nucleases, solvents, and detergents); and removal or reduction of product-related impurities such as empty capsids.

Vector formulation strategies to ensure minimal final product immunogenicity include minimizing formulation of the components used—each component present in the vector excipient should be justified and present at the appropriate concentration. Formulation should be compatible with the route of parenteral administration because undesirable physicochemical features of the vector formulation, such as pH or osmolality, may potentiate immune responses. Specific consideration for AAV vectors must also be taken into account. AAV tends to undergo concentration-dependent aggregation, and large, particulate antigens are more readily phagocytosed by macrophages and processed or presented by

MHC Class I; therefore, final clinical vector product formulation should be optimized to avoid vector aggregation.

Dr. Wright also described the evaluation by real-time quantitative polymerase chain reaction (QPCR) of the clinical lot administered to Subject G in the Hemophilia B trial. Both *in vitro* (human hepatocyte cell culture) and *in vivo* (mouse tail vein injection) studies showed an absence of AAV *cap* expression at a multiplicity of infection that was thirtyfold to sixtyfold higher than the clinical high dose.

Potential Contaminants in Certain Types of AAV Vector Preparations: Implications for Immunogenicity

Presenter: Dr. R. Jude Samulski

Dr. Samulski described some of the vector system modifications designed to decrease the potential for generation of rcAAV or other contaminants such as by removal of p5 sequence or increasing the size of helper plasmids beyond AAV packaging capacity.

He then reviewed the kinetics of the immune response encountered by two research participants in Dr. High's clinical trial in the context of the AAV life cycle. In AAV, transgene expression occurs after the virus is uncoated to release the viral DNA. Capsid processing and presentation occurs before transgene expression. After uncoating, antigen is presented on the cell surface but will probably only persist 24-72 hours unless there is a renewable source of capsid antigen. The kinetics of the liver damage in the trial is more consistent with presentation of transgene antigen; however, Dr. High's studies indicated no T cell response to F.IX. This raised the possibility of a cryptic epitope derived from an alternative open reading frame (ORF). Cryptic epitopes could be derived from defective ribosomal products or cryptic translation products resulting from such events as incomplete splicing, ribosomal frameshifting, or readthrough. A 65 amino acid ORF with three candidate antigens was identified within the F.IX transgene. The ova antigen (SIINFEKL) presentation assay was used to study the immune response to the alternative ORF peptide. The kinetics of the response to the alternative ORF peptide would be more consistent with the response observed in the hemophilia B trial research participants.

Recently double-stranded AAV vectors have been developed. Because these vectors bypass second strand synthesis, transgene expression occurs sooner following vector administration. The vectors are twentyfold to a thousandfold more efficient in all tissues tested, and are compatible with all AAV serotypes. Because of increased efficiency, it is possible to reduce the vector dose by a factor of from 50 to 100. At a dose in mice at which single-stranded vectors show no F.IX gene expression, the optimized double-stranded vectors show production of therapeutic product. Using double-stranded vector will likely allow the use of lower dose and hopefully will result in researchers being able to dose research participants within the therapeutic window while also being safe.

DISCUSSION SESSION

The discussion session was structured around a set of 11 questions related to the four presentation sessions of this AAV safety symposium.

Session I. Discussion moderated by Drs. Ertl, Muzyczka, Leonard B. Seeff, and Jonathan Yewdell

Question 1:

Is the approximately 4-week delay before decline of transgene expression and the laboratory evidence of transaminitis (seen in Dr. High's trial) consistent with destruction of hepatocytes by AAV capsid-specific CD8+ memory T cells reactivated upon gene transfer?

Dr. Yewdell agreed with Dr. Samulski regarding the kinetics issues raised by persistence of capsid presentation for four weeks. He noted that not much is known about how exogenous presentation works transiently. The turnover time of Class I molecules on hepatocytes is unknown. It is possible they would last for a long time after loading, but typically Class I molecules have a half-life of about 12 hours. However, the information about antigen-processing class I molecules is derived from tissue culture cells, which may not be relevant.

