Replication-Competent Retroviruses in Gene-Modified T Cells Used in Clinical Trials: Is It Time to Revise the Testing Requirements?

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Adoptive T-cell transfer is recognized as an innovative treatment strategy for various malignant diseases.[1,2] To improve the efficacy and sometimes the safety of this approach, T cells can be genetically manipulated to modify their antigen specificity, to enhance their *in vivo* survival and trafficking to specific tissues, or to be eliminated in the event of undesired toxic effects.[3] γ-retroviral vectors are frequently used to obtain robust and stable genetic modification of human T lymphocytes because these vectors can efficiently integrate within the genome and ensure that the inserted transgene is passed to the progeny of infected cells.

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Although integration is the desired effect of retroviral-mediated gene transfer, it carries the risk of insertional mutagenesis, which can result in dysregulated gene expression and subsequent malignant transformation.[4] The potential for insertional mutagenesis and malignant transformation may be increased in cases where replication-competent retroviruses (RCRs) are present in the vector products, as continued viral replication could result in multiple integrations within the host-cell genome. When a high-titer vector-producer packaging cell line (VPC) with a known level of RCR contamination of 10^3–10^4 virions/ml was used to transduce stem cells in 10 nonhuman primates, three animals developed virus-induced T-cell lymphoma.[5] This observation led to extensive public discussions and the adoption of the current US Food and Drug Administration (FDA) guidance for RCR testing.

Almost all γ-retroviral vectors manufactured for clinical use are produced using stable VPCs derived from murine or human cell lines. The risk of RCR generation is minimized during the production of the retroviral vector supernatant by segregating vectors encoding the exogenous gene of interest from sequences encoding the viral proteins *gag*, *pol*, and *env*. These sequences are provided *in trans* by VPCs that stably express *gag*, *pol*, and *env* genes. Early VPCs that contained *gag*, *pol*, and *env* genes expressed from a single plasmid had a high incidence of RCR generation due to recombination between the vector and viral component plasmids.[6] The risk of RCR generation was greatly reduced by segregating each of the viral components into separate plasmids with minimal homology, one containing *gag-pol* and the other *env,* and by minimizing homology between the vector and packaging sequences.[6–8]

Although current packaging cell line and vector designs make the generation of RCRs extremely unlikely, present FDA guidelines require extensive testing at multiple levels for the presence of RCRs, beginning with screening of packaging cell lines and vector products, followed by testing of the final gene-modified cell product, and culminating with patient monitoring after cell infusion. The extended S^+L^- assay—in which test material is added to a susceptible cell line that allows virus replication (amplification), after which the medium from these cells is assayed for helper virus—is commonly used to detect RCRs within packaging cell lines, viral lots, and cell products.[9] Despite negative *in vitro* testing results for RCRs, it is further required that patients be regularly monitored postinfusion using polymerase chain reaction (PCR) or serological methods, and positive results obtained by these assays must then be confirmed by biological assays such as the S^+/L^- assay.

RCR testing is labor-intensive, time-consuming, and extremely costly, which severely limits the translational applicability of genetically modified cell therapies by most not-for-profit medical centers. For more than a decade, our own centers have focused on adoptive transfer of gene-modified T cells for the treatment of malignancies; other investigators, nationally and internationally, have followed similar practices. Despite the widespread and extensive use of resources to detect RCRs, there has been no systematic study examining the incidence of RCR positivity in these therapeutic products or their recipients. Here, we report a large cohort of RCR testing results from clinical trials carried out at Baylor College of Medicine, St Jude Children's Research Hospital, the National Cancer Institute, the University of Pennsylvania, and Indiana University.

Overall, 30 master cell banks (MCBs) have been generated, from which 42 viral supernatant lots have been produced

