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Review

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Ludwik Hirszfeld Memorial Lecture: HIV-1 reservoirs: major molecular obstacles to viral eradication*

Roger J. Pomerantz

Center for Human Virology and Biodefense, Thomas Jefferson University, Philadelphia, PA 19107, USA

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Summary

Over the last 18 years of study in one of our laboratories, we have observed the development of residual disease and latent reservoirs as major problems in the long-term therapy of HIV-1-infected individuals on highly active antiretroviral therapy (HAART). It was shown in the early 1990's that HAART, as it is presently configured, is unlikely to lead to viral eradication due to several mechanisms of viral persistence. The two general mechanisms involved with persistence during HAART include low-level residual, cryptic replication and proviral latently-infected cells. As such, these are key areas of potential study for depletion and, hopefully in the future, eradication of residual disease in patients on suppressive HAART. To deplete these residual disease mechanisms will require multipronged approaches. These will include induction of HIV-1 latent proviruses, suppression of residual viral replication and destruction of long-lived cellular sanctuaries, such as tissue-bound macrophages.

Key words: **HIV-1 • eradication • reservoirs • latency**

* Dedicated to Professor Ludwik Hirszfeld on the 50th anniversary of his death.

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Author's address: Roger J. Pomerantz, M.D., F.A.C.P., Professor of Medicine, Biochemistry and Molecular Pharmacology, Director, Division of Infectious Diseases and Environmental Medicine, Director, Center for Human Virology and Biodefense, Thomas Jefferson University, 1020 Locust Street, Suite 329, Philadelphia, PA 19107, USA, tel.: +1 215 503-8575, fax: +1 215 503-2624, e-mail: Roger.J.Pomerantz@jefferson.edu

INTRODUCTION

The era of highly active antiretroviral therapy (HAART) now allows both clearer investigations of classical questions in human retrovirology, as well as the generation of new clinical problems[41, 32, 35, 61]. Mechanisms of: 1) viral latency plus, 2) hidden or "cryptic" viral replication can also now be addressed without the "noise" of active virally-producing cells and high levels of cell-free virions[41], as virally-suppressive HAART "unveils" viral persistence. Furthermore, approaches toward possible viral eradication or at least long-term remission can be rationally studied. Nonetheless, it is critical to note that true viral eradication may be extremely difficult for many reasons (see below), including the finding that cells other than those in the immune system (e.g. central nervous system, kidney, heart, and others) also appear to be infected at low-levels with HIV-1 *in vivo*[19, 81, 110].

