

Measuring covert HIV replication during HAART: the abundance of 2-LTR circles is not a reliable marker

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The development of highly active anti-retroviral therapy (HAART) has conferred great benefits to patients, but treatment failures are common. This leads to the question of what is going on with the virus during the period of successful control. Is there ongoing 'covert' viral replication, allowing potential accumulation of drug resistance mutations, or is replication effectively abolished, requiring other explanations for viral breakthrough? Crucial to understanding this issue is the development of methods for detecting low-level viral replication during successful HAART. A recent study suggested that the abundance of 2-long terminal repeat (LTR) circles was a convenient marker [1]. This study suggested that 2-LTR circles, once formed in cells, were quite short-lived. Thus if 2-LTR circles could be detected in well suppressed patients, then they must indicate recent new infection resulting from ongoing replication. Unfortunately, more recent work, including a study by Brussel *et al.* in this issue of *AIDS*, indicate that this marker is in fact unreliable and should not be used in future studies. The data supporting the initial proposal and the subsequent reassessment are summarized below.

Many studies have characterized the forms of retroviral complementary DNA (cDNA) present in cells after infection [2]. The immediate product of reverse transcription is a linear cDNA molecule which is the

substrate for integration to form the provirus which supports subsequent viral replication. The viral cDNA can also undergo other transformations which yield dead-end products (see [3] and references therein). The viral cDNA can be circularized by: ligation of the ends of the viral cDNA by the host non-homologous DNA end-joining pathway [4] to yield 2-LTRs circles; homologous recombination between LTR to yield 1-LTR circles [3]; autointegration, in which the integration reaction uses the viral cDNA itself as an integration target yielding a rearranged circle. Other more complex pathways can also yield aberrant forms, and much of the viral cDNA is in fact degraded after synthesis without doing anything (at least in high titer infections of cultured cells [5–7]).

The idea that 2-LTR circle abundance might be useful as an *in vivo* marker came primarily from Sharkey *et al.* [1], who began by studying experimental infections of cultured cells. They infected cells with HIV-1, added a reverse transcriptase inhibitor to prevent viral spread, then monitored the abundance of viral cDNA forms produced by a PCR assay. In their cultures the numbers of 2-LTR circles per cell declined with time, suggesting to them that the 2-LTR circles within cells were being degraded at a high rate—within 48 h the abundance of 2-LTR circles apparently declined by 90%.

See also p645 and p679

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Two-LTR circles can be readily measured in clinical samples. If they are quite short lived, then detection of 2-LTR circles would indicate new infection, providing a convenient assay. Such analysis was applied to samples from patients who had undergone long term successful HAART, and 2-LTR circles were detected in the peripheral blood mononuclear cells (PBMC) of 76% of the patients. The inference was that this indicated ongoing covert replication despite successful HAART, as the detection of 2-LTR circles appeared to require *de novo* infection in patients with good viral control. Following this report, measuring the presence of 2-LTR circles has come into use as a marker for ongoing covert replication in clinical studies (see, for example [8,9]).

The idea began to unravel when the stability of 2-LTR circles was looked at more closely. It seemed odd from the start that 2-LTR circles should be unstable, as other extrachromosomal circular DNAs were known to be quite stable, such as the TREC circles generated during DNA rearrangements at the T-cell receptor locus [10,11].

In 2002, Pierson *et al.* reported a very careful reinvestigation of 2-LTR circle stability, in which they carried out experimental infections of cultured cells with HIV, blocked further infection with a protease inhibitor, and quantified both the rate of decrease of 2-LTR circles and the rate of cell division [12]. They found that 2-LTR circles per cell did decline with abundance over time, but at a rate that could be fully accounted for by division of the infected cells. Evidently a 2-LTR circle – once formed – persisted indefinitely. Another study of cultured cells reached a similar conclusion. Butler *et al.* also found that 2-LTR circles declined in abundance about as expected by the rate of cell division and also added another experiment. They found that when cell division was stopped with aphidicolin, the abundance of 2-LTR circles per cell did not change over time [13]. Together these thorough studies indicate that 2-LTR circles are in fact quite stable, dissolving the premise of the Sharkey analysis.

The Brussel *et al.* study in this issue of AIDS extends these findings in the context of clinically relevant models of HIV infection. Brussel *et al.* carried out a longitudinal study of patients on successful HAART and found scarcely any decay of 2-LTR circles over time in patients' PBMC. Assays of plasma viral RNA levels and infectious cell frequencies, in contrast, showed the expected decline with effective therapy. In five of the eight patients on HAART studied, 2-LTR

circles showed an unmeasurably long half-life. In the other three patients, the half-lives were variable but were never less than 3 months. Analysis of 2-LTR circles in PMBC cultured from HIV-infected patients supported similar conclusions. Thus 2-LTR circle frequencies were quite stable under successful HAART and discordant with measurements of viral RNA or infectious cells, which fell as expected.

It is unclear how Sharkey *et al.* obtained their anomalous results. Possibly there was more loss of 2-LTR circles due to death of infected cells in their initial cultures than they realized. Whatever the explanation, it is safe to conclude that recent data weighs against the idea that 2-LTR circles provide a reliable marker of *de novo* infection during successful HAART.

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