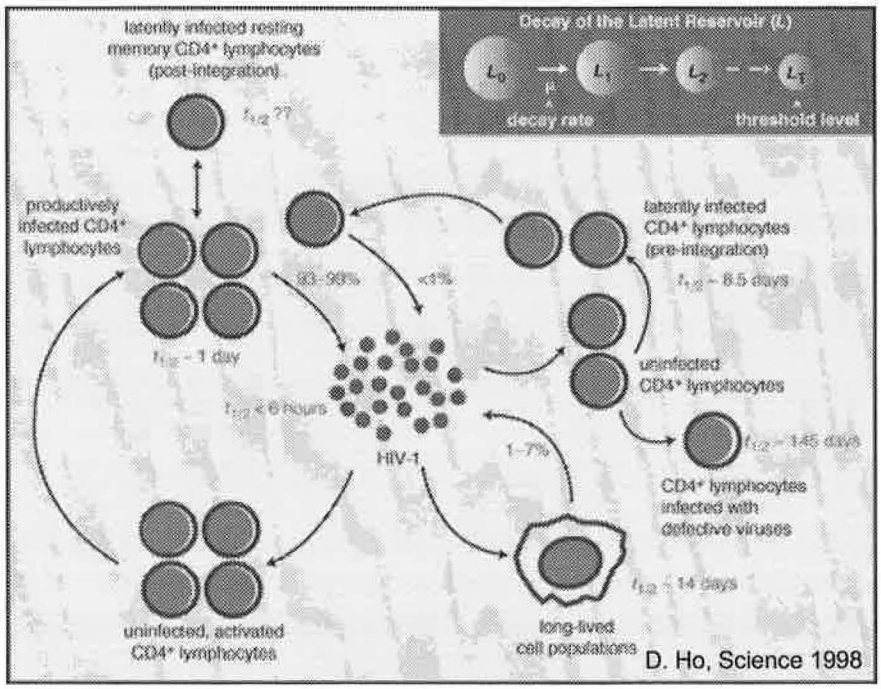


# Eradicating the latent reservoir of HIV: finding light at the end of the tunnel

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## Introduction

HIV-1 may be the most devasting zoonotic infection in human history, dwarfing the influenza pandemic of 1918. More than twenty years into the worldwide AIDS epidemic, the progress of medicine and society in coping with HIV can be viewed as spectacular in some regards, but inadequate in others. Drug therapy, known as highly active antiretroviral therapy (HAART), has made it possible to control viremia, allow reconstitution of the immune system, and arrest the progression of disease. However, the benefits of HAART are difficult to achieve and maintain. Therapy can fail due to the selection of drug resistant mutants without strict adherence to medication, or medication-associated toxicity can make therapy intolerable. When interrupted, virus contained in stable reservoirs rapidly rebounds and disease progression resumes [Blankson 2002].

Taking a more global perspective, tiny minorities of those with HIV infection worldwide enjoy the benefits of HAART. Although advances have been made recently towards developing infrastructures and expanding the availability of HAART, in the developing world therapy is still restricted to those with the most advanced disease. Due to the advanced stage of the epidemic in many countries, the economic and social impact of HIV is already certain to be devastating in the near-term. To allow control of the HIV epidemic over the next two decades, advances must be made simultaneously in the prevention of HIV infection, the prevention of disease in those who acquire infection, and, ultimately, the eradication of infection.

## Controlling the HIV Epidemic

It is nearly a cliché to say that a simple and effective vaccine capable of preventing infection by the many strains and recombinant species of HIV circulating in the human population is needed to control the HIV epidemic. The obstacles, both scientific and practical, to the attainment of this goal are considerable [Letvin 2004; Pantaleo & Koup 2004; Garber 2004]. These challenges are well illustrated by an anecdotal case reported by XXX et al.:

A recently HIV-infected volunteer enrolled in a vaccine trial. In this study, the volunteer initiated potent HAART within 6 months of the estimated date of HIV infection. After prompt and successful suppression of viremia, the volunteer received several doses of an experimental HIV vaccine. As per protocol, after the detection of a broadened HIV immune response following vaccination, the volunteer stopped HAART therapy. Off HAART viremia appeared to be well-controlled, and CD4 counts stable. The volunteer then apparently had an unprotected sexual encounter, acquiring a new strain of HIV. Despite prior exposure to the ultimate live virus "vaccine," a subsequent experimental vaccine, and the demonstration of a multi-epitope anti-HIV immune response. Not only did the volunteer acquire new HIV infection, but this infection was marked by high-level viremia and CD4 decline [xxxxx 2003].

Simpler and more nearly attainable advances in the prevention of HIV infection include the advancement of the science of behavioral prevention, and the development of

safe and effective microbicides and post-exposure prophylactic therapies that would allow those at risk, particularly women, to protect themselves. The urgency of this need is reflected in the number of research programs recently funded to test daily single-drug prophylactic antiretroviral therapy in high-risk populations such as commercial sex workers and men-who-have-sex-with-men. Investment is such approaches, unthinkable a short time ago, reflect the acknowledgment that even sexually transmitted diseases that are easily curable (e.g. syphilis) have proven difficult to control over decades using current approaches [see http://www.cdc.gov/hiv/PUBS/TenofovirFactSheet.htm].

