

the MoFlo high-speed cell sorter. However, please note that while these “containment systems permit sorting of materials classified as BSL-2 using BSL-2 practices, the effectiveness of aerosol containment should be verified through rigorous testing before sorting any potentially infectious samples” (Schmid et al., 2007).

Auditing Safety Measures

This new ISAC Standard emphasizes the importance of audits to verify that containment measures are effective. ISAC advises:

- Testing the sort chamber with bottled smoke and sealing any leaks found
- Using splash shields for the sample uptake area and/or the sort chamber
- Accessing and inspecting fluidic tubing that is under pressure before infectious sorts
- Using sizing nozzles appropriate for the cells to be sorted. At a minimum, the aperture should be four times greater than the cell size; six times the size of the cells to be sorted is listed as the ideal (Schmid et al., 2007).

Some manufacturers recommend cleaning the nozzles by sonication—biosafety professionals will have opinions on where that is done! Details such as proper disinfection of the equipment according to manufacturer’s directions, sink discharge of disinfected waste, and the use of personal protective equipment and handwashing after removal of personal protective equipment should all be evaluated during an audit. If a risk assessment warrants the wearing of respiratory protection, staff should be enrolled in a respiratory protection program. The ISAC standard contains a great deal of substantive, updated information that will be of assistance to biosafety professionals whose responsibilities require them to review the

sorting of unfixed biohazardous samples. Sharing the ISAC guidelines with staff performing this procedure is an important first step for safe cell sorting.

References

- Aloisio, C. H., & Nicholson, J. K. A. (1990). Recovery of infectious human immune deficiency virus from cells treated with 1% paraformaldehyde. *Journal of Immunological Methods*, 128(2), 281-285.
- Ghidoni, D. A., Eagleson, D. A., Lockhart, E. X., & Zarembo, M. J. (2006). Biosafety enclosures for automated laboratory equipment. *Anthology of Biosafety IX: Exploring the Performance Envelope for BSL-3 and BSL-4 Laboratories* (pp. 167-175).
- Lennartz, K., Lu, M., Flasshove, M., Moritz, T., & Kirstein, U. (2005). Improving the biosafety of cell sorting by adaptation of a cell sorting system to a biosafety cabinet. *Cytometry, Part A*, 66, 119-127.
- Oh, M., Kim, N., Huh, C., Choi, C., Lee, E., Kim, I., et al. (2001). Scrub typhus pneumonitis acquired through the respiratory tract in a laboratory worker. *Infection*, 29, 54-56.
- Perfetto, S. P., Ambrozak, D. R., Koup, R. A., & Roederer, M. (2003). Measuring containment of viable infectious cell sorting in high-velocity cell sorters. *Cytometry, Part A*, 52A, 122-139. Available at: www3.interscience.wiley.com/cgi-bin/fulltext/104084100/HTMLSTART
- Ruprecht, R. M., Baba, T. M., Liska, V., Ray, N. B., Martin, L. N., Murphey-Corb, M., et al. (1999). Oral transmission of primate lentiviruses. *Journal of Infectious Diseases*, 179, Suppl 3:S408-12.
- Schmid, I. C., Lambert, D., Ambrozak, D., & Perfetto, S. P. (2007). Standard safety practices for sorting of unfixed cells. In *Current Protocols in Cytometry*, Section 3.6.1-3.6.20. New Jersey: John Wiley & Sons, Inc. Available at: www.mrw.interscience.wiley.com/emrw/9780471142959/cp/cpcy/article/cy0306/current/pdf

Molecular Biosafety

Margy S. Lambert

University of Wisconsin—Madison, Madison, Wisconsin

The molecular biology and biotechnology fields are growing by leaps and bounds. Molecular Biosafety aims to shed light on how these cutting-edge techniques impact safety. Please e-mail your insights and questions to Margy Lambert at mlambert@fpm.wisc.edu or Co-Editor Barbara Johnson at barbara_johnson@verizon.net or Co-Editor Karen B. Byers at karen_byers@dfci.harvard.edu.

A Reassessment of Adeno-Associated Virus Vector Risks That Takes New Information on Insertional Mutagenesis into Account

A recent *Science* article implicated insertional mutagenesis as a potential mechanism for induction of liver cancer in mice by adeno-associated virus (AAV) vectors (Donsante et al., 2007). The results of this study, as discussed by Kay 2007, have resurfaced concerns that AAV vectors may not be as safe as previously thought and may influence how AAV and recombinant AAV will be handled in research laboratories in the future.

