Can HIV be Cured? Mechanisms of HIV Persistence and Strategies to Combat It

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Abstract: Stable remission is the ultimate goal of HIV therapy. A review of recent studies on the ability of HIV to persist despite highly active antiretroviral therapy (HAART) and immune stimulation suggests that achieving this goal will require four developments in basic and clinical science. First, more effective antiretroviral therapies, targeted at proteins other than reverse transcriptase and protease, in order to eliminate the cryptic replication that continues despite best available HAART. Second, agents that activate latent HIV gene expression in quiescent CD4 memory T cells, thereby exposing this viral reservoir to therapeutic intervention by a "shock and kill" strategy. Third, molecules such as immunotoxins that specifically recognize HIV-encoded membrane proteins and thereby potentiate the destruction of infected cells. Fourth, and still most distant, novel approaches such as genetically engineered cytotoxic T lymphocytes or anti-HIV microbes to suppress rekindling of infection by residual virus sequestered in anatomical and cellular reservoirs. Although each of these steps will be difficult to achieve, the many benefits of a cure for HIV make this a worthwhile pursuit.

Keywords: HIV-1, Latency, Viral Reservoir, Immunotoxin

INTRODUCTION

More than twenty years into the AIDS epidemic, there is still not a single person who has been cured of HIV infection. True, the development of highly active anti-retroviral therapy (HAART) has made it possible to control viremia, partially reconstitute the immune system, and delay disease progression in most individuals who are fortunate enough to have access to the drugs [67,70,122]. But the relief afforded by HAART is contingent on continued medication. If therapy is discontinued or becomes ineffective, virus contained in stable reservoirs rapidly rebounds and disease progression resumes, leading eventually to immune failure, opportunistic infections, AIDS and death (reviewed in [12,32,90,131,139,140, 157,165]).

This leaves infected individuals, even if they have access to sophisticated medical care, between a rock and a hard place: death or lifelong HAART. The later option is unappealing at many levels. HAART is expensive, difficult to adhere to, and has multiple serious side effects including hyperlipidemia, hyperglycemia, lactic acidosis, lipodystrophy, hypersensitivity reactions, neuropathies, hepatotoxicity, pancreatitis, anemia, cardiovascular disease, and type 2 diabetes [20]. Moreover, because of HIV's exceptionally high mutation rate, drug-resistant viral variants can readily arise. Thus, although it is now commonplace to say that HAART has rendered HIV infection a "chronic but manageable disease", we do not actually know the life expectancy of an HIV-infected individual under best available clinical management. It is certainly longer than before the HAART era, but still probably shorter than for an uninfected person. Of course, for the large majority of HIV infected individuals who do not have access to expensive antiretroviral drugs, even the option of lifelong HAART is not available; AIDS and death are the only prospects.

For these reasons, the ultimate goal for HIV therapy must be to cure the infection rather than to simply treat it. In this review, I define cure to mean stable remission of viremia, immune deterioration and disease progression without ongoing medication. Viral eradication, meaning elimination of all HIV genetic information from an infected individual, would be an even more desirable goal, but also more difficult given HIV's ability to persist in many different cell types and anatomical locations.

Attitudes about the possibility of curing HIV have swung back and forth over the two decades since the discovery of the virus. At the beginning of the epidemic, when the time between diagnosis and death was typically counted in months, pessimism reigned. Then, when HAART was introduced in the late 1990s, there were optimistic predictions that the virus could be eliminated by 2 to 3 years of treatment [127]. The discovery of stable viral reservoirs and cryptic replication, however, soon dashed such thoughts. Reports of a patient in Berlin who appeared to achieve remission following several cycles of intermittent HAART led to a second wave of hope, but attempts to repeat his experience through deliberate structured treatment interruptions were unsuccessful and the patient himself disappeared to follow-up [66].