Dr. Muzyczka asked, given the prevalence of endogenous AAV seen in NHP, why did the immune response arise upon the introduction of a lower amount of capsid from the vector? Humans have latent genomes, with a frequency of about 1 in 1,000 cells in liver, which may become reactivated upon gene transfer. Dr. Wilson indicated that they plan to study possible reactivation further.

Dr. High described a study by Avigen, Inc., which detected AAV capsid in liver beyond four weeks. This study was conducted with an AAV-2 injection into a dog, and 11 weeks later the liver was harvested and stained with an antibody to intact capsid, which were present in the liver 11 weeks after vector injection. Other data in the literature also point to this type of persistence with other types of viruses for months after a viral infection. The participants discussed possible experiments and their limitations to pursue this type of study further.

Dr. High clarified that Subject G's liver function tests (LFTs) were beginning to rise at two weeks postinfusion; the LFTs were not suddenly abnormal at week 4. Dr. Ertl asked how much liver damage must occur before a rise in LFTs is detected. Dr. Seeff responded that in studies of drug-induced liver injury, it has been observed that some drugs may lead to enzyme elevations without necessarily toxicity. Recent reports from Spain and Sweden suggested that ten percent of people who develop enzyme elevation as a consequence of hepatotoxicity associated with jaundice are likely to die if they also develop hyperbilirubinemia. It would be difficult to determine the relationship between hepatocyte destruction and enzyme elevation. Dr. Seeff noted the difficulty in arriving at any decisions because at this point it is not possible to know whether the elevated enzymes would be associated only with a mild transaminitis or were a signal that liver failure could occur depending on the subject's genetic background. Better understanding of the pathogenesis is needed. Genetic studies are being conducted to help identify individuals who may have such an adaptation phenomenon.

Dr. Federoff noted the two explanations suggested for the immune response: AAV capsid from whatever source which may warrant immune suppression or an epitope from an alternative ORF epitope which may be eliminated by sequence changes. He asked for suggestions to clarify the data and if necessary modify the F.IX trial. No liver biopsy data were available because it is difficult to biopsy hemophilia subjects. Some muscle biopsy data were available from the first hemophilia B and the LPL trial with intramuscular administration; however, it would be difficult to obtain sequential data because most participants are reluctant to have more than one muscle biopsy. The subject in the LPL trial was HLA-B0702 positive, so Dr. High offered to use her soluble T-cell receptor assay on the biopsied material. Drs. Wright and Samulski suggested that one way to distinguish between the alternative explanations would be to study the effect of empty capsids. An ongoing trial for muscular dystrophy may provide some answers because the subjects in this blinded trial would be receiving vector in one limb and empty capsid in the other.

Assuming an immune response to capsid did occur in the hemophilia trial, Dr. Muzyczka asked what would be an appropriate approach to immunosuppression. Dr. Crispe responded that at this time it is not known whether there is a cytotoxic T cell effect destroying transduced cells or an interferon effect suppressing transgene expression. Therefore, global immunosuppression may be a disservice to research participants because it would be suppressing modalities not actually causing a problem.

Question 2:

Are there unique aspects of vector metabolism or immune responses in the liver that may have contributed to the clinical outcome of the hepatic AAV-2-F.IX trial in hemophilia patients? Would a similar response be expected in other organs, such as the muscle, heart, lungs, spleen, etc.? Is there any evidence that relatively "immune-privileged" environments such as the nervous system would be insulated from this immunological response?

Dr. Crispe responded that the issue of immune privilege in the liver is a very complicated. The liver can prime an effective immune response, in some cases to eradicate viruses such as hepatitis A. However, there are other factors, such as cytokines and cell surface molecules, that predispose the liver to a low level of immunity. The environment is distinctive, possibly because of continuous exposure to endotoxin that comes up the portal vein from the intestinal flora. Liver is a tolerogenic environment, but many immune responses can be launched there successfully; there is no simple model to explain all of this at present.