HIV-1 LATENCY AND PERSISTENCE

Interest in retroviral latency or, more generally, persistence preceded the AIDS epidemic. Although retroviral latency was initially studied in avian retroviruses, the understanding of these processes *in vivo* has significantly increased in recent years, utilizing HIV-1 as a model[59]. Viral latency is a general property of many viruses, including herpes viruses. Retroviral latency is defined as integrated provirus with no active transcription. Low-level chronic yet productive viral expression best characterizes persistent or cryptic replication. In the case of HAART, virus replication from previously infected cells is not fully ablated. Viral persistence takes place after initially successful virally-suppressive therapy or effective anti-viral immune responses in the infected host. Such cryptic viral replication may also continue in immunologically privileged sites, such as brain and testes. As well, transcriptionally active but non-productive viral infections (i.e. certain viral mRNA are expressed but not intact virions) may also occur (see below). Many studies have demonstrated that "clinical" HIV-1 latency, when defined as no viral expression in an entire untreated infected individual, does not exist at the host level in any stage of disease[23, 62, 68]. In the great majority of HIV-1-infected individuals, some cultivable virus may be recovered at all stages of disease[23, 62, 68]. Nevertheless, data demonstrate that in the HIV-1-infected individual, some cells contain proviral DNA but express little or no viral RNA and produce few or no virions[23, 64, 65]. In addition to post-integration latency, various states of pre-integration HIV-1 latency have been described both *in vitro* and *in vivo*[5, 99–101, 107, 108]. As such, latency at a cellular level exists *in vivo*, and the number of latently-infected cells may vary based on the stage of disease. As HIV-1 infects, *in vivo*, $CD4^+$ T cells, monocyte/macrophages, and other non-immune-based cells[42–44], the virus may maintain cellular latency by different mechanisms in differing cell types. This possibility presents important research opportunities in further elucidating mechanisms of HIV-1 latency *in vivo*. HIV-1 replicates at a rapid rate in infected-individuals, with a virion half-life in plasma of hours[66]. The vast majority of this viral replication (over 99%) occurs in activated and productively-infected $CD4^+$ T cells in the peripheral blood and lymphoid tissue. Analysis of plasma viral decay characteristics in patients initially treated with potent combinations of anti-retroviral drugs demonstrates a rapid first phase decay of productively infected cells, a second phase decay of long-lived cells (possibly from tissue-bound macrophages), and then a third phase decay of persistently infected cells (e.g. resting $CD4^+$ T cells)[66]. Effective HAART blocks the virus from infecting healthy $CD4^+$ T cells and the initial drop in HIV-1 levels in the peripheral blood reflects the life-spans of cells that were infected before treatment was initiated. Based on these complex viral and cellular kinetics with slow decay of viral infected cells in several phases, most HIV-1-infected individuals would require complete suppression of viral replication for years if one were even going to consider viral eradication. However, this goal is hindered by several factors, including less than optimal treatment, HIV-1 infection in immunologically-privileged sites, and partial drug sanctuary compartments *in vivo* (see below). Recent studies have also suggested that monocytes of HIV-1-infected patients on virally-suppressive HAART may harbor low-level replicating virus[98, 112]. These cellular sites of residual disease may also include virions sequestered in multivesicular bodies of macrophages[30, 53, 89].

RESIDUAL HIV-1 PROVIRUS *IN VIVO* DURING HAART

Persistently-infected, non-activated $CD4^+$ T cells have been demonstrated in the peripheral blood of HIV-1-infected individuals, after treatment effectively suppresses most productive viral infection[9]. Replication-competent viruses can be recovered from these proviral-positive cells after $CD8^+$ T cell depletion *in vitro*[13, 26, 102]. The vast majority of these viruses are CCR5-tropic[69]. In addition, this cell reservoir is established soon after primary HIV-1 infection[10], and can be activated by pro-inflammatory cytokines *in vitro* and potentially *in vivo*[11] (Fig. 1). Suppressive HAART initiated before primary HIV-1 seroconversion and during perinatal HIV-1 infection was also shown to be unable to halt the development