At present, the idea of "curing AIDS" is seen as unrealistic by many investigators and is rarely even mentioned in the scientific literature. Instead, there is increasing emphasis on finding preventions for HIV infection such as vaccines. While this is an important goal, it won't do any good for the 40 million people in the world who are already infected with the virus. The goal of this review is to focus attention on the key basic and clinical

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1570-162X/04 $45.00+.00

questions that need to be addressed in the search for a strategy to achieve stable remission of HIV infection.

THE PROBLEM 0F COVERT REPLICATION

The ability of HAART to reduce viral loads by more than 1000-fold (i.e., from greater than 100,000 copies/ml to less than 50 copies/ml, the usual limit of detection in clinical assays) often leads to the erroneous conclusion that combination therapy inhibits viral replication by more than 99.9%. In fact, large decreases in viral load are expected for any treatment that reduces the basic reproductive number, which is the average number of infected cells produced by one initially infected cell, to less than 1, even if there is substantial ongoing viral replication. Multiple experimental approaches have shown that such covert replication occurs in many if not all HAART-treated individuals.

One clear cut indication of ongoing replication is the continued presence of circulating virus despite extensive HAART. Dornadula and colleagues 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 [41]. 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 [39]. It is important to recognize that even at <5 copies/ml of viral RNA, an infected individual could easily contain more than 10^4 virus particles, which is probably sufficient to rekindle infection in the absence of continuous therapy.

Residual HIV replication has also been detected by analysis of intracellular HIV RNA and DNA. Sensitive assays based on in situ hybridization or real-time PCR with molecular beacons have documented the presence of HIV transcripts in lymph tissue and peripheral blood mononuclear cells (PBMC) in many subjects on HAART; positive signals are most common in subjects who experience "blips" of plasma virus, indicating incomplete drug action, but are also found in some individuals who appear to be well suppressed [61,93,97,124,183]. Other studies of HAART-treated individuals have found unintegrated HIV DNA in resting CD4 T cells [33] and 2-LTR circles in PBMC [40,117,119,162]. Because 2-LTR circles are formed only after completion of viral cDNA synthesis and translocation to the nucleus and appear to be unstable in cultured cells, they are thought to serve as a marker for recent synthesis and nuclear entry of pre-integration complexes; however, this interpretation has been questioned [18,19,132].

Some of the most convincing evidence for covert replication despite HAART comes from studies of sequence evolution. Because HIV reverse transcriptase has a high error rate, viral replication rapidly leads to the generation of quasispecies with multiple sequence alterations [113]; these can be detected by sequencing proviral DNA even when plasma virus is undetectable. Such sequence changes have been observed in variable proportions of HAART-treated individuals [11,109,183,184], and in some cases can be attributed to positive selection driving adaptive evolution [59]. Recently Frenkel and colleagues [58] 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.

Indirect evidence for cryptic HIV replication has been obtained by fitting data on post-HAART decreases in viral RNA levels to mathematical models of HIV infection dynamics. Rather than making the usual but crude assumption that HAART is completely effective, Ferguson, Fraser and colleagues simultaneously fit data from multiple subjects to estimate drug efficacy separate from the clearance rates of actively infected cells and free virus. They estimated that HAART reduced viral replication by only 50 to 80%, even in subjects who were receiving potent drug regimens that suppressed plasma viral loads below the limit of detection [51,57]. These results were interpreted to indicate that HAART pushed the viral reproduction number only slightly below the critical threshold of 1, perhaps reflecting the implicit use of this criterion to define the minimum inhibitory concentration and clinical dose.

Taken together, these studies indicate that substantial 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 [75] suggests that this is not simply due to the occasional spontaneous activation of 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 [78,109,113]. Rather, it appears that current HAART is intrinsically incapable of completely inhibiting viral replication.

STRATEGIES FOR IMPROVING HAART

Attempts to cure HIV are doomed to failure without improved forms of HAART that can completely block new rounds of infection by virus released from the latent reservoir [139]. This will require the development of new types of antiretroviral drugs.