Infected individuals, if they have access to medication, are left with an unappealing choice: advancing AIDS or lifelong HAART. Lifelong therapy is difficult to adhere to, and even relatively brief lapses in adherence can lead to drug resistance and treatment failure. Increasingly, multiple serious side effects are recognized with the long-term administration of HAART. These include hypersensitivity reactions, neuropathies, hepatotoxicity, pancreatitis, anemia, cardiovascular disease, hyperlipidemia, hyperglycemia, lactic acidosis, lipodystrophy, and type II diabetes [Carr 2003]. HAART is said to have transformed HIV infection into a chronic, manageable disease. However, the life expectancy of an HIV-infected individual under best available clinical management is likely shorter than an uninfected person's.

However, advances in the prevention of disease in those who acquire infection are foreseeable. Recent improvements in antiretroviral formulations now allow once a day combination antiretroviral therapy. Currently, seven of the 11 available reverse transcriptase inhibitors can be administered once a day. Five of the available protease inhibitors can be given once a day, most often when given with subtherapeutic doses of the protease inhibitor ritonavir that serves to slow protease inhibitor metabolism. Fixed-dose combination formulations have also decreased the number of pills that must be taken to receive HAART. Although not now available in the U.S., a joint venture between Merck and Gilead will bring to the international market a complete HAART combination product (tenofovir-emtricitabine-efavirenz) that is administered in one tablet as a single daily dose [Morningstar Business Wire, Dec. 20, 2004].

Due to the costs and toxicities of even the simplest forms of HAART, alternatives to standard multi-agent antiretroviral therapy are desirable. An induction/maintenance therapeutic strategy, unsuccessful when tested in the past, is being retested with improved therapeutics. Following successful initial therapy with standard HAART (the induction phase) the ability of less potent but simpler therapy to maintain suppression of viremia, allow clinical stability, and minimize exposure to antivirals is being tested. In a small AIDS Clinical Trials Group pilot study, 33 volunteers with at least 1 year of complete suppression on standard HAART regimens (undetectable plasma viremia, < 50 copies HIV-1 RNA/ml) will simplify therapy to the once-a-day protease inhibitor combination of atazanavir 300 mg/ritonavir 100 mg. A similar study several years ago using the protease inhibitor indinavir failed when virologic rebound was observed in many patients on maintenance therapy, but it is hoped that the improved pharmacodynamic characteristics of ritonavir-enhanced atazanavir will allow successful maintenance therapy.

Immunotherapies are an alternative approach that may allow control of HIV replication but limit exposure to antiretroviral therapy. Prophylactic vaccination before infection may improve immune control, even if infection occurs despite vaccination. Therapeutic vaccination after infection might limit viremia, and delay the need to implement antiretroviral therapy [Wahren 2004]. A recent small, uncontrolled study suggested that this could be achieved with a dendritic cell-based vaccine, but these preliminary observations require confirmation and extension [xxxxx}

It is conceivable that other immunomodulatory therapies might allow long-term clinical stability despite viremia. The biological precedent for this is found in simian immunodeficiency virus infection, in which profound subspecies-specific differences in the pathogenic potential of a given SIV strain are seen [Silvestri 2003]. Similarly, human "long-term non-progressors" are recognized to tolerate HIV infection for decades without clinical progression [Mikhail 2003]. Although viral genetic mutations sometimes appear to contribute to this phenomenon, multiple human genetic markers have been linked to a reduced risk of progressive AIDS following HIV infection.

Nevertheless, if HIV infection is not prevented, the ultimate goal for HIV therapy must be to cure the infection. Viral eradication, meaning elimination of all HIV with the ability to replicate from an infected individual, would avoid the risks, costs, and toxicities of long-term antiviral therapy. Attitudes towards "the cure for AIDS" have varied over the years since the discovery of HIV. In the 1980's and early 1990's, when the time between diagnosis and death could often be measured in months, even control of infection seemed unattainable. The introduction of HAART in the late 1990s led to expectations that 2 to 3 years of therapy would lead to eradication of infection [Perelson 1997]. However, we have since learned that the terminology "highly active" contained within the acronym HAART is a relative one, and viral replication is ongoing in many patients despite HAART.

At present, the idea of "curing AIDS" is seen by many as an unrealistic holy grail. However, given our considerable detailed understanding of HIV replication and pathogenesis, and the human toll this virus will inevitably extract over the decades to come, a careful analysis of the obstacles to eradication of HIV from infected individuals is warranted.

## Problem #1: HAART is not active enough

The ability of HAART to reduce viral loads by more than 3 logs to less than 50 copies/ml, the usual limit of detection in clinical assays, often leads to the erroneous conclusion that combination therapy inhibits all significant viral replication. In fact, in the face of what is now called "highly active" antiretroviral therapy (HAART), cryptic viral replication below the limits of detection of most clinical assays persists. Numerous studies have documented persistent rounds of new infection that may be related in part to both inadequate drug potency and suboptimal antiviral pharmacodynamics [Dornadula

1999; Gunthard 1999; Zhang 1999; Fischer 2000; Lewin 2000; Ramratnam 2000; Sharkey 2000; Pierson 2001; Pomerantz 2003].

