Guidance for Working with Adeno-Associated Virus (AAV) Vectors

Guidance

Background to Adeno-Associated Virus (AAV)

AAV is a small, stable virus that has never been shown to cause disease in humans even though a majority of the population has been exposed to it. The naturally occurring form of the virus contains only two genes, rep and cap, encoding for the regulatory and structural proteins, respectively, flanked by 145bp Inverted Terminal Repeats (ITRs). The virus cannot reproduce itself except in the presence of a helper function, usually provided by another virus such as adenovirus or herpes simplex virus. Recombinant AAV vectors (rAAV) are derived from the wild type virus by removing the two virus genes and replacing them with the gene under study otherwise known as the transgene. The vector when purified is unable to grow on its own (replication defective) but retains the virus' ability to enter cells because the AAV capsid is provided in trans. Once the vector has entered a cell, the transgene is expressed from the transcriptional regulatory signals supplied with the gene.

The advantages of this vector system is the stability of the viral capsid, its low immunogenicity, the ability to transduce both dividing and non-dividing cells, the potential to achieve long-term gene expression even *in vivo*, and its broad tropism allowing the efficient transduction of diverse organs including the skin. Indeed many *in vitro* and *in vivo* studies have demonstrated that these vectors can efficiently introduce foreign genes into various cell types leading to long-term expression of the gene in tissues such as the skeletal muscle, the liver, the brain, and the retina.

Human clinical gene therapy trials with these vectors have mainly been attempts to correct single gene defects. Although the trials have had varying degrees of success in terms of the management of disease over a hundred patients have been treated with AAV vectors, without contraindication, indicating the basic safety of the system.

Vector System

The first step in producing an AAV vector is to engineer in bacteria, a recombinant AAV genome (contained within a plasmid). This is achieved by replacing the Rep and Cap genes with the gene under study (often referred to as the transgene). Virus can then be produced from the purified plasmid by several different protocols all of which require transfection of a continuous cell line with the plasmid. Initially rAAV vectors were produced by transfection of the vector plasmid (transgene flanked by inverted terminal repeats – ITRs) and an AAV helper plasmid (provides the Rep and Capsid proteins) into HeLa or 293 cells followed by infection with helper virus. More recently a

second procedure has been developed where the helper virus can be substituted by plasmids designed to express the complementing adenovirus genes, avoiding potential helper virus contamination in the final preparation. A further development for industrial scale preparation combines these two protocols and generates a stable packaging cell line that also contains the rAAV vector. Superinfection with adenovirus allows the rAAV vector particles to be made. With improvements in the purification protocols it is now possible to generate rAAV preparations of high purity and titre (>10^{10} transducing units/ml). This area is one of active research and among other systems developed one utilizing co-infection of insect cells with 3 different recombinant baculoviruses looks promising.

Safety Issues with AAV Vectors

In effect the basic AAV vector system uses a defective (gene deleted), replication defective virus (i.e. one that requires a helper to replicate) to deliver the gene of interest. Consequently spread is very unlikely even where helper virus is present because a source of AAV Capsid and Rep would be required to generate packaged recombinant vector. DNA packaging constraints of the virus particle mean that even if illegitimate recombination occurred between plasmids containing the transgene and the Rep/Cap plasmid a recombinant virus containing the transgene is unlikely to be viable. Therefore the main concern regarding AAV vector work is likely to arise from the nature of the gene being expressed and its direct potential effect on an accidentally infected individual.

Control of the most likely route of infection i.e. via sharps is important and measures to reduce exposure to any aerosol generated also seem appropriate.

Questions to be Asked for Work with AAV

1. Does the experimental protocol use co-infection with helper virus? If so BSL2 should be used as a minimum.

2. Is there residual helper virus left in the AAV preparations? If so BSL2 should be applied to the experiments/animal work as a minimum. This will depend on the method of production and the extent of the purification protocols.