AAV is a parvovirus that can be aerosol-transmitted and a dependovirus that normally requires another virus such as adenovirus or herpes simplex virus (HSV) to supply factors that support replication (Hamilton et al.,

2004). AAV is generally considered nonpathogenic; therefore, AAV has been assessed as a risk group 1 (RG1) virus with the use of biosafety level 1 (BSL-1) conditions considered adequate (NIH Guidelines for Research Involving Recombinant DNA Molecules: www4.od.nih.gov/oba/rac/guidelines/guidelines.html).

On the other hand, some characteristics of AAV may increase risks, including the following: detection in embryonic tissue (Burguete et al., 1999; Dutheil et al., 1997; Kiehl et al., 2002), an association with male infertility (Erles et al., 2001; Mehrle et al., 2004), the ability to replicate in some cases without a helper virus (Meyers et al., 2000), and the capability of integrating into the host genome (Miller et al., 2002). AAV is an unusual virus that can not only integrate into the host genome, but also is inserted preferentially at a specific site (on human chromosome 19). AAV integration translates to AAV having the potential for insertional mutagenesis, and for latency where AAV can be reactivated to a productive infection at a later time when a helper virus is present.

Based on risk information available before the 2007 Donsante article was published, the University of Wisconsin-Madison's Institutional Biosafety Committee (IBC) instituted a policy in 2006 (modified in early 2007) deeming that some procedures involving AAV and AAV vectors should be conducted using biosafety level 2 (BSL-2) precautions and containment (www2.fpm.wisc.edu/biosafety/ibc/docs/OppportunisticPathogens_Jn07final.pdf).

Earlier research conducted by the authors of the 2007 *Science* article indicated the same type of results: liver cancer in mice associated with AAV vector treatment (Donsante et al., 2001). The main drawbacks of this study were the evaluation of only one knockout strain of mice and small numbers. This article raised sufficient concerns; however, that the Food and Drug Administration (FDA) temporarily halted two clinical trials using AAV vectors in 2001.

Some research studies indicate that there is no association between AAV vectors and hepatocellular carcinoma (HCC). One significant research paper, in particular, concluded that there was no evidence for AAV vector induction of liver cancer in a large-scale study in mice (Bell et al., 2005). This analysis was a compilation of many individual studies so a variety of different vectors, mouse strains, and experimental conditions were employed. One tumor was observed in 695 mice receiving AAV vectors while none were observed in the 226 control mice. The primary mouse strain used was C57Bl/6, which is a strain classified as having a low frequency of spontaneous liver tumor formation. Drawbacks of this study included: using mice of a broad range of ages (it's conceivable that more tumors would have appeared if the mice were allowed to age longer), evaluation of only part of each liver (an average of one third of each), and using a variety of AAV constructs including different transgenes

and regulatory sequences. The authors recommend that future studies use mice matched for age, vector, dosage, strain, and other relevant parameters.

A symposium entitled "Safety Considerations in the Use of AAV vectors in Gene Transfer Clinical Trials," jointly sponsored by the National Institutes of Health (NIH) and the FDA, was held in March 2001 (www4.od.nih.gov/oba/rac/Transcript3-7-011.pdf). The conflicting research results for an association between AAV vectors and liver cancer were discussed, and the NIH Recombinant Activities Committee (NIH RAC) concluded that the tumors observed in the 2001 murine liver cancer study were not likely caused by AAV vectors. It was suggested that the tumors were probably due to the knockout strain of mice used in the study being especially susceptible to cancer. As a result, the human gene therapy trials utilizing AAV vectors that had been put on hold were allowed to continue.

The 2007 Donsante study tested the association of liver cancer with AAV vectors using several strains of neonatal mice, including wild type mice, and found similar results as their previous research (56% or 33% of treated mice [depending on strains of mice and specific AAV constructs used] developed liver cancer as compared to 8%, or 4% for the untreated controls). In addition, the current study detected AAV vector integration events in the liver tumor tissue and mapped the integration site. Surprisingly, an evaluation of the tumors demonstrated that integration of AAV vectors was at a common site on mouse chromosome 12 rather than at random sites in the genome. For humans, altered gene regulation, including down regulation of a putative tumor suppressor gene at the syntenic (homologous) site on human chromosome 14 has been associated with several types of cancer (Astuti et al., 2005; Kawakami et al., 2006).