Table 1 RCR results from T-cell products and patient-monitoring samples

Trial ID	Cell product	Transgene	Disease	T cells	≤3 mo	3–6 mo	6–12 mo	≥12 mo
NCT00001832	TILs	Neo	Melanoma	0/10	0/7	0/6	0/2	
NCT00019136	ATCs	CAR-FBP.Fcg	Ovarian cancer	0/6				
NCT00058617	EBV-CTLs	Neo	Hodgkin's disease	0/8	0/8	0/5	0/3	0/2
NCT00058773	EBV-CTLs	Neo	Heme malignancies post-HSCT	0/26	0/41	0/28	0/49	0/82
NCT00062036	TILs	IL-2	Melanoma	0/5	0/4	0/2		
NCT00085462	ATCs	TCR-gp100-209	Melanoma	0/14	0/8	0/4	0/2	
NCT00085930	ATCs/EBV-CTLs	CAR-GD2.ζ	Neuroblastoma	0/36	0/19	0/19	0/17	0/14
NCT00091104	ATCs	TCR-MART-1 (DMF4)	Melanoma	0/31	0/24	0/14	0/5	0/1
NCT00368082	LMP2a-CTLs	TGFβRIIdcyt (DNRII)	EBV$^+$ lymphoma	0/4	0/7	0/4	0/2	
NCT00393029	ATCs	TCR-p53	p53$^+$ malignancies	0/11	0/6	0/2	0/1	
NCT00509288	ATCs	TCR-MART-1 (DMF5)	Melanoma	0/20	0/14	0/12	0/6	0/1
NCT00509496	ATCs	TCR-gp100-154	Melanoma	0/16	0/5	0/4	0/3	0/1
NCT00586391	ATCs/EBV-CTLs/ multivirus-CTLs	CAR-CD19.ζ CAR-CD19.CD28.ζ	CD19$^+$ leukemia/ lymphoma	*	0/3	0/3		
NCT00610311	ATCs	TCR-gp100-154	Melanoma	0/2	0/1	0/1		
NCT00612222	ATCs	TCR-MART-1 (DMF5)	Melanoma	0/2	0/2	0/3		
NCT00670748	ATCs	TCR-NY-ESO-1	NY-ESO-1$^+$ malignancies	0/19	0/14	0/11	0/9	
NCT00704938	ATCs	TCR-p53	p53$^+$ malignancies	0/3	0/2	0/2		
NCT00706992	ATCs	TCR-MART-1 (DMF5)	Melanoma	0/41	0/25	0/26	0/31	
NCT00709033	ATCs/EBV-CTL/ multivirus-CTLs	CAR-CD19.ζ	CD19$^+$ Leukemia/ lymphoma	*	0/1	0/1		
NCT00710892	Allodepleted T cells	iCaspase9.ΔCD19	Heme malignancies haplo-HSCT	0/11	0/3	0/3		
NCT00840853	ATCs/EBV-CTL/ multivirus-CTLs	CAR-CD19.ζ	ALL	*				
NCT00889954	ATCs/EBV-CTLs	CAR-HER2.CD28.ζ TGFβRIIdcyt (DNRII)	HER2$^+$ lung cancer	*				
NCT00902044	ATCs/EBV-CTLs	CAR-HER2.CD28.ζ	HER2$^+$ sarcoma	*				
NCT00923195	ATCs	TCR-MART-1 (DMF5) TCR-gp100-154	Melanoma	0/4	0/1		0/1	
NCT00923390	ATCs	TCR-2G1	Renal cancer	0/5	0/4	0/1	0/1	
NCT00923806	ATCs	TCR-CEA	CEA$^+$ malignancies	0/3	0/2	0/2		
NCT00924287	ATCs	CAR-ERBB2.CD28.41BB.ζ	HER2$^+$ malignancies	0/1				
NCT00924326	ATCs	CAR-CD19.CD28.ζ	B-cell malignancies	0/8	0/2	0/3	0/1	
NCT01013415	ATCs	CAR-CD4-ζ	HIV-1 infection	0/11	0/10	0/10	0/10	0/6
Total				**0/297**	**0/213**	**0/166**	**0/143**	**0/107**

ALL, acute lymphoblastic leukemia; ATC, activated T cells; CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; CTL, cytotoxic T lymphocyte; EBV, Epstein–Barr virus; HSCT, hematopoietic stem cell transplantation; IL-2, interleukin-2; TCR, T-cell receptor; TGF, transforming growth factor; TIL, tumor-infiltrating lymphocyte. *Pending samples (n = 15).