Current HAART regimens usually consist of three agents: two nucleoside reverse transcriptase inhibitors (NRTIs) plus either a nonnucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (PI). The simplest theory for intensifying HAART is "more is better". For example, scientists in the Netherlands have extensively

studied a small group of patients who started a triple class five-drug antiretrovial 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 [57,63,158,173,174,176].

Another strategy is to add drugs that improve the potency of standard HAART. For example, low doses of ritonavir improve the pharmacokinetics of other PIs by inhibiting P450 3A4 isoenzymes [170]. Mycophenolic acid, an inhibitor of inosine monophosphate dehydrogenase, enhances the activity of abacavir and other NRTIs by depleting intracellular dGTP [25,76,106,107]. One of the most extensively studied enhancements is hydroxyurea, an inhibitor of ribonucleotide reductase that inhibits reverse transcriptase by decreasing dNTP pools and acts synergistically with didanosine [53,54,101,102]; note, however, that hydroxyurea may have toxic effects and decrease immune function in some individuals [72]. Recently Kulkosky et al [89] 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 virological rebound when HAART was discontinued.

Several agents that interrupt stages of the HIV replicative cycle other than reverse transcription and proteolysis are under development. Most advanced are inhibitors of viral entry into host cells, which involves three stages: attachment, which is mediated by the binding of the viral envelope glycoprotein external subunit gp120 to the surface receptor CD4; engagement of a chemokine coreceptor, usually CXCR4 or CCR5, which leads to conformational changes in the gp41 ectodomain; and fusion, in which the C-terminal regions from three gp41molecules pack as amphipathic α-helices against a central trimeric coiled coil formed by three N-terminal regions, thereby forming a trimer of hairpins that brings the viral and cellular membranes into close proximity [23,46]. During the latter process, gp41 forms a prehairpin intermediate in which the N-terminus is inserted in the target cell membrane and the N-terminal coiled coil is transiently exposed to inhibitory compounds [62,115]. Peptides derived from the gp41 C-terminal region (denoted C-peptides) can bind to the exposed coiled coil and block the proper formation of the trimer-of-hairpins, thus preventing membrane fusion [22,80,177]. One such C-peptide, known as Enfuvirtide, shows nanomolar potency against HIV-1 in vitro, decreases viral load in humans, and was recently approved by the FDA for use as a salvage therapy in individuals failing conventional HAART [85,86]. N-terminal peptides and a 5-helix protein that inhibit HIV-1 fusion by binding to the C-terminal region of gp41 are also under development [45,103,154]. Because the C-terminal and N-terminal peptides recognize different portions of gp41, they should be synergistic with one another as well as with reverse transcriptase and protease inhibitors.

In an exciting new development, scientists at Bristol-Myers Squibb recently unveiled the first small molecule inhibitor of the interaction between gp120 and CD4, BMS-378806 [98]. This 4-methoxy-7-azaindole derivative inhibits a wide spectrum of laboratory and clinical isolates of HIV with mid-nanomolar to micromolar potency and, unlike the peptide compounds described above, is orally available. Surprisingly, viral resistance mutations mapped at many different locations in gp120. The precise site and mechanism of binding, and its relationship to the crystal structure of the gp120:CD4 complex [92], will be of interest. Antagonists of the chemokine coreceptors for HIV entry are also under preclinical and clinical development. The observation that individuals homozygous for a deletion in the CCR5 gene are completely resistant to HIV infection, yet fully viable, has made CCR5 an especially attractive candidate, and several small molecule antagonists have been described [13,48,160].

Also in the pipeline 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. The two zinc fingers of the viral nucleocapsid protein NCp7, which is involved in both the uncoating and packaging of HIV, has been targeted by zinc-ejecting compounds. Still lacking are agents that specifically inhibit HIV transcription and mRNA production, which utilize the virally encoded TAT and REV proteins as well as numerous cellular components.