A common theory has been that if AAV genes (e.g., rep genes) that target integration to human chromosome 19 are removed, integration will occur at random sites in the genome (Kearns et al., 1996). Evidence from the 2007 murine liver cancer study contradicts that assumption. AAV vectors (at least in some tissues) appear to integrate preferentially at specific sites, potentially leading to altered regulation of nearby key genes and disease outcomes. This result could be due to either a site-specific recombination mechanism, or to random integration followed by selection for cells containing AAV vectors inserted at that site.

This research provides evidence that AAV vector insertional mutagenesis risk is higher than previously thought. Removing genes responsible for insertion of AAV at the preferred site on chromosome 19 does not eliminate the potential for insertional mutagenesis. In fact, insertion of AAV vectors at sites near high hazard genes may carry more risk than insertion at the chromosome 19 site preferred by wild type AAV containing functional rep genes.

Key points from the 2007 *Science* article are the following: 1) The results of the previous study were repeated and the criticism of the earlier study that the results would likely not apply to wildtype mice was refuted; 2) A mechanism for the association between AAV vectors and liver cancer (insertional mutagenesis) was postulated; and 3) the surprising result that AAV vector integration occurred at a specific site rather than at random sites in the host genome was observed.

What explains the conflicting results of different studies on the association of AAV vectors with insertional mutagenesis and cancer? This may be a case of, "The devil is in the details." The specific experimental conditions of the study may define whether tumorigenesis occurs. Important factors that should be studied in more detail include: genetic susceptibility, AAV construct specifics including transgenes and regulatory sequences, characterization of AAV vector integration sites, involvement of DNA repair factors, tissue specificity, the presence of environmental triggers, such as DNA-damaging chemicals or radiation, and identification of disease genes near insertion sites with analysis of whether the expression of these host genes is altered.

The genetic background of different murine species could make a difference in whether AAV vectors can induce liver cancer through insertional mutagenesis. Other host variables such as age and gender could also influence whether liver cancer can be induced by AAV vectors. Thus, it may not be surprising that liver cancer was not seen in murine strains with a low rate of spontaneous tumorigenesis, while liver cancer was detected in other strains that have a higher susceptibility to carcinogenesis. A similar dichotomy is seen in humans with some individuals showing a higher tendency to get cancer due to their genetic background.

The 2007 Donsante study showed that the frequency of liver cancer varied depending on the AAV construct used: 56% of mice developed liver cancer when the β -actin promoter was present in the AAV construct, while 33% of mice developed liver cancer when this promoter was absent from the construct. In this case, the different frequencies of liver cancer occurred when the same transgene, but different regulatory sequences were present in the AAV constructs. The same research group that concluded there was no evidence for tumorigenesis of AAV vectors in a large-scale murine study in 2005, reported research indicating an association of tumor formation and liver cancer with AAV vector delivery to the liver, which was dependent on the transgene delivered. The authors conclude that the expression of some transgenes alone, or in combination with the AAV vector may be problematic (Bell et al., 2006).

Other studies support the Donsante 2007 article's conclusion that AAV vector integration is not random. Actively transcribed genes are targeted in the liver (Russell, 2003). A large scale characterization of integra-

tion sites in fibroblasts (Miller et al., 2005) and murine liver (Nakai et al., 2005) shows preferential integration near transcription start sites and regulatory sequences; the presence of hotspots for integration, deletions and other rearrangements in host chromosomes at insertion sites; and a 3.5% integration frequency in cancer-related genes (in the liver). The Nakai study concludes that gain of function, in addition to loss of function mutations in disease-related genes, can be caused by AAV vector-mediated insertional mutagenesis.

The involvement of DNA repair in AAV integration needs further study. AAV appears to use some host DNA repair factors in the integration mechanism. Unlike retroviral vectors, the AAV integration mechanism exploits aspects of DNA repair pathways with integration at chromosomal double-stranded breaks (Russell, 2003; Miller et al., 2004). *In vitro* and *in vivo* studies indicate that inhibition of DNA repair enzyme DNA-PK results in an increased level of AAV integration (Song et al., 2003).