(**Supplementary Table S1** online). The great majority of clinical-grade MCBs (28/30) were generated using the PG13 VPC, which provides viral particles pseudotyped with the gibbon ape leukemia virus (GALV) envelope.[7] Two MCBs were generated using the PA317 VPC. In most cases, the SFG (10/30) or MSGV1 (15/30) retroviral vector was used. Single-cell clones from transduced packaging cells were expanded to produce an MCB, from which clinical-grade retroviral supernatant was produced and collected. RCR screenings performed by amplification in HEK 293 cells and analyzed using the S^+L^- focus assay were consistently negative from all 30 MCBs and 42 viral supernatant lots.

Representative data from 29 clinical studies using gene-modified T-cell products manufactured with γ-retroviral vectors from the tested MCBs are shown in **Table 1**. These studies utilized either polyclonal activated T cells (ATCs); cytotoxic T lymphocytes (CTLs) specific for antigens encoded by Epstein–Barr virus (EBV), cytomegalovirus, or adenovirus; or tumor-infiltrating lymphocytes (TILs). As shown, a variety of transgenes were expressed from marker proteins (Neo, ref. 10) to tumor-antigen receptors (chimeric antigen receptors (ref. 11)) or transgenic αβ-T-cell receptors (ref. 12) to cytokines (interleukin-2, ref. 13) and suicide genes (iCaspase9, ref. 14). S^+L^- focus-forming assays or PG-4 plaque assays were performed to detect RCR generation within 297 T-cell

products. RCRs were absent in all T-cell products thus far tested, with 15 results still pending. Three additional T-cell products were generated but not tested for RCRs, as the cells were maintained in culture for less than 4 days after retroviral transduction. Current FDA recommendations do not require RCR testing when cells are cultured under these short-term conditions.

FDA guidelines require patient follow-up analysis for RCRs to be performed at 3, 6, and 12 months and yearly thereafter postinfusion. RCR screening was performed by PCR analysis of peripheral blood mononuclear cells to detect the presence of the GALV, amphotropic envelope, or *gag-pol*. A total of 629 follow-up samples have been analyzed, ranging from 1 month to 8 years after infusion (**Table 1**). In most studies occurring since 2001, yearly samples collected from 1 to 15 years postinfusion were banked but not analyzed for RCRs. Thus far, we have not detected RCRs in any patient sample.

Although the results of RCR testing have consistently been negative, the costs of the procedure are considerable. Certain tests were performed in-house or through the National Gene Vector Biorepository, which defrayed a significant part of the trial cost. Because estimating true cost to academic institutions can be difficult, we utilized prices provided by two separate commercial sources to estimate the costs incurred by RCR testing. As indicated in **Supplementary Table S2**, the commercial cost of testing 30 MCBs plus 42 retroviral supernatant lots ranges between $1,497,036 and $1,593,726. The total cost of testing 312 T-cell products ranges between $3,088,800 and $3,092,856, and the total in-house cost of testing 629 patient samples is around $314,500.

We believe that the data reported herein have significant implications for the conduct of clinical trials using T cells genetically manipulated with γ-retroviral vectors. FDA requirements for RCR screening have changed little over the past 20 years since this novel therapy was first introduced into the clinic. Our analysis indicates that modern VPCs such as PG13, which are characterized by independent *gag-pol* and *env* components and pseudotyped viral particles, significantly reduce the risk of RCR generation. In addition to screening the MCBs, investigators must screen 5% of the final vector product and 10^8 end-of-production cells of each vector lot. These requirements were set somewhat arbitrarily, balancing the need to provide rigorous screening with the technical and practical implications of testing a large percentage of a vector lot. In an effort to ensure that the RCR assays and sample sizes adequately screened for RCRs, the FDA further required testing of *ex vivo* manipulated cell products and patient samples. Our data demonstrating the lack of RCRs in *ex vivo* γ-retroviral-transduced T-cell products and patient samples over 29 trials, including HIV-infected patients, suggest that the current FDA requirements for screening MCBs and viral lots are adequate and appropriate.

Our data also question the need for continued testing of T-cell products and patient samples for RCRs. This testing does not appear to provide additional safety assurances above those obtained by the screening of vector products. Given the lack of apparent benefit, one must also consider the associated high costs of testing. Because many of these therapies have already produced well-documented, complete, and long-term responses in otherwise lethal disorders,[12,15] the significant expense of testing will impede translation of this therapy into the clinic.