Dornadula and colleagues [1999] studied 22 subjects receiving suppressive HAART using a supersensitive modification of the reverse transcriptase polymerase chain reaction assay capable of detecting cell-free virion RNA down to 5 copies/ml. Residual viral RNA was detected in the peripheral blood plasma of every subject, with a mean level of 17 copies/ml. Given the short half-life of HIV virions in serum, this implies a constant replenishment by ongoing replication. In a follow-up study, only 3 selected subjects out of an overall cohort of 80 showed a statistically significant decay in plasma viral RNA from 50 to <5 copies/ml [Di Mascio 2003]. This finding suggests that there may be biological limits to the potency of the antivirals that currently constitute HAART. It is important to recognize that even at <5 copies/ml of viral RNA, an infected individual could harbor more than $10^4$ virus particles, likely sufficient to rekindle infection in the absence of continuous therapy.

A more recent research assay with 5- to 10-fold improved sensitivity has been reported that can detect HIV-1 RNA at levels as low as 0.4 copies/ml [Palmer et al 2003]. Initial studies in 15 patients whose plasma HIV-1 RNA levels were suppressed to less than 50 to 75 copies/ml revealed persistent viremia in all 15 patients, with HIV-1 RNA levels ranging from 1 to 32 copies/ml (median, 13 copies/ml). However, recent studies finds that a subgroup of patients can be identified with persistently undetectable viremia at the < 1 copy/ml limit [Palmer, personal communication].

Convincing evidence for covert replication despite HAART emerge from studies of sequence evolution. Quasispecies with multiple sequence alterations can be detected by sequencing proviral DNA even when serum virus is undetectable. He appearance of sequence changes over time in proviral DNA has been observed in variable proportions of HAART-treated individuals [Zhang 1999; Zhu 2002; Martinez 1999; Birk 2000], and in some cases can be attributed to positive selection driving adaptive evolution [Frost 2001]. Recently Frenkel and colleagues [2003] examined *env* and *pol* sequences from a group of HIV-infected children prior to and during HAART and showed that the ability to detect viral replication depended on the type of analysis employed. The least sensitive method was standard phylogenetic analysis, which gave a positive result for 1 out of 10 subjects, whereas the most sensitive method was maintenance of genetic distance from the most recent common ancestor of infection, which gave a positive result for 6 out of 10 subjects. Given that only a portion of all proviral sequences can be sampled in this type of experiment, it is conceivable that all of the children would have displayed some level of cryptic replication if examined in sufficient detail.

Taken together, these studies indicate that viral replication continues even in individuals who faithfully adhere to the best available HAART regimens. The observation that "successfully" treated subjects contain as many as $10^5$ productively infected cells [Hockett 1999] suggests that this is not simply due to the occasional spontaneous activation of cells containing quiescent provirus to produce virions (the "latent reservoir"). Nor can it be attributed to drug resistance since the proviral and

replication-competent viral sequences isolated from PBMC of HAART-treated subjects usually have a wild-type genotype [Martinez 1999; McGrath 2001; Izopet 2002]. Rather, it appears that current HAART is not now capable of completely inhibiting viral replication.

## Solution #1: Improved Therapies

Nevertheless, the fact that viral replication is currently suppressed to such low levels is encouraging. Current HAART regimens target only two steps of HIV replication, reverse transcription and processing of virion proteins by viral protease. HAART usually consist of three agents: two nucleoside reverse transcriptase inhibitors (NRTIs) plus either a nonnucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (PI). Testing the old notion that "more is better" the Lange group in the Netherlands studied a triple class five-drug antiretroviral regimen consisting of three NRTIs, an NNRTI, and a PI during primary HIV infection. Although this intensive treatment resulted in more rapid suppression of plasma viremia compared with standard drug regimens, it still allowed some viral replication and had no effect on the size or composition of the resting CD4 T cell reservoir. Furthermore, subjects who ceased therapy experienced immediate rebound of plasma virus [Fraser 2000; Sankatsing 2003; Ghani 2002; Weverling 1998; van Rij 2002]

Another strategy is to add drugs that improve the potency of standard HAART. Protease inhibitors are now widely prescribed with low doses of ritonavir, given to improve the pharmacokinetics of the "active" protease inhibitor through inhibition of p450 metabolism [van Heeswijk 2001]. Our laboratory and others have shown that mycophenolic acid, an inhibitor of inosine monophosphate dehydrogenase, enhances the activity of abacavir and other NRTIs by depleting intracellular dGTP [Margolis 1999; Chapius 2000; Hossain 2002; Margolis 2002]. Although clinical antiviral effect can be demonstrated with the use of mycophenolate [Margolis 2002; Garcia 2004], a long-term clinical advantage of the use of mycophenolate has not yet been demonstrated

Hydroxyurea, an inhibitor of ribonucleotide reductase that inhibits reverse transcriptase by decreasing dNTP pools and acts synergistically with didanosine can also augment antiviral activity [Foli 1997] but may blunt immune reconstitution and increase drug-related toxicicites [Frank 2004]. Recently Kulkosky and colleagues [2002] added hydroxyurea plus didanosine to the antiretroviral regimen of three HIV-infected men participating in an intensification and stimulation therapy trial and observed decreases in plasma viral RNA to <5 copies/ml and absence of replication-competent virus in PBMC co-culture assays. Disappointingly, however, all three experienced virologic rebound when HAART was discontinued.