The participants discussed the trials involving administration of AAV vectors to muscle, heart, and lung. Dr. Federoff summarized several AAV protocols involving the central nervous system in which no immune responses or SAEs have been reported to date.

Question 3:

Please discuss any data available about seroprevalence rates in humans to the different AAV serotypes.

Dr. Wilson discussed his screening of approximately 800 individuals from various countries. Although there have been suggestions that seroprevalence may be higher with AAV-2 and possibly higher with AAV-1, there is some level of cross-reactivity when looking at related clades. No clades appear to be clean of neutralizing antibodies, so that switching serotypes will be of limited advantage over seroprevalence.

In evaluating the feasibility of AAV as a vaccine, Dr. Wilson and colleagues conducted passive transfer studies of neutralizing antibody; however, the neutralizing assay was not sufficiently sensitive to detect which animals would inhibit an *in vivo* effect of the vector. Therefore, the assays are not sensitive enough to screen potential research participants. He noted that the mechanism of neutralization *in vivo* is not known. There are other ways in which viruses can be prevented from initiating a productive infection other than occupying the receptor or by exclusion, but those mechanisms are unknown at present.

Question 4:

Please discuss the immune responses observed in two of the studies presented today in the context of the other clinical trials in which immune responses were not reported. What factors may have contributed to the different results—AAV vector system, transgene, dose, route of administration and target cell, underlying disease, immune status of the research participants, age of research participants, and types of assays used to monitor and timing of sample analysis.

Dr. Yewdell responded that in addition to the factors listed, the genetics of the subjects could contribute, particularly polymorphisms in immune response genes.

Regarding monitoring assays, Dr. Ertl explained that while the ELISPOT assay is probably the most sensitive to show a T-cell response, it does not allow distinguishing between CD4+ and CD8+ T cells. Biopsies to look for vector genome copies require at least an early and a late biopsy to draw any conclusions, and most research participants would probably not agree to undergo more than one muscle biopsy. Lymphoproliferative assays are fairly insensitive. She recommended that AAV protocols include conducting ELISPOT (against the capsid and against the transgene product) before infusion and then at several time points thereafter. For researchers who do not know how to do ELISPOT assays, many contract labs offer this service.

Regarding alternative ORFs, Dr. Samulski noted that during his tenure as a member of the RAC, the committee frequently recommended that every transgene be screened for potential ORFs. Dr. Takefman explained that the FDA has recommended for the past three years that all vectors be fully sequenced and analyzed for potential alternative ORFs. The best way to look for ORFs is now under discussion at the FDA. The FDA has also been recommending that researchers monitor for T-cell responses and use the

ELISPOT assay, although the reproducibility, precision, and operator-to-operator variability are somewhat unclear with that assay.

As to timing of samples, in general, more sampling is better, but there is currently not enough experience to recommend the timing of sample analysis.

Session II. Discussion moderated by Drs. Ertl, Glen R. Nemerow, and Yewdell

Question 1:

Please discuss possible factors that may contribute to the different results observed regarding the detection of immune response and elimination of transduced cells in humans and the various animal models, including nonhuman primates.

As Dr. Ertl presented, the initial mouse models were done with effector T cells. Her more recent studies involving memory T cells should be a better model. It may be useful to prescreen animals for neutralizing antibodies and T cells in designing experiments.

Question 2:

Please suggest preclinical experiments that would help clarify the immunogenic potential of AAV vector systems.

Studies should be focused on determining if the response was due to the vector or the transgene. The vector dose could be lowered to lower capsid. To study the transgene effect, the transgene could be expressed in a non-AAV vector, such as Ad or plasmid.