The new antiretroviral drugs will first be used in patients who are failing conventional HAART – a population that continues to grow due to the increasing transmission of drug-resistant HIV variants [100]. In a sense, this is fortunate since it provides a commercial incentive for the development of new drugs. However, the only way to test whether these agents can reduce covert viral replication will be to combine them with reverse transcriptase and protease inhibitors in drug-responsive patients, then perform ultra-sensitive assays of viral load, latent reservoirs, and sequence evolution. This should be a priority as soon FDA approval of the new agents is achieved.

THE PROBLEM OF RESERVOIRS

Will more effective forms of HAART allow HIV to be cured? Not necessarily. The second major obstacle to eliminating HIV is its ability to hide out in reservoirs, which have been defined as "a cell type or anatomical site in association with which a replication-competent form of the virus accumulates and persists with more stable kinetic properties than the main pool of actively replicating virus" [12]. The stability of these reservoirs, which is based in the normal physiology of the immune system, may make HIV infection intrinsically incurable by antiretroviral therapy alone [128].

The most extensively studied reservoir for HIV consists of latently infected resting memory CD4 T cells harboring integrated proviral DNA that is potentially functional but not expressed without stimulation. This compartment 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 [27,31,33]. The estimated average size of this reservoir is 10^6 cells, assuming that memory T cells are limited to the circulation and peripheral lymphoid organs of spleen, gut-associated lymphoid tissue and lymph nodes; if memory cells are also common in nonlymphoid tissue, as recently found in mouse [146], the reservoir may be substantially larger.

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 of 6 months in individuals with optimal suppression of viral replication [39,145,183] to more than three years in most HAART-treated people [52,164]. 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, making eradication unlikely if not impossible [164]. Consequently, 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 [155]. The stability of the latent reservoir is expected from the physiological function of memory CD4 T cells in providing long-term immunological memory.

How does HIV become integrated into the genome of resting CD4 memory T cells given the preference of the virus for infecting actively replicating T cells [180]? One possibility is that a fraction of infected lymphoblasts returns to a resting state; however, it is unclear how such cells could escape the cytopathic effects of infection and host cytolytic effector mechanisms. A second scenario, supported by recent work from Stevenson's group [168], is that HIV has evolved mechanisms to render even resting cells permissive for viral infection. The new work shows that HIV, through a signaling pathway involving the accessory protein Nef, induces the release of sCD23 from infected macrophage. This soluble factor upregulates the expression of the co-stimulatory receptors CD22 and CD58 on B cells, which in turn interact with their corresponding ligands on T cells. This "tickles" the T cells into becoming permissive for HIV entry and gene expression, but not virion release, even in the absence of proliferation, thereby leading to the establishment of a non-productive but inducible reservoir of infected resting cells. HIV-infected macrophages also produce sICAM, which upregulates the expression of CD80 on B cells and leads to T cell replication and productive infection. These complex networks of cell-to-cell communication illustrate the sophisticated strategies that have been harnessed by HIV to ensure its own persistence.

Macrophage/monocytes are another potential cellular reservoir for HIV. Because macrophage/monocytes are more resistant than T cells to the cytopathic effects of HIV and to antiretroviral treatment, they may serve as a hiding place for the virus in patients receiving clinically suppressive HAART. This has been confirmed by the isolation of replication-competent virus from highly purified monocytes of HAART-treated individuals following in vitro stimulation [94,166]. 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 [184]. 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.

HIV can persist in several anatomical reservoirs outside of the lymphoid tissue. In the central nervous system, HIV is found in macrophage, microglial cells, and astrocytes, especially in perivascular areas of the brain. A recent study by Polis and colleagues [138] detected >50 copies/ml of HIV RNA in CSF after two months treatment with a potent four drug cocktail. Although HAART has greatly reduced the incidence of HIV encephalitis, the presence of detectable HIV in cerebrospinal fluid even in individuals with undetectable plasma viral loads suggests that the central nervous system could act as a viral reservoir – a possibility exacerbated by the inability of many antiretroviral drugs to penetrate the blood-brain barrier. HIV also persists in the genitourinary tract and can be found in T cells and macrophage isolated both from both semen and cervix. Furthermore, replication-competent virus has been recovered from semen of HAART-treated men with undetectable viral loads, indicating that the male reproductive tract is both a potential source of rebound virus in the infected individual and of new infection in his sexual partners [50,119,181].