However, several agents that interrupt stages of the HIV replicative cycle other than reverse transcription and proteolysis are entering the clinical arena. An inhibitor of the fusion step of viral entry into host cells is now available. Enfuvirtide, shows nanomolar potency against HIV-1 in vitro, potently decreases viral load, and is approved by the FDA for use as a salvage therapy in individuals failing conventional HAART

[Lalezari 2003]. Although its high cost may limit its widespread use, other inhibitors of viral attachment to the surface receptor CD4 and viral engagement of a chemokine coreceptor are in development [Este 2003]. It is hoped that the ability to block the fragile, cooperative process of viral entry at multiple steps will lead to unprecedented synergy of antiviral activity.

Many new therapies may lie farther over the horizon. Under development are inhibitors of the viral enzymes integrase, which is required for integration of proviral DNA into the host cell chromosome, and RNAase H, which is necessary for reverse transcription, and virion maturation required to produce an infection-competent particle. Intensive studies are now also underway to enhance the activity of recently discovered host factors that restrict retroviral replication such as TRIM5α and ApoBEC3G [Bieniasz 2004]. RNA interference has been shown to potently interrupt replication in culture, and in time may become a viable therapeutic tool [Berkhout 2004].

## Problem #2: Persistent Viral Production

Productively infected lymphocytes generally have life spans measured in days, HIV replication has cytopathic effects, and the antiviral immune response can induce cell death in productively infected cells. Therefore if new rounds of HIV infection can be inhibited with sufficient potency, viral replication should eventually be snuffed out. However, some cell types may exist with longer half-lives and with intrinsic resistance to HIV-induced cytolysis.

As macrophage/monocytes are more resistant than T cells to the cytopathic effects of HIV and as some nucleoside analogs are poorly metabolized in these cells, they may serve as a hiding place for the virus in patients receiving clinically suppressive HAART. This has been suggested by the isolation of replication-competent virus from highly purified monocytes of HAART-treated individuals following in vitro stimulation [Lambotte 2000; Sonza 2001]. Interestingly, measurements of sequence evolution and of the concentrations of unspliced and multiply spliced mRNA suggest that HIV replication is more pronounced in monocytes than in resting CD4 T cells [Zhu 2002]. Thus, macrophage/monocytes probably represent a site for ongoing growth of the low levels of virus that continue to be released in patients on HAART rather than a true latent reservoir as found in resting T cells. Nevertheless, because macrophage/monocytes have a relatively long half-life of approximately two weeks, they are likely to serve as a clinically important source of viral production and evolution in HAART-treated individuals.

Other cells with longer lifespans may contain and produce HIV. In the central nervous system, HIV is found in macrophage, microglial cells, and astrocytes, especially in perivascular areas of the brain. Further some antiretroviral drugs penetrate the blood-brain barrier poorly. Similarly, poor drug penetration may allow HIV to persist in the genitourinary tract and can be found in T cells and macrophage isolated both from both semen and cervix [Nunnari 2002; Zhang 1998]. HIV RNA has been detected in renal

tubule epithelial cells [Winston et al. 2001] and in adipose tissue [Hazan and Leibowich 2003], but the role of these cell types as a true viral reservoir is unclear.

## Solution #2: Killing Infected Cells

If viral replication can be completely blocked, it may not be necessary to destroy all remaining infected cells. Viral- or immune-mediated apoptosis or natural cell turnover may destroy infected cells given sufficient time. However, if sufficiently potent therapy must be maintained for long periods of time or if HIV-specific cytotoxic T cells have been lost during infection it may be clinically impractical to wait for all infected cells to die [Atlfeld 2000]. Immune-mediated clearance may be enhanced by therapeutic vaccination. Further, if eradication of infection is the goal, the current paradigm of withholding therapy until advanced immunodeficiency is impending might change, and therapeutics might be implemented before HIV-specific cytotoxic T cells are depleted.

Targeted toxins, or immunotoxins, represent an approach for killing activated HIV infected cells [Berger 1998]. These toxins are bifunctional molecules that consist of two domains: a binding domain that recognizes the HIV envelope glycoprotein Env or another target expressed on the surface of the infected cell, and a cytotoxic domain that kills the cell once internalized. Typically the cytotoxic domain is derived from a naturally occurring protein toxin such as ricin, diptheria toxin, or *Pseudomonas aeruginosa* exotoxin A (PE), and is joined to the binding domain either by protein engineering or chemical linkage. For targeted toxins to be clinically useful, they must bind with high affinity to the target. In addition, they must display suitable pharmacokinetic properties including minimal nonspecific toxicity to uninfected cells and stability in the circulation.

Another approach to the strategy of targeted killing focused on resting CD4+ cells has been advanced at UTSouthwestern. Drs. Vitteta, Ramillo, and colleagues have demonstrated that the use of an anti-CD45RO immunotoxin can reduce the target population of resting cells that harbor latent HIV infection in laboratory model systems [McCoig 1999]. In a follow-up study using resting CD4 cells harvested from HIV-infected patients, latent infection in CD4+ cells was depleted but CD8+ memory cells unharmed [Saavedra-Lozano 2002].

## Problem #3: Virologic Latency

True virological latency exists within a subpopulation of HIV-infected CD4+ lymphocytes that enter the resting state, avoiding viral or immune cytolysis. These infected cells cannot be recognized by the antiviral immune response, even when antiviral immunity is optimal. Once established, potent antiretroviral therapy appears to have little impact on this quiescent reservoir of HIV infection [Wong 1997; Finzi 1997; Chun 1997]. This population of cells appears to be the most difficult obstacle to eradication of established HIV infection.