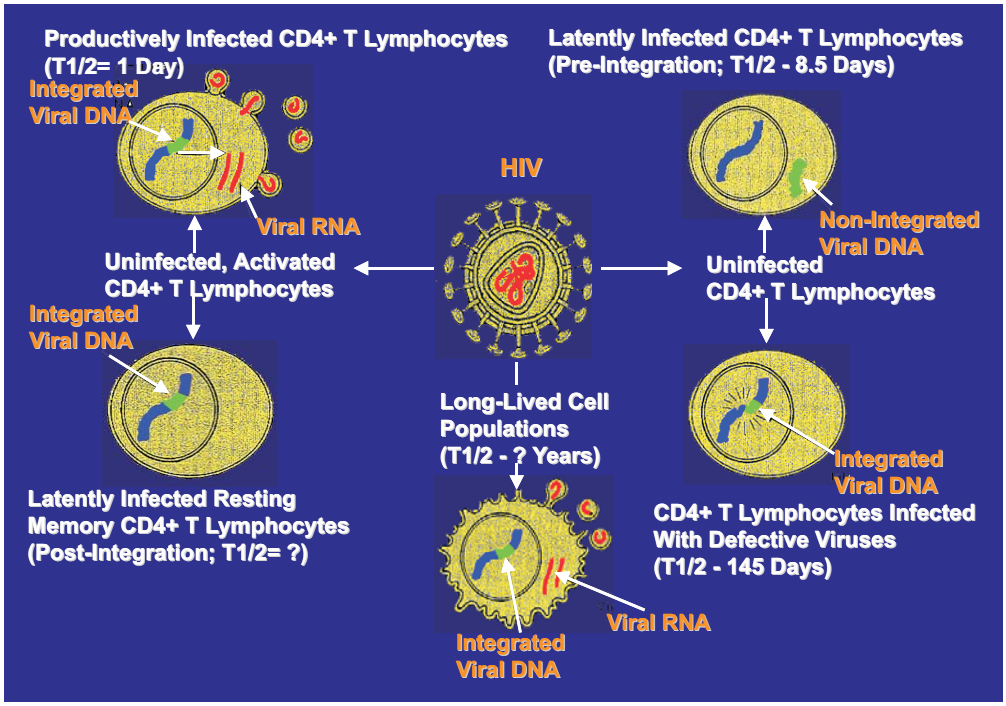

Figure 1. HIV-1: cell interactions

of this replication-competent virus reservoir in CD4+ T cells[10, 67, 71].

Most viruses isolated from resting CD4+ T cells from the peripheral blood of patients with undetectable levels of viral RNA in plasma have few mutations that confer anti-retroviral drug resistance[106]. As resistance mutations in the reverse transcriptase (RT) and protease genes of HIV-1 are correlated with on-going viral replication despite treatment[33, 54], this finding suggests that these viral strains may represent "archival" species from a time before treatment. Data from the above studies demonstrated that although defective proviruses accumulate in CD4+ T cells *in vivo* (i.e. "viral graveyard sequences")[92], replication-competent provirus still exists in resting CD4+ T cells, which may hinder attempts at reducing the viral reservoir and re-seed the body with virus if HAART is discontinued. These possibilities, therefore, confound our attempts for pharmacologically-mediated viral eradication. In addition, recent studies have also demonstrated replication-competent HIV-1 in peripheral blood monocytes of patients on virally-suppressive HAART[98, 112]. This latent reservoir in resting CD4+ T cells is also found in infants after vertical transmission[90] and may also be important as an archive for drug resistant HIV-1 quasi-species[111]. Our laboratories have recently demonstrated, though, that peripheral blood dendritic cells are not a major reservoir for residual HIV-1 in patients on virally-suppressive HAART[60]. Finally, it is important to note that recent data have suggested that much of the latent HIV-1 provirus may resides in HIV-1-specific CD4+ T lymphocytes, and therefore may reactivate when these cells are exposed to HIV-1-specific antigens[3, 20, 49].

Active replication of HIV-1 during HAART

Cell-associated virus

Persistently-infected CD4+ T cells containing non-defective but quiescent HIV-1 proviral DNA and low-levels of viral replication could account for the replication-competent virus within the peripheral blood CD4+ T cells and seminal cells of infected individuals on HAART[110]. Viral replication could occur at such low-levels that the virus would not be detectable in peripheral body fluids with standard clinical assays. Low-level productively infected cells might infect small numbers of target cells, in the surrounding cellular microenvironment.

In certain studies[29], and confirmed by another group[96], measures of the "footprints" of persistent viral replication were used to evaluate HIV-1 mRNAs species and HIV-1 long-terminal repeat (LTR) DNA circles, which are formed by self-ligation of proviral DNA by cellular nuclear ligases after transport of the viral pre-integration complex to the nucleus. LTR-DNA circles were demonstrated in the CD4+ T cells of most patients on suppressive HAART. The LTR-DNA circles along with quasi-steady state levels of HIV-1 mRNA demonstrate the

existence of low-level viral replication at some time in the recent past. Although a short *in vivo* half-life for HIV-1 2-LTR DNA circles has been suggested[96], this is now very controversial[7, 70], and thus 2-LTR DNA circles may be most useful in assaying cytoplasmic to nuclear transport of the viral pre-integration complex in newly-infected cells.

A further complexity towards characterizing HIV-1 persistence during HAART was also demonstrated[29], as certain patients had cells with multiply-spliced RNA out of proportion to unspliced viral RNA, in agreement with *in vitro* models[6,57,85]. As multiply--spliced viral RNA encodes regulatory proteins, while unspliced RNA encodes structural proteins, this pattern suggests transcriptionally-active but non-productive infection. These patterns of viral RNA species expression may be based on the state of host cell activation. Thus, several forms of cryptic replication might "reset the virological clock" by infecting previously uninfected cells in localized microenvironments. Recent data also suggest that active, low-level replication is a dynamic steady state in most patients on HAART[28, 39]. This is interesting in light of new data demonstrating HIV-1 integration in active genes, both utilizing cell-lines and in resting $CD4^+$ T lymphocytes *in vivo*[36, 42, 94].