3. How will work with AAV be segregated from adenovirus (Ad) or herpes simplex virus (HSV) work? This question addresses the possibility of cross contamination of cultures. AAV preparations can be extremely high titre and there is potential to contaminate Ad or HSV stock viruses that may be used for other work. For example other workers in the department may be using Ad as a vector. Ideally separate hoods and incubators should be used for Ad (or HSV) and AAV work but this may not be practicable and it is not an absolute requirement (effective measures can be taken to clean hoods appropriately and segregate AAV work in incubators).

4. What detrimental effects (if any) is the gene expressed by the vector likely to have – is it an allergen, oncogene or cytokine? If so BSL2 should be used as a minimum.

5. Are any sharps going to be used? For animal work this may be unavoidable but should be controlled carefully with a standard operating procedure. Harvest of gradients used in purification is for example another point where sharps may be used and should

be controlled with a standard operating procedure.

6. Is insertional mutagenesis a potential problem (as for retroviruses)? Unlikely - see Appendix 1 for discussion.

7. The table that follows is a summary of the recommended biosafety levels for handling AAV and vectors derived from AAV.

Virus System	Recommended Biosafety Level/Comment
Wild type AAV (all serotypes)	**1**
Wild type AAV grown with helper virus (eg Ad or HSV)	**2**
AAV vector with marker gene or other innocuous molecule e.g. EGFP, β-galactosidase or inactive fragment of a gene	**1**
AAV vector expressing a biologically active molecule	**1 or 2** (depending on the gene) e.g. CFTR (the gene in cystic fibrosis patients that is non-functional) would be level 1; highly biologically active molecules such as oncogenes (including siRNA to a tumour suppressor) allergens or cytokines would be level 2
Any AAV vector used in conjunction with helper virus	**2**. Ensure sharps procedures rigidly adhered to, especially if animal work is to be carried out.

Appendix 1. Insertional Mutagenesis and AAV

Some concern has been expressed over the potential for AAV to cause cancer (Nature 423, 573–574: 2003 – news story). Nakai et al. (Nature Genet. 34, 297–302: 2003) demonstrated that AAV vector DNA will preferentially integrate into active genes when delivered into the livers of mice. Part of the following is a synopsis of a comment from these authors (Nature. 424, 251: 2003).

"Concerns over AAV vectors have been raised because of reports of leukaemia in patients treated with a recombinant retroviral vector for a lethal genetic disease, X-linked severe combined immunodeficiency disorder (SCID). The leukaemia was caused at least in part by the retroviral insertion and activation of an oncogene (insertional mutagenesis) in bone-marrow progenitor cells. Because retroviral vectors preferentially integrate into intragenic regions of the chromosome, the Nature news story quoted suggests that recombinant AAV vectors may pose similar risks in gene-therapy trials."

The conclusion of a symposium entitled "Safety considerations in the use of AAV vectors in gene transfer clinical trials", jointly sponsored by the NIH and the FDA, held in March 2001 was that, on the basis of data from hundreds of normal

mice treated with AAV vectors, there was no evidence to suggest that the vector caused cancer.

It is also worth noting that there are substantial differences between retroviral and AAV-mediated integration. First, unlike retroviral vectors, AAV-mediated vector integration is relatively uncommon. Second, retroviral vectors contain additional regulatory elements that are more likely than AAV vectors to activate a gene that they insert next to. Third, retroviral vectors contain the protein machinery needed to cause host chromosomal DNA breaks, whereas AAV does not. It is possible that AAV preferentially integrates into DNA regions that are already damaged within treated cells.

In addition, the leukaemia found in patients treated with X-linked SCID gene therapy may be unique to this particular disease because of the unusual physiological events that occur after treatment. In X-linked SCID, the genetic reconstitution of a very few precursor cells results in the selective proliferation of immune cells genetically corrected with the vector. Any additional proliferation stimulus, such as the activation of an oncogene, may result in the further growth and expansion of these cells. This type of growth advantage is not a factor in most gene-therapy trials and is also unlikely to be an issue in accidental infections.