Latently infected resting memory CD4 T cells strictly defined are those cells harboring integrated proviral DNA that is potentially functional but not expressed without stimulation. These cells can be detected and quantitated by purifying resting CD4 T cells on the basis of surface markers, incubating limiting dilutions with a general activator of T cell proliferation, and amplifying the output virus by addition of CD4 lymphoblasts from uninfected donors. Latently infected resting CD4 T cells represent only a small fraction of the total infected cells in untreated individuals, but become a progressively more important component as HAART reduces overall viral load [Chun 1997a; Chun 1997b; Chun 1998].

Based on estimates of a total body mass of ca. 1000 billion resting memory T cells, and a frequency of infection of these cells of one per million, the estimated average size of this reservoir is approximately 1 million cells. This assumes that memory T cells are limited to the circulation and peripheral lymphoid organs of spleen, gut-associated lymphoid tissue and lymph nodes. However, studies of patients durably and successfully treated with HAART suggest that the frequency of latency in some patients may be as much as two logs lower, leaving as few as 10,000 resting infected CD4+ T cells to be cleared [Ylisastigui 2003].

The resting T cell reservoir is established early in infection, as shown by its presence even in individuals who received HAART prior to seroconversion, and is extremely stable, having an estimated mean half-life that varies from 6 months in individuals with intensified HAART and optimal suppression of viral replication [DiMascio 2003; Zhang 1999; Ramratnam 2003] to more 44 months in most HAART-treated people [Finzi 1999; Siliciano 2003]. This reservoir contains an archive of all viral species that have arisen over the course of infection. For example, one study demonstrated the presence of wild-type, drug-sensitive HIV in resting CD4 T cells even in individuals who developed drug resistance and were exposed to drugs selecting that resistance for more than 10 years [Ruff 2002]. Therefore, while there is hot debate between the laboratory groups involved as to the true half life of this reservoir during HAART, there is little doubt that decay is slow enough to require years of intensive, potent therapy to effect clearance.

A recent study showed that even in treated patients who have had no detectable viremia for as long as 7 years, the reservoir decayed with an estimated half-life of 44 months [Siliciano 2003]. The stability of the latent reservoir is expected from the physiological function of memory CD4 T cells in providing long-term immunological memory. However, other studies have suggested that the decay of the infected resting CD4 cell pool slows over time [Strain 2003], reflecting the selection of a population of increasing stability, from which it may be more difficult to induce viral replication. This may mean that some virus that can be detected in the laboratory is unlikely to clinically re-ignite HIV infection in a patient, or alteratively that specific strategies may be needed to purge this "stable latent reservoir."

But how does HIV become integrated into the genome of resting CD4 memory T cells and establish latency given the preference of the virus for infecting actively replicating T cells [Zack 1990]? One scenario is that HIV has evolved mechanisms to render even resting cells permissive for viral infection. Through a signaling pathway involving the accessory protein Nef, it has been suggested that B cell co-stimulatory receptors CD22 and CD58 may signal T cells. This specific mode of signaling is thought to allow HIV entry and gene expression, but not virion release, leading to the establishment of a non-productive but inducible reservoir of infected resting cells [Swigler 2003].

The most likely possibility is that a fraction of infected lymphoblasts return to a resting state, escaping the cytopathic effects of infection and host cytolytic effector mechanisms, and enacting the natural program that a minority of activated T cells must carry out to preserve immunological memory. However, during this process HIV gene expression and virion production must be dampened.

There are two fundamental mechanisms for the repression of HIV gene expression in latently infected cells. One is simply the lack of sufficient factors required for HIV gene expression, a *trans*-dominant mechanism. In the *trans* mechanism, viral gene expression is blocked due to a shortage of the cellular or viral proteins that are required for the efficient initiation, elongation or transport of HIV mRNA. However, recent studies of unstimulated primary lymphocytes have shown that they are capable of supporting HIV promoter expression and activation [Martin-Serrano 2002].

By contrast, a *cis* mechanism of latency would be driven by integration of the provirus into a region of chromatin that either is or becomes inhospitable to proviral gene expression. The most extensively studied mechanism of *cis*-acting repression of HIV transcription is histone deacetylation. Acetylated histones are required for activation of the HIV LTR by NF-κB [Van Lint 1996; Sheridan 1997; El Kharroubi 1998; Benkirane 1998]. It has been known for some time that inhibitors of histone deacetylation, such as sodium butyrate, derepress HIV replication both in latently infected cell lines and in PBMC from infected individuals [Kashanchi 1997; Laughlin 1993, 1995]. This implies a role for the human enzyme family of histone deacetylases (HDACs) in the establishment or maintenance of proviral quiescence [He 2002b].

We have defined the first host cell mechanism that maintains quiescence of HIV gene expression in infected resting CD4+ lymphocytes harvested from patients. Repression is mediated by the recruitment of histone deacetylase 1 (HDAC1) to the LTR by the host factors LSF and YY1, and the formation of condensed heterochromatin [Margolis 1994; Romerio 1997; Coull 2000; He 2002a; Coull 2002]. To demonstrate the relevance of this mechanism in primary T cells we utilized pyrrole-imidazole polyamides, small molecule DNA-binding reagents that directly block LSF binding to its LTR site. If LSF-directed recruitment of HDAC contributes to HIV quiescence within resting cells, exposure of HIV+ donor resting CD4+ cells to LSF-blocking polyamides should induce HIV outgrowth [Coull 2002].

