False-Positive HIV PCR Test Following *Ex Vivo* Lentiviral Gene Transfer Treatment of X-linked Severe Combined Immunodeficiency Vector

To the editor:

An increasing number of clinical trials of *ex vivo* gene transfer into hematopoietic stem cells (HSCs) involve HIV-derived lentiviral vectors (LVs) to treat patients with monogenetic blood[1] and metabolic disorders,[2,3] cancers,[4] and infectious diseases, including HIV infections.[5–7] We have begun a LV-mediated *ex vivo* CD34$^+$ HSC gene transfer study in which older children and young adults are treated for X-linked severe combined immunodeficiency (X-SCID). These patients have significantly impaired immunity despite parental-source haplo-identical bone marrow transplant in infancy, which leads to recurrent infections[8] (Institutional Review Board–approved National Institutes of Health (NIH) Protocol 11-I-0007). Preliminary results from this study have been presented in abstract form.[9] Our LV is self-inactivating and uses a short internal promoter derived from the human elongation factor alpha (EF1α) gene to express a codon-optimized human γc therapeutic gene. The vector also contains a 400–base pair insulator derived from the chicken β-globin gene (CL20i4-EF1α-hγcOPT).[10–12]

We have enrolled and treated two young adult men with X-SCID (both aged 24 years at the time of their treatment) who are alive and stable without adverse events at 1 year and at 4 months since treatment, respectively. Subjects must be HIV-negative at study entry. Because our protocol includes subjects who are sexually mature, the virus screening and monitoring procedures in the NIH protocol include periodic tests for HIV infection. Monitoring their HIV status following gene transfer employs a widely used molecular assay for detection of HIV RNA by polymerase chain reaction (PCR): the Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, v2.0 (hereafter called the Roche COBAS AmpliPrep HIV-1 test). Here we show that in both subjects, this reverse transcription PCR test for HIV gave positive results early after gene transfer. Despite extensive search for presence of wild-type infectious HIV-1 or vector-derived replication-competent virus, none was detected. Our data demonstrate that the Roche COBAS AmpliPrep HIV-1 test gave false-positive results for our two patients; this finding has important social, economic, and informed-consent implications for the subjects of other LV-based clinical studies.

As stated earlier, subjects must be HIV-negative to participate in our protocol. Subjects with X-SCID lack B-cell function, do not produce endogenous antibodies, and require frequent treatment with intravenously or subcutaneously administered pooled human gamma globulin. Their impaired B-cell function and accompanying failure to produce specific antibodies precludes detection of antibodies to HIV to determine their HIV infection status. Testing for HIV infection must rely on molecular assays; we used the Roche COBAS AmpliPrep HIV-1 test. This assay has a detection limit of 20 copies per milliliter of blood and is quantitative up to 1 million copies per milliliter. In a follow-up test on subject 1 performed 6 months after the infusion of the LV-modified cells, the assay detected 259 copies of HIV RNA per milliliter. This patient did not have any risk factors for HIV infection, and there was no clinical or laboratory evidence of infection. Subject 2 also had a negative HIV RNA test before treatment, but at 2 and 3 months following infusion of his LV-modified cells, his blood tests were also positive, at 11,000 and 19,890 copies per milliliter, respectively (**Supplementary Figure S1** online). To determine if residual vector components were carried over from the initial CD34$^+$ HSC transduction, we used the Roche COBAS AmpliPrep HIV-1 test, a sensitive reverse transcription PCR assay, to measure HIV RNA load on archived serial samples from subject 1 (**Supplementary Figure S2** online) and on fresh blood samples. These assays revealed increasing levels of HIV RNA over time, a trend that is contrary to the decline that is expected for carryover of vector components, but that is consistent with the increasing polyclonal marking in B cells and T cells following the LV gene transfer treatment in this subject. Of note, all fresh samples (**Supplementary Figure S1**) were processed at the NIH Clinical Center Laboratory, which is only authorized to process clinical samples. Thus, the archived plasma samples from subject 1 were measured at an independent research laboratory that runs the same assay; however, differences in laboratories can give rise to variation in the measured levels of viral RNA (**Supplementary Figure S2**).