The risk of cancer in current AAV gene therapy trials is negligible, on the basis of infrequent integration efficiency and the quiescent nature of the target tissues. On the same basis risks from insertional mutagenesis in the laboratory are also limited."

Following these initial considerations further papers seemed to cast doubt on these conclusions. Donsante et al [1] and two other papers [2, 3] describe hepatocellular carcinomas in murine models of AAV. However the fact that Bell et al. (2005) [4] looked at nearly 700 mice treated with AAV vectors mostly by intra-portal vein inoculation and did not find increased liver tumour formation and that there were technical problems analyzing the structure of the integrated AAV in the Donsante paper further confuses the issue. It is worth noting one of the papers quoted by Donsante and also by Bell et al (2006) [3] concludes that AAV vectors alone do not contribute to the formation of tumors in these strains of mice although the expression of LacZ alone or in combination with vector may be problematic. Interestingly the construct used in the other paper quoted [2] contains a portion of the woodchuck hepatitis virus post transcriptional regulatory element that has been implicated in liver tumourogenicity in other studies [7]. In the Donsante paper the CMV enhancer element may also have played some part in the activation of gene expression when integrated. The supplementary material in the Donsante paper shows that the CMV enhancer element remains in the promoter negative construct.

A commentary by one of the authors on the Donsante paper [5] does elaborate and makes some valuable points coming to the conclusion that clinical trials involving AAV which target the liver should not be carried out. A more recent commentary by the same author (2009) [6] is also available.

A recent abstract presented at the annual conference of the American Society for Haematology: http://ash.confex.com/ash/2009/webprogram/Paper22600.html appears to contradict the Donsante view. (The study describes a huge and very

thorough piece of work from authors carrying out human gene therapy trials.)

The three (or four – if you count the original Donsante paper) examples of liver tumour formation must be considered in light of the many studies done in rodents, dogs, and primates (including the human gene therapy trials) where no increased incidence of tumors was noted. It is possible that features of the AAV constructs used or the genetic makeup of the mice studied contributed to tumour formation. However it is not possible to be definitive about which elements should be avoided to decrease risk. In contrast to retrovirus vectors the case for insertional mutagenesis by AAV vectors is far from proven. In biological safety terms the University of Hong Kong Biosafety Committee will continue to advise that work with AAV can be carried out safely at Class 1. The only slight concern would be the high titres involved and worst case scenario accidents such as 50 µl of vector prep being injected into a finger or an aerosol generated by a centrifuge failure. Even in these situations very low amounts of vector would reach the liver and it is unlikely these incidents would be a serious risk to the health of the individuals concerned.

1. "AAV Vector Integration Sites in Mouse Hepatocellular Carcinoma" Donsante et al. Science 317, 477 (2007)

2. "Long Term Portal Vein Administration of AAV-WPRE Vector Results in Increased Incidence of Neoplastic Disease and Hepatic Pathology" J. E. Embury et al., Mol. Ther. 13, S83 (2006). Abstract 216.

3. "Analysis of Tumors Arising in Male B6C3F1 Mice with and without AAV Vector Delivery to Liver" P. Bell et al., Mol. Ther. 14, 34 (2006).

4. "No evidence for tumorigenesis of AAV vectors in a large-scale study in mice." Bell, P., et al. (2005). Mol. Ther. 12: 299 – 306. 695

5. AAV Vectors, Insertional Mutagenesis, and Cancer D.W. Russell Mol. Ther. 15:1740-43

6. "Adeno-associated virus vector integration." Deyle DR, Russell DW. Curr Opin Mol Ther. (2009) 11(4):442-7.

7. "Oncogenesis Following Delivery of a Nonprimate Lentiviral Gene Therapy Vector to Fetal and Neonatal Mice" Themis et al. (2005) Molecular Therapy 12, 763–771.