Cell-free virus

Using a laboratory-based, super-sensitive RT-PCR assay which can quantify at least 5 copies/ml of plasma viral RNA[110], low but detectable levels of virus were demonstrated in all HIV-1-infected patients studied with clinically undetectable levels of plasma HIV-1 RNA (i.e. less than 50 copies/ml)[19]. Of importance, this on-going viral replication may infect cells in local sites, as well as cells at a distance within the body[39, 110]. Of note, though, the quantities of defective virions in residual plasma HIV-1 is still unknown[92] and remains important for understanding possible viral spread within an infected host. Recent data suggest, though, that residual viral replication during virally-suppressive HAART may not lead to resistance[43].

Some patients with less than 50 copies/ml of blood plasma viral RNA on HAART still have low-level bursts of viral replication leading to more than 50 copies/ml in transient "spikes"[21, 104], which may carry risks of viral resistance evolution, although this was not demonstrated in a recent study[37]. The mean decay half-life of latent replication-competent proviral reservoirs is longer in patients with transient plasma viral RNA spikes, as compared to those patients who consistently maintain plasma HIV-1 RNA levels below 50 copies/ml[88]. It will be important to determine whether these viral spikes are due to insufficient plasma drug levels and/or depressed drug penetration into unique reservoir sites, or just continued HIV-1 replication in the lymphoidal tissues, as well as peripheral blood cells.

The findings that 60–70 years of suppressive HAART would be necessary to possibly allow latent reservoir eradication, were suggested to be due more to the slow decay of a truly latent proviral state rather than to on-going viral replication[25, 97]. The other study mentioned above might lead to some optimism, as a mean half-life of approximately 6 months of the latent viral reservoir in patients who have no spikes above 50 copies/ml of plasma viral RNA on HAART may allow viral eradication in this very select group of patients in a shorter time-period[88]. Nonetheless, once again this possibility assumes that even in these patients there exists no other viral reservoir(s) in peripheral blood, lymphoid tissue, or other solid organs (such as the central nervous system)[59], and that sub-clinical viral replication did not occur. Our laboratories have been interested and actively studying HIV-1 latency and residual disease for over 18 years. Our labs were one of the first groups to address the molecular mechanisms of HIV-1 latency in cell culture[85]. The viral regulatory protein Rev, which rescues unspliced HIV-1-specific RNA from the nucleus of infected cells, may be involved in certain forms of viral persistent infection. Our laboratories' work and other studies have suggested that Rev-negative or Rev-impaired viral phenotypes may be demonstrated in persistently-infected cells both *in vitro* and *in vivo*[6, 38, 57, 85]. This is characterized by multiply-spliced viral mRNAs and their protein products (i.e. Nef, Tat, and Rev) being expressed out of proportion to unspliced viral RNA (i.e. genomic RNA and *gag-pol* mRNA)[29]. We have had a long-standing interest in the effects of Rev on HIV-1 latency and persistence[59], and previously demonstrated that an intracellular threshold exists for Rev function. Low-level HIV-1 transcription can lead to a subthreshold level of Rev[84], and thus viral persistence.

INTENSIFICATION AND STIMULATION THERAPY TO ERADICATE LATENT VIRAL RESERVOIRS

Some current clinical strategies approach the challenge of eliminating low-levels of on-going replication-competent virus through an intensification protocol that relies on administration of additional viral inhibitors apart from the combination of drugs that typically constitute HAART. Candidate drugs used in intensification with HAART include hydroxyurea (HU), although this should only be used in intensifi-

cation research protocols based on its toxicity especially in combination with the RT inhibitor, didanosine (ddI)[38]. Hu inhibits the cellular enzyme, ribonucleotide reductase, thereby decreasing intracellular deoxyribonucleotide pools which indirectly impedes viral RT activity[52]. Recently it has also been demonstrated that mycophenolic acid, together with the RT inhibitor, abacavir, may be similarly effective at blocking residual HIV-1 replication as ddI and Hu[8]. Of note, a small cohort of patients on ddI and hydroxycarbamide did not rapidly experience viral rebound after discontinuation of HAART[102].