From a clinical standpoint, the most important aspect of reservoirs is their capacity to contribute to viral rebound following discontinuation of HAART. To date there have been four studies that have addressed this issue by sequence comparisons of virus isolated from plasma of rebounding individuals versus rescued from resting T cells of the same subjects prior to treatment interruption. Each study has generated different and sometimes contradictory results.

Chun et al. [28] used heteroduplex and mobility tracking assays to study nine individuals who ceased treatment after an average of 22 months of suppressive HAART. In 2 of these individuals, the plasma rebound virus was identical or very similar to the replication-competent virus derived from resting T cells. In the remaining 7 subjects, however, the two compartments were clearly different. Zhang et al [182] examined virus from eight individuals who initiated HAART within 3 months of seroconversion and were well suppressed for 30 to 40 months prior to treatment cessation. In 5 of these subjects the plasma rebound virus and latent reservoir virus were indistinguishable, but in 3 individuals the rebound virus was genetically distinct and more closely resembled minor variants found in lymphoid tissue. Interestingly, 2 of the 3 subjects with different rebound and latent viral profiles showed evidence for cryptic replication during HAART, suggesting different origins of rebound virus in the two groups of subjects. A third study by Imamichi et al [77] focused on three patients who started

HAART during chronic infection and were virally suppressed for at least one year prior to treatment interruption. In this study, the sequences found in rebounding plasma virus from all subjects were also found in virion RNA derived from PBMC coculture and were closely related to the replicating plasma virus present before starting HAART. Different results were recently obtained by Kulkosky and coworkers [91], who studied three individuals who received intensified HAART and immune stimulation with anti-CD3 antibody and IL-2 prior to treatment withdrawal [89]. The rebound virus from the first patient most resembled that found in PBMC prior to treatment whereas that found in the second patient was related to the virus induced by anti-CD3 and IL-2. By contrast, the rebound virus in the third patient was unique, suggesting that it emerged from a latent viral pool present in PBMC at low frequency or in a distant tissue site.

These inconsistent results probably reflect genuine heterogeneity in the treatment history, immune status, and viral quasispecies present in different infected individuals. However, there are two important caveats in interpreting such data. First, sequence identity alone cannot prove that the latent reservoir is the source of rebound virus since both pools could have arisen from a different but still unidentified source. Second, lack of sequence identity is also inconclusive due to the technical limitations of fully sampling the latent reservoir. Moreover, there may be rapid *in vivo* evolution of the rebound virus, thus obscuring its relationship to the latent reservoir virus, which is propagated in vitro.

Summing up, it is now well established that there are significant reservoirs of HIV in resting CD4 T cells and in various anatomical sanctuaries, and that these persist despite lengthy and intensive HAART. It also appears that these reservoirs can kindle viral rebound, although the quantitative extent of their contribution is unclear. Still unexplained, however, is the finding that some rebound virus can *not* be traced to any known cellular or anatomical compartment. That suggests that there are still unidentified sources of virus lurking in the body of the infected person.

STRATEGIES TO INDUCE LATENT PROVIRUS

Because latently infected resting CD4 T cells do not express viral mRNA or proteins, they are impervious to standard therapy and the immune system. Eliminating this reservoir will therefore require agents that can induce the transcription of latent integrated provirus. Such agents could