Dr. Ertl planned additional studies based on her mouse memory T cell model. Dr. Wilson would like to conduct NHP studies in animals with memory T cells but without neutralizing antibodies to AAV capsid and use sufficient vector to exceed any threshold effect in monkeys.

Dr. Yewdell suggested studies to determine if AAV triggers any innate immune responses.

Question 3:

Which animal models are the most appropriate for such studies?

In a comparison of gene transduction in airways of Old World monkeys to New World monkeys, John Engelhart's study (published in *Molecular Therapy*) suggested that cynomolgus monkeys and rhesus macaques have different transduction patterns by different vectors. The species and country of origin of the monkey should be considered. For liver, the transduction efficiency does not appear to be species dependent. The age of the animals may also have a role in the immune response.

Mice are appropriate models because their genetics have been well defined and they are not expensive; monkeys require considerable additional effort. However, with respect to the issue of liver toxicity or persistent expression, the mouse model is not necessarily going to predict the experience in larger animals.

Public Comment:

Samuel C. Wadsworth, Ph.D., Genzyme Corporation, asked why expression of the alternative ORF does not eliminate transgene expression in animals. Dr. Yewdell suggested that the rules of translation may differ between tissues. Even if aberrant protein is produced, a second signal, such as innate immunogenicity, may be needed to trigger the immune system.

Session III. Discussion moderated by Drs. Stephen Dewhurst, Muzyczka, and Nikunj V. Somia

Question 1:

Please discuss available assays for detection of replication-competent AAV or other capsid-expressing recombinants and their relative strengths.

Dr. Samulski noted that the AAV reference standard currently being generated will be helpful in addressing many of these questions. As part of the reference standard effort, Richard O. Snyder, Ph.D., University of Florida, compiled recommendations on the type of assays that should be conducted. Attendees at the ASGT meeting asked that those recommendations be published in *Molecular Therapy*.

Given the detection of capsid sequence contaminants in vector preparations that could contribute to the CD8+ T cell response observed, Dr. Muzyczka suggested that assays should be considered for the detection of capsid. Dr. Wright suggested clinical lots could be assayed for capsid DNA by QPCR. Dr. Wilson pointed out that wild-type AAV2 would not be an adequate control for assays of vectors derived from other AAV capsid serotypes.

Dr. Takefman explained that the FDA requires an rcAAV assay for lot release; however, due to the nonpathogenic nature of the virus, some clinical lots have been allowed to proceed with low levels of rcAAV contamination. The participants discussed the difficulty in setting requirements or limits. Dr. Muzyczka suggesting monitoring rcAAV levels which may provide useful information about correlations with CTL responses.

Question 2:

Please discuss various methods to detect neutralizing antibodies to different AAV serotypes.

Dr. Wilson suggested transduction inhibition assays. However, the *in vitro* assay does not predict outcome *in vivo*, so it may be useful to develop an *in vivo* assay to be able to predict such an event. Dr. High suggested that with the use of alternative capsid vectors, the assays be able to account for cross reactivity of antibodies.

Question 3:

Please discuss possible modifications to either vector design or production that may decrease the potential for an immune response to the vector, including vector design, alternative viral serotypes, packaging system, or purification.

Dr. Samulski emphasized the need to optimize expression from the transgene cassette. This would allow administration of lower doses with less capsid. Double stranded vectors may allow for more efficient transgene expression with more rapid onset.

Dr. Wilson suggested that it would be worth pursuing production systems that are not transfection based. Systems using baculovirus, herpes virus, or Ad have been developed that may generate a cleaner product. In the next year or so, some of those production systems will be used in the clinic.

Dr. Wright suggested that if the response is to capsid protein, it might be beneficial to remove empty capsids from vector preparations. However, this is difficult to do with large scale preparations.