Different tissues show different patterns of AAV transduction and integration and of tumorigenesis. AAV vector integration in the liver has been shown to occur preferentially in actively transcribed regions of the genome (72%), to cause deletions and other rearrangements at chromosomal integration sites (100%), and 100% of the targeted genes analyzed were found to be genes expressed in the liver (Nakai et al., 2003).

DNA-damaging agents (chemicals, gamma radiation, and ultraviolet radiation) can increase the rate of AAV transduction (infection of cells) and the frequency of AAV integration (Alexander et al., 1994; Russell et al., 1995; Peng et al., 2000). Some DNA-damaging agents were able to induce transduction by 750-fold. The liver is, of course, the primary site for detoxification of chemicals so it is a tissue where the opportunity for chemical induction of AAV transduction and AAV integration would be greater.

If a disease association (e.g., cancer types other than liver) is detected in future studies involving AAV vectors, similar testing, as was done in the 2007 *Science* article, should be carried out. If possible, host chromosome insertion sites should be identified and expression levels of nearby genes analyzed. This sort of investigation could determine whether other disease outcomes are associated with AAV vectors, under what experimental conditions, and what disease genes are involved in the process.

A July 2007 death in a gene therapy trial using AAV vectors was investigated by NIH RAC with their findings detailed in their September 2007 and December 2007 meetings (webcast: videocast.nih.gov/default.asp). The main cause of death was apparently an overwhelming infection with *Histoplasma capsulatum*. The expert panel deemed it unlikely that gene therapy contributed to the death, but stated that this possibility cannot be ruled out definitively. A potential role for insertional mutagenesis was not discussed.

What does the above information mean for the bio-

safety community? In conducting risk assessments, investigators should be aware of the recent information on insertional mutagenesis. Factors considered in risk assessments of *in vitro* AAV vector projects should include: aerosol route of transmission, aerosol-generating activities, quantity and concentration of the virus, and transgene expressed. Additional factors evaluated for *in vivo* experiments should include: dosage, route of administration, organ targeted, and the ability of AAV vectors to migrate from the target site. Some tissues could present higher risks (e.g., the lungs, because of aerosol transmission, and the liver, because of the recent data indicating an association between AAV vector integration and liver cancer).

The 2007 Donsante study provides a compelling argument for insertional mutagenesis being a potential risk in AAV vector research. Based on this new information, IBCs may want to consider setting policies designating the use of BSL-2, rather than BSL-1 precautions and containment, for some research projects involving AAV vectors.