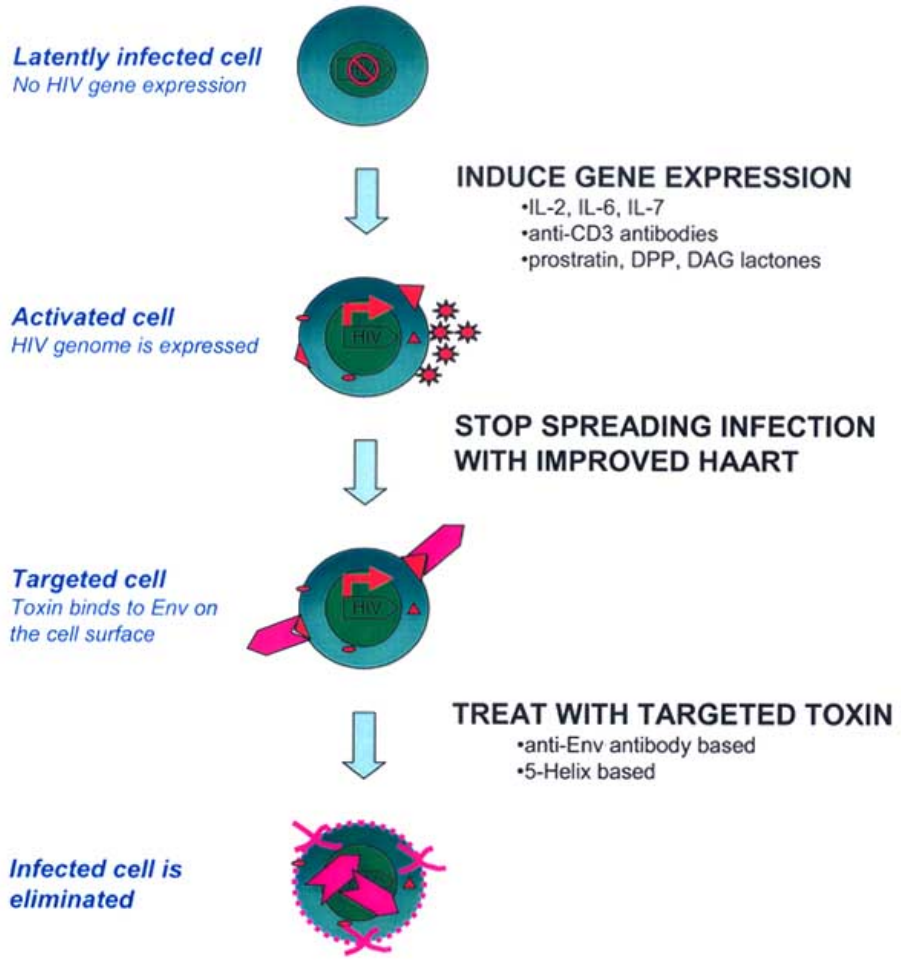

Fig. (1). The "shock and kill" strategy to reduce latent reservoirs of HIV. The first step of the strategy is to reactivate HIV gene expression in latently infected cells. This is performed in the presence of HAART to prevent spreading infection by the newly released virus. The second step of the strategy is to selectively eliminate the HIV infected cells using chimeric toxins targeted to the HIV Env glycoprotein.

be administered in combination with effective HAART to prevent spreading infection by the newly released virus as the first step in a "shock and kill" strategy to reduce the reservoir of latent HIV (Fig. 1). Developing such inducing agents will require better understanding of the mechanism of postintegration latency, an area of research that has been hindered by the low frequency of latently infected cells in infected persons but has recently been given a boost by the development of relevant *in vitro* [81] and animal models [163].

There are two fundamental mechanisms for the repression of HIV gene expression in latently infected cells, *trans*-dominant and *cis*-dominant, that will require different strategies to overcome. 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. By contrast, the lack of HIV gene expression in the *cis* mechanism of latency is due to integration of the provirus into an inactive region of the genome.

Trans-activation

The initiation of HIV gene transcription is regulated by the host transcription factors NFκB, NFAT and Ets family members, which recognize promoter and enhancer sequences in the viral LTR [126,150]. Because all of these factors are more active in proliferating than in resting T cells, latent provirus gene expression can be induced by cytokines, antibodies and drugs that stimulate T cells to divide [169].