We recruited volunteers from our clinics with prolonged and consistent suppression of plasma HIV-1 RNA to less than 50 copies/ml. Lymphopheresis this population provided large numbers of resting infected cells, enabling us to definitively analyze these rare cells. As viremia was been durably suppressed in this population by antiretroviral therapy, recently infected cells (termed "pre-integration latency") contributed little to viral outgrowth in this system. Following exposure to any of four distinct and specific polyamides that block HDAC1 recruitment, replication-competent HIV emerged from cultures of resting CD4+ cells in 6 of 8 HIV+ patients whose viremia was suppressed by therapy. In comparison, HIV was recovered from 7 of 8 of these patients' samples following non-specific T cell activation, but not following exposure to control scrambled polyamides. These studies demonstrated that HDAC recruitment to the LTR plays a central role in restricting HIV expression in vivo and allowing persistence of integrated HIV [Ylisastigui 2004a].

## Solution #3: Terminating Virologic Latency

Because latently infected resting CD4 T cells do not express viral mRNA or proteins, they are unaffected by HAART and unrecognized by the immune system. Eliminating this reservoir will therefore require agents that induce expression of latent integrated provirus. Such agents most likely should be administered in combination with effective HAART to prevent spreading infection by the newly released virus.

*Shock and Awe:* The most obvious approach to depleting the latent resting cell reservoir, and the first one attempted, is to "purge" the reservoir *in vivo* with reagents that induce T cell activation and viral outgrowth from latently infected cells. Chun and colleagues at the NIH analyzed a series of patients who received intermittent IL-2 therapy in addition to HAART, and found that resting CD4 cell infection was less frequent than a control group who received HAART alone. Subsequently, however, the same researchers analyzed a series of recently infected individuals who were treated with IL-2 plus HAART within 6 months of diagnosis and found no reduction in the pool of HIV-infected resting CD4 T cells. Unfortunately, IL-2 treatment had no effect on the re-emergence of viremia upon cessation of HAART in two studies [Chun 1999; Dybul 2002].

Another technique attempted in a clinical trial was the stimulation of the T cell receptor complex with antibodies to CD3. A Dutch group treated three patients on highly suppressive HAART with IL-2 plus a high dose (5 x 5 mg) of OKT3, a mouse anti-CD3 monoclonal antibody approved for human clinical use, plus IL-2 [Prins 1999]. The experiment was "successful" in the sense that OKT3 induced a strong but transient release of serum cytokines and chemokines, CD4 T cell division, and increased HIV RNA in lymph nodes and serum. However, the treatment was highly toxic. All of the patients had serious side effects, including one who nearly died due to renal failure and seizures. Moreover, the subjects experienced long-lasting CD4 T cell depletion that was not restored even two years after treatment [Prins 1999; Van Praag 2001; Stellbrink 2002].

Kulkosky and colleagues [2002] then conducted a careful trial of anti-CD3 therapy on 3 patients who were first given didanosine and hydroxyurea in addition to HAART as an intensification strategy to minimize cryptic viral replication. The subjects were then administered a single low-dose infusion of OKT3 (400 mcg), followed by a course of IL-2. This treatment was well tolerated, and as hoped plasma viral RNA remained <5 copies/ml and replication-competent virus was undetectable after treatment. However, upon cessation of HAART, all of the subjects developed plasma viral rebound, indicating that even this sophisticated combination of treatments was insufficient to eradicate the virus.

Several other stimulatory reagents have been tested in model systems. Our laboratory reported that IL-7 can induce latent HIV expression from patient's cells, a finding recently confirmed by the Pomerantz group, and has the added benefit of stimulating T cell generation [Fry 2003; Lehrman 2004; Wang 2005]. Activators of protein kinase C, a family of enzymes that lies downstream of the T cell receptor signaling complex, can induce HIV expression. 1,2-diacylyglycerol (DAG), prostratin (12-deoxyphorbol 13-acetate), a nontumor promoting phorbol ester from *Pimela prostrata*, and DPP (12-deoxyphorbol 13-phenylacetate), a non-tumor promoting phorbol ester isolated from the West African "candle plant" *Euphorbia poissonii* can induce expression of HIV in various model systems of latency [Kulkosky 2001; Korin 2002; Hamer 2003].

Although cytokines, CD3 antibodies and PKC activators are potent inducers of latent HIV expression, they all suffer the drawback of non-specific T cell activation of both infected and non-infected cells. Of particular concern is the ability of these compounds to release inflammatory cytokines, potentially leading to respiratory distress syndrome, hypotension and other toxicities. It is not yet clear whether the transcription factors and signaling molecules that induce HIV replication and cytokine production are precisely the same or partially distinct. If the former is the case, it is unlikely that any of these non-specific T cell activators will ever be useful in humans.

Further, modeling studies suggest that global T cell activation may induce viral replication and increase the number of susceptible uninfected target cells beyond the threshold that can be contained by antiretroviral therapy [Fraser 2002]. As an alternative approach, reagents that selectively induce the expression of quiescent proviral genomes but have limited effects on the host cell might allow outgrowth of latent HIV and avoid the pitfalls of global T cell activation.