References

- Alexander, I., Russell, D., & Miller, A. (1994). DNA-damaging agents greatly increase the transduction of nondividing cells by adeno-associated virus vectors. *J. Virol.*, 68(12), 8282-8287.
- Astuti, D., Latif, F., Wagner, K., Gentle, D., Cooper, W., Grundy, R., et al. (2005). Epigenetic alteration at the DLK1-GTL2 imprinted domain in human neoplasia: Analysis of neuroblastoma, phaeochromocytoma and Wilms tumor. *Br J Cancer*, 92, 1574-1580.
- Bell, P., Moscioni, A., McCarter, R., Wu, D., Gao, G., Hoang, A., et al. (2006). Analysis of tumors arising in male B6C3F1 mice with and without AAV vector delivery to liver. *Mol. Ther.*, 14(1), 34-44.
- Bell, P., Wang, L., Lebherz, C., Flieder, D., Bove, M., Wu, D., et al. (2005). No evidence for tumorigenesis of AAV vectors in a large-scale study in mice. *Mol. Ther.*, 12(2), 299-306.
- Burguete, T., Rabreau, M., Fontanges-Darriet, M., Roset, E., Hager, H., Koppel, A., et al. (1999). Evidence for infection of the human embryo with adeno-associated virus in pregnancy. *Hum. Reprod.*, 14(9), 2396-2401.
- Donsante, A., Miller, D., Li, Y., Vogler, C., Brunt, E., Russell, D., et al. (2007). AAV vector integration sites in mouse hepatocellular carcinoma. *Science*, 317, 477.
- Donsante, A., Vogler, C., Muzyczka, N., Crawford, J., Barker, J., Flotte, T., et al. (2001). Observed incidence of tumorigenesis in long-term rodent studies of rAAV vectors. *Gene Therapy*, 8, 1343-1346.
- Dutheil, N., Malhomme, O., Provost, N., Becquart, P., Burgutte, T., Schlehofer, J., et al. (1997). Presence of integrated DNA sequences of adeno-associated virus type 2 in four cell lines of human embryonic origin. *J. Gen. Virol.*, 78(Pt. 11), 3039-3043.
- Erles, K., Rohde, V., Thaele, M., Roth, S., Edler, L., & Schlehofer, J. (2001). DNA of adeno-associated virus (AAV) in testicular tissue and in abnormal semen samples. *Hum. Reprod.*, 16(11), 2333-2337.
- Hamilton, H., Gomos, J., Berns, K., & Falck-Pedersen, E. (2004). Adeno-associated virus site-specific integration and AAVS1 disruption. *J. Virol.*, 78(15), 7874-7882.
- Kawakami, T., Chano, T., Minami, K., Okabe, H., Okada, Y., & Okamoto, K. (2006). Imprinted DLK1 is a putative tumor suppressor gene and inactivated by epimutation at the region upstream of GTL2 in human renal cell carcinoma. *Hum. Mol. Genet.*, 15(6), 821-830.
- Kay, M. A. (2007). AAV vectors and tumorigenicity. *Nat. Biotechnol.*, 25(10), 111-1113.
- Kearns, W., Aflone, S., Fulmer, S., Pang, M., Erikson, D., Egan, M., et al. (1996). Recombinant adeno-associated virus (AAV-CFTR) vectors do not integrate in a site-specific fashion in an immortalized epithelial cell line. *Gene Therapy*, 3(9), 748-755.
- Kiehl, K., Shlehofer, J., Schultz, R., Zugaib, M., & Armbruster-Moraes, E. (2002). Adeno-associated virus DNA in human gestational trophoblastic disease. *Placenta*, 23(5), 410-415.
- Mehrle, S., Rohde, V., & Shlehofer, J. (2004). Evidence of chromosomal integration in human testis tissue. *Virus Genes*, 28(1), 61-69.
- Meyers, C., Mane, M., Kokorina, N., Alam, S., & Hermona, P. (2000). Ubiquitous human adeno-associated virus type 2 autonomously replicates in differentiating keratinocytes of a normal skin model. *Virology*, 272, 338-346.
- Miller, D., Petek, L., & Russell, D. (2004). Adeno-associated virus vectors integrate at chromosome breakage sites. *Nat. Genet.*, 36(7), 767-773.
- Miller, D., Rutledge, E., & Russell, D. (2002). Chromosomal effects of adeno-associated virus vector integration. *Nat. Genet.*, 30(2), 147-148.
- Miller, D., Trobridge, G., Petek, L., Jacobs, M., Kaul, R., & Russell, D. (2005). Large-scale analysis of adeno-associated virus vector integration sites in normal cells. *J. Virol.*, 79(17), 11434-11442.
- Nakai, H., Montini, E., Fuess, S., Storm, T., Grompe, M., & Kay, M. (2003). AAV serotype 2 vectors preferentially integrate into active genes in mice. *Nat. Genet.*, 34, 297-302.
- Nakai, H., Wu, X., Fuess, S., Storm, T., Munroe, D., Montini, E. et al. (2005). Large-scale molecular characterization of adeno-associated virus vector integration in mouse liver. *J. Virol.*, 79(6), 3606-3614.
- Peng, D., Qian, C., Sun, Y., Barajas, M., & Prieto, J. (2000). Transduction of hepatocellular carcinoma (HCC) using recombinant adeno-associated virus (rAAV): In vitro and in vivo effects of genotoxic agents. *J. Hepatol.*, 32(6), 975-985.
- Russell, D. (2003). AAV loves an active genome. *Nat. Genet.*, 34, 241-242.
- Russell, D., Alexander, I., & Miller, A. (1995). DNA synthesis and topoisomerase inhibitors increase transduction by adeno-associated virus vectors. *Proc. Natl. Acad. Sci. USA*, 92, 5719-5723.
- Song, S., Lu, Y., Choi, Y., Han, Y., Tang, Q., Berns, K. et al. (2003). DNA-dependent PK inhibits adeno-associated virus DNA integration. *Proc. Natl. Acad. Sci. USA*, 101(7), 2112-2116.