We make the following proposals for future T-cell gene therapy protocols. We believe it is important that the MCB, end-of-production cells, and viral lots continue to be rigorously analyzed to exclude the presence of RCRs before they are released for clinical use; the current FDA recommendation for screening γ-retrovirus lots for RCRs should continue unchanged. Final T-cell products that incorporate γ-retroviral vectors, together with samples from treated patients, should be collected and archived for retrospective studies, but need not be actively screened for RCR contamination. It should be noted that the majority of retroviral vectors used in this analysis were generated using the PG13 cell line. Currently, we do not know whether these data can be extrapolated to other packaging cell lines, other methods of vector production, or other vector systems (e.g., lentiviral vectors). Such virus-production methods will need to be tested experimentally. Future efforts may reasonably be dedicated to the development of rapid and inexpensive assays for the detection of RCR contamination of manipulated T-cell products, but for the present, our data show that the changes we propose can be introduced without measurably affecting patient safety and, by reducing the cost of each protocol, will allow further effective treatments to be safely developed.

SUPPLEMENTARY MATERIAL

Table S1. RCR results from packaging cell lines and viral supernatant lots.
Table S2. Cost analysis of RCR testing.

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REFERENCES

1. Rosenberg, SA and Dudley, ME (2009). Adoptive cell therapy for the treatment of patients with metastatic melanoma. *Curr Opin Immunol* **21**: 233–240.
2. Brenner, MK and Heslop, HE (2010). Adoptive T cell therapy of cancer. *Curr Opin Immunol* **22**: 251–257.
3. Vera, JF, Brenner, MK and Dotti, G (2009). Immunotherapy of human cancers using gene modified T lymphocytes. *Curr Gene Ther* **9**: 396–408.
4. Hacein-Bey-Abina, S, von Kalle, KC, Schmidt, M, Le Deist, F, Wulffraat, N, McIntyre, E *et al.* (2003). A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* **348**: 255–256.
5. Donahue, RE, Kessler, SW, Bodine, D, McDonagh, K, Dunbar, C, Goodman, S *et al.* (1992). Helper virus induced T cell lymphoma in nonhuman primates after retroviral mediated gene transfer. *J Exp Med* **176**: 1125–1135.
6. Lynch, CM and Miller, AD (1991). Production of high-titer helper virus-free retroviral vectors by cocultivation of packaging cells with different host ranges. *J Virol* **65**: 3887–3890.
7. Miller, AD, Garcia, JV, von Suhr, N, Lynch, CM, Wilson, C and Eiden, MV (1991). Construction and properties of retrovirus packaging cells based on gibbon ape leukemia virus. *J Virol* **65**: 2220–2224.
8. Miller, AD and Chen, F (1996). Retrovirus packaging cells based on 10A1 murine leukemia virus for production of vectors that use multiple receptors for cell entry. *J Virol* **70**: 5564–5571.
9. Chen, J, Reeves, L and Cornetta, K (2001). Safety testing for replication-competent retrovirus associated with gibbon ape leukemia virus–pseudotyped retroviral vectors. *Hum Gene Ther* **12**: 61–70.
10. Heslop, HE, Slobod, KS, Pule, MA, Hale, GA, Rousseau, A, Smith, CA *et al.* (2010). Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood* **115**: 925–935.
11. Pule, MA, Savoldo, B, Myers, GD, Rossig, C, Russell, HV, Dotti, G *et al.* (2008). Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nat Med* **14**: 1264–1270.
12. Morgan, RA, Dudley, ME, Wunderlich, JR, Hughes, MS, Yang, JC, Sherry, RM *et al.* (2006). Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* **314**: 126–129.
13. Heemskerk, B, Liu, K, Dudley, ME, Johnson, LA, Kaiser, A, Downey, S *et al.* (2008). Adoptive cell therapy for patients with melanoma, using tumor-infiltrating

lymphocytes genetically engineered to secrete interleukin-2. *Hum Gene Ther* **19**: 496–510.
14. Di Stasi, A, Tey, SK, Dotti, G, Fujita, Y, Kennedy-Nasser, A, Martinez, C *et al.* (2011). Inducible apoptosis as a safety switch for adoptive cell therapy. *N Engl J Med* **365**: 1673–1683.
15. Porter, DL, Levine, BL, Kalos, M, Bagg, A and June, CH (2011). Chimeric antigen receptor–modified T cells in chronic lymphoid leukemia. *N Engl J Med* **365**: 725–733.