An extensive search for the presence of full-length wild-type HIV RNA sequences, using multiple sets of nested primers to probe cells and plasma from subject 1, did not detect any evidence of the presence of complete genomic RNAs corresponding to HIV-1 types A, B, C, D, F, and G.[13–15] PCR assays for the HIV proviral DNA (Roche COBAS AMPLICOR HIV-1 MONITOR Test, v1.5) and p24 enzyme-linked immunosorbent assay (ELISA) performed on subject 1 were both negative. Standard surveillance for replication-competent lentiviruses (RCLs) based on molecular assays testing for vesicular stomatitis virus-G envelope sequence were or will be performed at 3, 6, 9, and 12 months, then yearly thereafter according to US Food and Drug Administration recommendations, and all the tests that have been performed for subject 1 have been negative. However, because of the initial positive results with the Roche dual-target HIV-1 test, we used a very sensitive co-culture cell culture assay to attempt to detect infectious replication-competent virus. Patient mononuclear cells were incubated with C8166-45, a cell line that is permissive for HIV infection and replication. Supernatants from this co-culture were then applied to naive C8166-45 cells, and the presence of RCL was evaluated by a p24 protein ELISA–reverse transcriptase assay.[16] The assay can detect infectious virus present at 12.5 pg/ml of p24 or 100 reverse transcriptase molecules per microliter. To improve the sensitivity of detection of replication-competent virus, if any was present, purified CD4 T cells from subject 1 were expanded using

a cocktail of interleukin-2, phytohemagglutinin, antibody to CD3, and CD8 cells for 21 days.[16,17] No RCL was detected in samples of the patient's blood with either assay. This assay would also have detected wild-type HIV.

Next, we sought to determine whether positive results obtained with the Roche COBAS AmpliPrep HIV-1 RNA PCR assay were due to the primer pairs used in the assay detecting sequences present in the vector. Healthy donor CD34 cells transduced with the clinical vector CL20i4-EF1α-hγcOPT were spiked into a sample of healthy donor's blood before running the Roche COBAS AmpliPrep HIV-1 RNA PCR assay, which revealed millions of copies per milliliter. This showed that the assay detects sequences in the vector used in this trial. Because of the proprietary nature of this assay, we were unable to obtain information from the manufacturer about the specific sequence detected. To identify the vector components that may have been detected by this assay, a quantitative real-time PCR was performed on subject 1's serum using primers specific for the HIV codon–optimized gagpol sequences present in the LV production system, or packaging sequences ("psi") in the therapeutic vector genome. Consistent with a lack of helper sequence transfer, the gagpol sequence PCR result was negative and the vector psi sequence PCR was positive. Efforts to specifically identify the amplified products from the Roche assay are ongoing.

The detection, as a false positive, of clinical lentiviral vectors by a conventional high-sensitivity HIV test has potentially important social, economic, and informed-consent consequences. Given the increasing role of LVs in gene therapy, clinicians should be aware that false positives can arise during the testing of samples from patients who have been treated with these vectors. In patients with a higher risk for HIV infection, definitive tests capable of distinguishing therapeutic vectors from bona fide wild-type HIV should be used. THE AMPLICOR HIV-1 DNA test and the p24 ELISA assay performed by Mayo Medical Laboratories were both negative with samples from our patients. Finally, consent documents should include language indicating that some HIV tests may become positive, and patients should receive counseling as to the likely permanent change in their apparent status in certain commonly used HIV tests.

SUPPLEMENTARY MATERIAL

Figure S1. Plasma HIV RNA load measured by Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 Test of two subjects after receiving lentivirus-mediated gene transfer into autologous hematopoietic CD34⁺ stem cells. 1, Subject 1; 2, subject 2.

Figure S2. HIV RNA load in archived frozen plasma samples from subject 1 using the Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 in an independent laboratory.

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