There is some evidence that eventually long-term remissions or eradication of residual virus may be achievable with clinical approaches referred to as stimulation therapy or more precisely immune activation therapy (IAT). This strategy aims to increase the turnover rate of the latent viral reservoir through activation of infected cells, which comprise this reservoir, in order to promote cell death and accelerate viral clearance. Prime candidates for use in IAT include the cytokine interleukin (IL)-2, alone or in conjunction with OKT3, as well as the myeloid cell activator, interferon (IFN)-γ[8, 12, 27, 50]. T cell activation is mediated directly through the T cell receptor (TCR) using the antibody, OKT3, which binds the TCR complex[22]. IL-2, in conjunction with OKT3, enhances OKT3-mediated T cell activation and proliferation although IL-2 alone can function in an autocrine fashion by interacting with the IL-2 receptor which is also up-regulated during T cell activation[56, 95].

In a study in which three patients on virally-suppressive HAART were treated with OKT3, there developed significant severe side-effects, including hypotension and adult respiratory distress syndrome[87]. This was likely due to the use of multiple, high-dose (5 mg) infusions of OKT3. We have completed a clinical study in which lymphostimulatory doses (400 μg), rather than lymphoablative doses (5 mg) of OKT3 were utilized in virally-suppressed HIV-1-infected individuals on HAART[46]. These patients only had modest side-effects, including headache and low-grade fever. This suggests that OKT3 can be given safely to HIV-1-infected individuals on HAART (see below for details).

The proof of concept for IAT has been successfully demonstrated *in vitro*[11]. The clinical use of viral stimulatory agents, such as OKT3 and IL-2, or IFN-γ, to activate viral expression from persistently infected cells to induce cell death, has also been reported subsequently[8, 12, 27, 50]. Viral spread is a diminished concern using these stimulatory compounds when administration is performed in the presence of HAART. Indeed, some patients on suppressive HAART, and treated with intermittent administration of IL-2 alone, exhibited decreased or even undetectable levels of resting $CD4^+$ T lymphocytes containing replication-competent virus[12]. Consistent, long-term benefits are crucial, and other reports, while showing an overall reduction in the HIV-1--infected T cell reservoir by IAT, have not demonstrated complete viral ablation[22, 27, 50]. The efficacy reported for such clinical interventions are unknown, with regard to the degree of success in reducing or eliminating latent virus reservoirs. Some treatments may also lead to a relatively long-lasting depletion of patient $CD4^+$ T lymphocytes in peripheral blood and lymph nodes[101]. Since clinical interventions using IAT are few in number, the results from additional efforts of this type are ultimately needed to ascertain whether IAT represents a clinically feasible approach to achieve the goal of long-term remissions off HAART, or complete viral eradication.

Of note, recent studies in our laboratories have demonstrated that IL-7 is a potent inducer of HIV-1 from latency *ex vivo*, using $CD4^+$ T cells from patients on virally-suppressive HAART. This cytokine may warrant clinical analysis in HIV-1 residual disease (manuscript submitted).

Potential adverse effects of IAT

Although HIV-1 eradication remains an important goal in this epidemic, in both the developed and the developing world, there are potential down-sides to IAT in patients. Firstly, the release of pro-inflammatory cytokines, as shown in an initial OKT3 and IL-2 trial[87], can lead to cytokine-induced sequelae of adult respiratory distress syndrome and hypotension. As such, approaches which release large quantities of pro-inflammatory cytokines may contraindicated clinically, although it may assist in induction of latent HIV-1 provirus. As well, the use of low-dose OKT3 and IL-2 may lead to an aseptic meningitis syndrome which, although not shown to be clinically serious, is nevertheless uncomfortable for these patients over several days[46].