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Signs of Progress in Gene Therapy for Muscular Dystrophy Also Warrant Caution

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AAV-based vectors have emerged as plausible candidates for clinical gene transfer to muscle, but they present several challenges in the context of Duchenne muscular dystrophy (DMD). These include the need to "miniaturize" an extremely large therapeutic gene, the development of strategies for effective regional and systemic vector delivery to a tissue mass the size of the musculature, and the avoidance of immune-mediated elimination of transgene in a degenerative disease. Leveraging previous progress on gene miniaturization,[1] in this issue of *Molecular Therapy* a multidisciplinary team of investigators provides important new perspectives on vector delivery[2] and an affiliated group of investigators from the same institution report on a safety trial intended to expedite translational studies of the immune response to a transgene.[3] The collaboration achieved thus far will require a future marriage of these three avenues of research to help set the stage for clinical efficacy with gene therapy for Duchenne and other muscular dystrophies. Nevertheless, important new questions arise about the potential escalation of risk to research participants in which a large volume of tissue is transduced with even a subtly immunogenic vector, and the advances presented in the two articles bring such bioethical concerns to center stage for discussion by the broader research community.

The DMD gene and its protein product dystrophin were at the epicenter of a revolution in human genetics 25 years ago, defining the birthplace of positional cloning.[4] We now recognize that this distinction was partially related to the extraordinary size of both the gene and its product, responsible for the high mutation rate that facilitated the genetic analysis but complicating the development of gene-based therapies. Measured from its promoter to the polyadenylation site at the 3′ end, the DMD gene is a staggering 2.4 megabases in length (11,057 base pairs complementary DNA), the longest gene fully characterized to date. If it were to be used in its unadulterated form as a molecular therapeutic, its molecular weight would be approximately 1.6 GDa and would require transfer to cells representing almost half of the body mass.

It was later recognized that much of the protein's 427-kDa molecular weight was attributable to 24 spectrin-like repeats.[5] With the discovery that a naturally occurring mutation in the mouse provided a convenient animal model,[6] it was possible to test by gene transfer the hypothesis that full-length and internally truncated versions of dystrophin might ameliorate the disease process.[7,8] Initially this research was guided by genotype–phenotype correlation related to the milder allelic form of disease at the dystrophin locus, Becker muscular dystrophy, in which patients are still ambulatory into adulthood.[9] However, in 2000, a team led by Xiao Xiao showed that an adeno-associated virus (AAV) vector could accommodate a microdystrophin retaining only five of the original 24 spectrin-like repeats and only the dystroglycan-docking half of a unique C-terminal domain.[1] Initial tests of this idea appeared promising after local intramuscular injections in mice and gained momentum after the eventual demonstrations of therapeutic systemic gene transfer in mice using pseudotyped vectors in serotypes 1 and 6 (refs. 10, 11).

Based on data such as these for preclinical efficacy (and data from other clinical studies), the US Food and Drug Administration authorized a phase I clinical study of intramuscular injection of an AAV serotypes 2 and 5–cytomegalovirus (AAV2.5-CMV) microdystrophin into the biceps muscle of six subjects with DMD.[12] The immunogenicity of the dystrophin transgene product in deletional-null recipients had been previously reported[12] but that paper left unaddressed the widely anticipated question of vector capsid immunogenicity in view of other preclinical and clinical studies.[13] Importantly, the vector capsid chosen for this study was an engineered chimera designed to gain the improved efficiency of AAV1 yet retain most of the protein sequence of AAV2. Additionally, these changes may circumvent some of the immune problems previously demonstrated for naturally occurring AAV vectors. In this issue, Bowles *et al.*[2] address some questions that can be reasonably answered by studying peripheral blood samples from this limited group of patients, as outlined below.

To put the accompanying article by Fan *et al.*[3] into perspective, it is worth revisiting an inconvenient truth about predictions on experimental scale in animal studies. The problem was first brought to public attention by J.B.S. Haldane, whose scaling concepts referred to as "Haldane's principles" address the observation that blood-vessel walls are stronger in larger animals,[14] reflecting the hemodynamic effects of gravity at greater body mass. Regarding vector biodistribution in gene therapy, the distinction between murine and canine models increases with age, as does the size discrepancy. AAV serotypes

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