*Coaxing virus out into the open:* As HDAC recruitment to the LTR appears to play a central role in maintaining HIV quiescence; we have hypothesized that HDAC inhibitors might be used therapeutically to disrupt HIV latency [Ylisastigui 2004a]. Valproic acid (VPA) has recently been demonstrated to be a direct inhibitor of HDAC, and this capability linked to the clinical effects of VPA [Phiel 2001]. VPA is in wide use and often given to HIV-infected patients to treat concomitant conditions. In early studies HIV gene expression and virus production were increased *in vitro* in the presence of VPA

[Moog 1996; Witvrouw 1997]. Literature prior to the era of HAART advised against the use of VPA as it might increase plasma HIV RNA, however a study recently observed no effect of VPA on plasma viremia in the presence of HAART [Maggi 2001].

To confirm that VPA could induce chromatin remodeling via HDAC inhibition that would allow HIV expression, we studied changes induced by VPA in histone H4 acetylation at the HIV LTR nucleosome 1. As this is not technically feasible in patient cells due to the rarity of the integrated promoter, we performed chromatin immunoprecipitation assays in Jurkat J89 T cell lines containing a single integrated HIV genome [Kutsch 2002]. In this T cell line model of latency, expression of integrated provirus can be induced by activation and quiescence is re-established when activating stimulus is withdrawn.

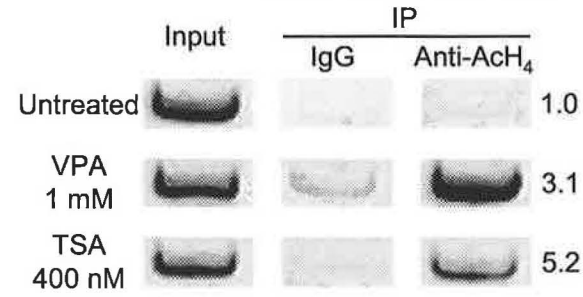


**Figure 1:** Chromatin immunoprecipitation showing increased HIV LTR acetylation at nucleosome 1 induced by VPA and the potent HDAC inhibitor trichostatin A. Fold increase denoted at right.

Using an antibody directed against acetylated histone H4, we precipitated DNA fragments associated with acetylated histone H4 from cell extracts. The precipitated HIV LTR DNA was quantified by PCR with primers spanning the nuc 1 region of LTR. Amplification of serial dilutions of DNA demonstrated that a two-fold increase in density units of PCR product represents at least a fourfold increase in target DNA [He 2002a]. As ChIP is a qualitative assay of factor occupancy at a specific DNA sequence, any 2-fold increase in PCR product observed in our assay conditions represents a significant qualitative increase in occupancy. Cells were assayed after only 4 hours of exposure to VPA, as longer periods of time might allow the observation of chromatin remodeling events that occur during cell cycle progression or due to the expression of other host genes. As shown in Figure 1 [Ylisastigui 2004b], exposure of cells to 1 mM VPA results in a significant increase in acetylated histone H4 at nuc 1.

We then tested the ability of VPA to allow expression of HIV from resting $CD4^+$ T cells of patients on HAART with plasma HIV RNA stably <50 copies/ml. We obtained 200-500 million resting CD4+ T cells from 5 HIV+ donors by leukopheresis and assessed the frequency of recovery of HIV (infectious units per million resting CD4+ cells; IUPM) following either activation with PHA (a method used to define the size of this reservoir) or exposure to concentrations of VPA that are employed clinically. Interestingly IUPM were identical VPA and PHA, with complete overlap of confidence intervals [Table 1].

As the goal of induction of latent HIV expression would be to allow eradication of chronic viral infection, reagents used to induce viral outgrowth should not simultaneously increase the likelihood of *de novo* infection. Global inhibition of HDAC activity is reported to significantly alter the expression of only 2% of host genes [Van Lint 1996]. To explore the global effect of VPA on peripheral blood mononuclear cells

in relation to HIV-1 infection, we performed cell surface phenotype analysis and measured *de novo* infection in the presence of VPA.

Table 1: **VPA allows HIV outgrowth from resting CD4+ cells of aviremic donors on HAART with a frequency similar to global activation with a mitogen (PHA)**

| Subject 1 | | Subject 2 | | Subject 3 | | Subject 4 | | Subject 5 | |
|---|---|---|---|---|---|---|---|---|---|
| PHA | VPA 1 mM | PHA | VPA 0.2 mM | PHA | VPA 0.2 mM | PHA | VPA 1 mM | PHA | VPA 0.2 mM |
| **0.44** (0.21-0.87) | **0.34** (0.15-0.68) | **0.06** (0.01-0.24) | **0.05** (0.01-0.14) | **0.06** (0.01-0.17) | **0.10** (0.03-0.27) | **0.17** (0.06-0.37) | **0.10** (0.02-0.26) | **0.09** (0.02-0.23) | **0.06** (0.01-0.19) |

Infectious units per million (IUPM) resting CD4 cells by maximum likelihood method, with 95% confidence intervals, as calculated by outgrowth frequency of HIV in high-input limiting-dilution resting CD4+ cell cultures. No HIV was recovered in IL2 control limiting-dilution cultures; a total of 112 to 150 million cells cultured per subject. No significant differences in IUPM between PHA and VPA within subjects. [Ylisastigui 2004b]