Session IV. Discussion moderated by Drs. Ertl, Federoff, and Muzyczka

Question 1:

Please discuss how the following issues should be considered in the RAC's future reviews of AAV clinical trials: vector quality control assays, dose escalation, subject population, clinical application, route of administration and target cells, inclusion/exclusion criteria (including clinical assays for preexisting immunity to AAV capsid), and monitoring of research participants (including assays for detection of immune response).

The participants discussed the issue of whether pre-existing AAV capsid-specific T cells should be an exclusion criterium. Dr. Wilson cautioned against excluding research participants on the basis of analysis of previous exposure to capsid T cells, at least at present. The assays to accomplish that analysis must be proven to be very robust and well validated, and that is not yet the case. In addition, anyone who had preexisting T cells might not have subsequent access to gene therapy. Dr. High responded that particularly for liver administration, she would find it preferable to have research participants without circulating T cells to AAV. Dr. Samulski noted that these types of discussions occurred while he was a RAC member and the consensus was that there was not enough experience to make recommendations until more data were accumulated. Rather than explicit recommendations, Drs. Federoff and Muzyczka suggested adopting the classical clinical approach to be as careful and quantitative as possible in identifying appropriate participants and utilizing available measures to characterize them. Dr. Samulski noted that benefit could be derived from including monitoring assays for detection of immune responses.

Regarding the earlier discussion of the unknown potential for liver failure in the "meltdown participant", Dr. Samulski wanted to consider the risk/benefit ratio. Is the hemophilia community the appropriate population? Hemophilia has at least some treatment options. Perhaps it would be more appropriate to proceed with gene transfer for a disease for which there is limited life expectancy and no alternative therapies. Dr. High recalled that hemophilia patients had presented at earlier RAC meetings regarding the limitations of current therapy and the desire for additional treatment options.

The participants also discussed the potential use of immunosuppression to allow gene transfer despite the immune response. Dr. Samulski cautioned that this might add another variable and risk to complex studies. Dr. High responded that when she and her colleagues were presented with the findings, the hepatologists stated that it looked like autoimmune hepatitis, which can be treated with steroids. An alternative would be to prevent the response by immunosuppression. Dr. Samulski suggested two other alternatives, both of which could be done relatively easily: (1) remove the ORF, do the same experiment again, and find out whether that is a variable and (2) switch from single strand to double stranded vectors to allow lower doses of capsids. Dr. High responded that following the T-cell responses while immunosuppressing would provide data on the adequacy of the regimen and correlations with transgene expression and transaminases.

Dr. Federoff concluded that many alternative approaches had been discussed for future research. He thanked the meeting organizers and the participants for sharing their expertise with the RAC.

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ACRONYMS LIST

AAT	alpha-1-antitrypsin
AAV	adeno-associated virus
Ad	adenovirus
ALT	alanine aminotransferase
CPK	creatinine phosphokinase
CTL	cytotoxic T-lymphocyte
FDA	U.S. Food and Drug Administration
F.IX	blood coagulation Factor IX
GFP	green fluorescent protein
hF.IX	human blood coagulation Factor IX
HLA	human leukocyte antigen
IFN- γ	interferon-gamma
IM	intramuscular
LPL	lipoprotein lipase
MHC	major histocompatibility complex
NEI	National Eye Institute, NIH
NIAID	National Institute of Allergy and Infectious Diseases, NIH
NIDCD	National Institute on Deafness and Other Communication Disorders, NIH
NIH	National Institutes of Health
OBA	Office of Biotechnology Activities, NIH
OD	Office of the Director, NIH
PBMC	peripheral blood mononuclear cell
PiZ	protease inhibitor phenotype Z
QPCR	quantitative polymerase chain reaction
rAAV	recombinant AAV
rAAV-2-hAAT	recombinant AAV-2-human alpha-1 antitrypsin
RAC	Recombinant DNA Advisory Committee, NIH
rcAAV	replication-competent AAV
SAE	serious adverse event
TCR	T-cell receptor
TNF- α	tumor necrosis factor-alpha
Treg	regulatory T cell