Finally, there is also the theoretical possibility that there may be spread of HIV-1 to other cells in the microenvironment surrounding originally latently--infected cells during IAT. As such, this may "reset the virological clock" with new cells being infected and able to produce virus, albeit at low levels, in patients on virally-suppressive HAART after IAT. Each of these potential problems must be evaluated closely in all clinical protocols, and some potential

Table 1. Approaches used for immune activation therapy in patients with HIV-1

Regimen	Potential mechanisms of action	Evidence of potential efficacy	Reference(s)
IL-2	direct T lymphocyte stimulation	increased latent T lymphocyte proviral reservoir *in vivo*	12
IL-2 and OKT3	direct T lymphocyte stimulation plus release of proinflammatory cytokines	increased HIV-1 replication from latent reservoirs *in vivo*	87, 27, 101
IL-2 and IFN-γ	direct T lymphocyte and macrophage stimulation plus release of proinflammatory cytokines	stimulated HIV-1 replication *in vivo* from T lymphocytes of patients receiving virally suppressive HAART	50
IL-2 and TNF-α	direct T lymphocyte and macrophage stimulation and release of proinflammatory cytokines	stimulated HIV-1 replication in cultures of PBMCs from patients receiving virally suppressive HAART	11

complications may also be modeled in *in vitro* systems.

In summary, HAART has led to dramatic, positive changes in the clinical management of HIV-1 infection. However, it is not likely that these regimens will lead to elimination of virus in their present forms for the majority of HIV-1-infected-individuals, at least within a clinically reasonable frame of time. Many questions remain open regarding the nature of HAART-persistent viruses and the cell and tissue-types in which they may be sequestered. For instance, do patients on virally suppressive HAART generally bear single or multiple persistent viral species? Are these viruses sequestered in single or multiple cell-types?

The activation of specific subsets of patient cells *in vitro*, with a variety of agents apart from phytohemagglutinin (PHA) and IL-2 (used in many T cell activation and proliferation studies in cell cultures), is warranted, as compounds such as phorbol myristic acid and prostratin (see below) likely induce viral expression through a pathway distinct from PHA/IL-2. This is important as latent virus may reside within multiple cell-types, and each could have a heterogeneous response in terms of viral expression induced by individual activating agents. This possibility supports the use of a more diverse panel of stimulatory agents in IAT studies as well, since certain mitogens, such as PHA, are restricted to use *in vitro*, and are not viable candidates for *in vivo* clinical application. Table 1 reviews the approaches used for IAT to date. Of note, each clinical trial, with different IAT approaches, demonstrated reduction but not eradication of residual HIV-1 disease.

A recent study in $SCID_{hu}$ mice has suggested that latent HIV-1 is stimulated via two major intracellular signaling pathways, via PKC or NF-AT[4]. Of note, MAP kinase and histone deacetylase did not induce viral expression. Nevertheless, this system may be somewhat different from proviral latency in human cells from patients on HAART, as the $SCID_{hu}$ cells are mainly naive rather than memory $CD4^+$ T lymphocytes with latent provirus, as found in humans. Thus, the $SCID_{hu}$ model may be more resistant to viral up-regulation, especially with agents that induce HIV-1 by targeting chromatin, which can induce latent virus from human cells[14]. These findings, though, may lead in the future to further rational design of drugs to induce HIV-1 replication[34].

Finally, it is important to determine whether currently devised and clinically applied viral intensification and IAT protocols are definitively aiding in the elimination of the latent viral pool. Can modifications to current protocols enhance their efficacy? Such modifications to IAT could include use of additional stimulatory compounds, if warranted, or alternatively hybrid immunotoxins which are specific recombinant proteins that exhibit preferential targeting and killing of HIV-1-infected cells[1, 2, 24, 31, 34, 55, 63, 91]. Of note, clinical studies utilizing IAT with intensified HAART are ongoing in our Center and others. Additional stimulatory compounds would broaden the spectrum of cell activation which is linked to latent viral expression and these compounds, perhaps combined with novel immune-based therapies[40, 51], could accelerate elimination of the virus so that complete withdrawal from HAART is possibly attainable, for at least some HIV-1-infected individuals.

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