PBMCs were pre-treated with IL2 20 U/ml alone, PHA 2 mcg/ml and IL2, or VPA 0.2 to 1.0 mM and IL2 for 3 days. Pre-treated PBMCs were then infected with a CXCR4-tropic clone (HIV-LAI) and p24 assayed over two weeks. In contrast to the effect on latently infected CD4+ cells, infection of seronegative donor cells in the presence of 0.2 to 1 mM VPA and IL2 did not result in a change in production of HIV compared to cells cultured with IL2 alone [Ylisastigui 2004b]. To eliminate the possibility that VPA exposure increased HIV entry and/or reverse transcription, despite the fact that it did not upregulate *de novo* productive infection, we performed HIV DNA PCR. Equivalent amounts of HIV DNA were detected in cells infected in the presence of VPA/IL2 or IL2, more than four-fold less than in PHA-activated cells. Findings using a CCR5-tropic viral clone (BaL) were similar.

Expression of activation markers on the surface of lymphocytes was unaltered by VPA. Exposure of peripheral blood mononuclear cells to up to 1 mM VPA and 20 units/ml IL2 did not significantly upregulate cell surface expression of CD38, CD69, CD25, or HLA-DR on CD3+/CD4+ or CD3+/CD8+ cells compared to cells cultured in 20 units/ml IL2 alone [Ylisastigui 2004b]. CCR5 and CXCR4 expression was also unaffected by VPA. Based on these encouraging pre-clinical data, and the long track record of the use of VPA in the HIV+ population, we have begun a pilot trial to measure the effect of intensified HAART and VPA on the frequency of resting CD4 cell infection.

## CONCLUSION

Although we are still far from a cure for HIV infection, our increasing knowledge of the mechanisms of viral persistence provide encouragement that this goal is actually achievable. Mechanistic understanding focuses our efforts on the advances that need to be made: better HAART, specific inducers of latent viral expression, and augmenting the host's capability to kill infected cells. Achieving these goals will not be simple. Due to

the

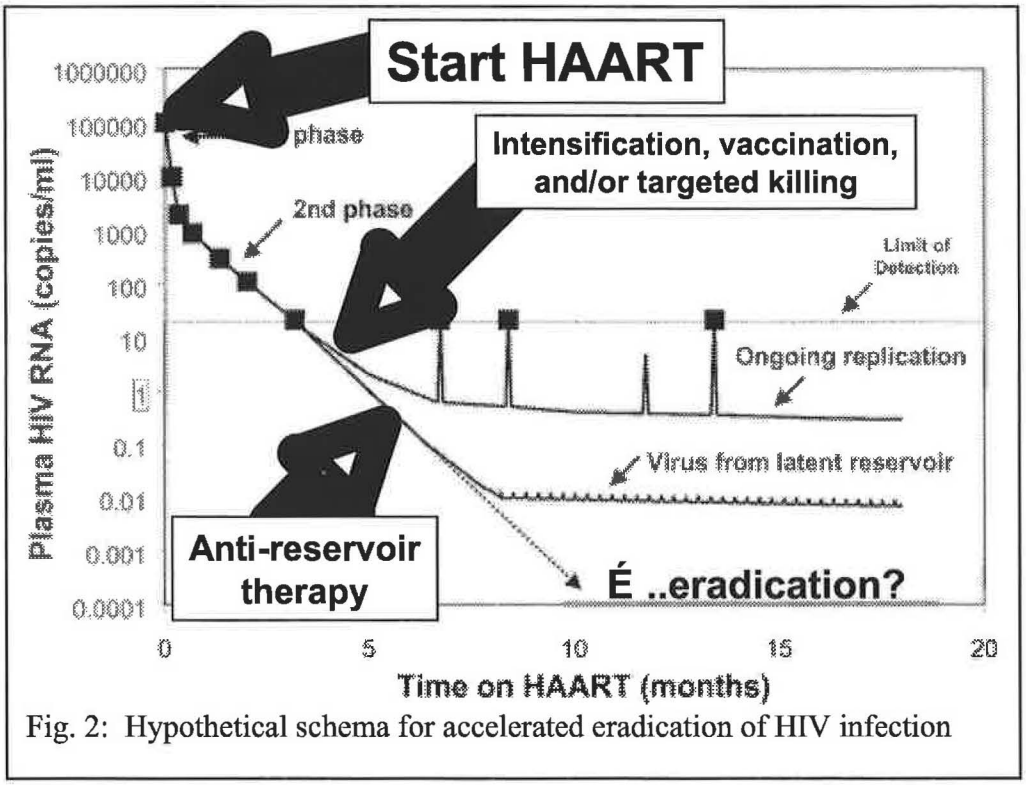


Fig. 2: Hypothetical schema for accelerated eradication of HIV infection

biological nature of lentiviral infections, and the sad irony that HIV targets the very cells most needed to defend against it, HIV is well designed to persist in man. Given its high replication and mutation rates, and the plasticity of its enzymes, it is not surprising that HIV is so far outwitting its human host. Nevertheless, the rapid advances in antiretroviral therapy and the understanding of HIV biology give us hope that HIV can be controlled, and eventually defeated.

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