Cytokines are key regulators of immune and inflammatory responses, T cell development and HIV gene expression. Chun and colleagues [29] found that a combination of the immunoregulatory cytokine IL-2, together with the proinflammatory cytokines IL-6 plus TNF-α, potently induced HIV replication *ex vivo* in highly purified, latently infected, resting CD4 T cells from both treatment-naïve and HAART-treated individuals. In hope that this would translate into a "flushing out" of the latent reservoir *in vivo*, the same group analyzed a series of patients who received intermittent IL-2 therapy in addition to HAART, and found that their latent reservoirs were significantly lower than a control group who received HAART alone [30]. 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 [44]. A study in Europe also found no effect of IL-2 on virus replication or proviral DNA in peripheral blood [167]. Most important, IL-2 treatment had no effect on the re-emergence of viremia upon cessation of HAART in two studies [89,167]. Thus, although IL-2 appears clinically useful for restoring CD4 T cell numbers and function [123,125], it does not reduce the size of the latent reservoir in any meaningful way. Perhaps a better prospect is IL-7, which in model systems induces latent HIV expression with minimal side effects and may have the added benefit of stimulating T cell generation [60,120,121,161].

Another way to induce T cell differentiation and latent HIV expression is to stimulate the T cell receptor complex with antibodies to CD3. This method is routinely employed *in vitro* and has also been attempted *in vivo* in two clinical trails. A group in the Netherlands 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 [142]. The experiment was "successful" in the sense that the treatment induced a strong but transient release of serum cytokines and chemokines, CD4 T cell division, and increased HIV RNA in lymph nodes and serum. From a clinical perspective, however, the treatment was a disaster. All of the patients experienced serious side-effects, including a near death due to renal failure and seizures. Moreover, even though there was no beneficial effect on the latent reservoir [171], the subjects experienced long-lasting CD4 T cell depletion which was not restored even two years after treatment – an iatrogenic case of an AIDS-like syndrome that might have been predicted in view of the ability of high dose OKT3 to deplete lymphocytes [147] and the lack of theoretical rational for high-level T cell stimulation [57]. Recently Kulkosky and colleagues [89] conducted a more carefully planned and monitored 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 infusion of 0.4 mg of OKT3, a low dose known to cause T cell activation but not depletion, 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. Interestingly, one of the subjects showed a fluctuating pattern of viremia following treatment interruption, suggesting that he was on the borderline between remission and relapse.

HIV gene expression can also be induced by protein kinase C (PKC), a family of at least 12 serine/threonine-specific isozymes that lies downstream of the T cell receptor complex in the T cell differentiation pathway. PKC activates both NFκB, which binds to the enhancer region of the HIV-1 LTR [118], and AP-1, which can bind either to the enhancer cooperatively with NFκB or to downstream sequence elements in concert with the CREB and ATF transcription factors [82,149,179]. It may also increase HIV-1 gene expression by phosphorylation of the virally encoded TAT transcription factor and cellular TAR-binding factors [71,79].

PKC is an appealing target for therapeutics because it can be activated by a number of small molecule analogs of its physiological regulator, 1,2-diacylyglycerol (DAG), which binds to the C1 regulatory domain of PKC, thereby exposing the catalytic domain by displacing a negative regulatory pseudosubstrate region and inducing translocation to the inner leaflet of the cellular membrane. One such analogue is prostratin (12-deoxyphorbol 13-acetate), a nontumor promoting phorbol ester from *Pimela prostrata* [21] that was subsequently reisolated and identified as an anti-viral constituent of the Samoan medicinal plant *Homolanthus nutans* [68]. Prostratin activates HIV-1 replication both in latently infected cell lines [65,68] and in

primary cells isolated from both HIV-1 infected humans [88] and SCID-hu (Thy/Liv) mice [87]. Moreover, although prostratin is a mitogen in mononuclear phagocytes [88], it can activate HIV-1 in quiescent T cells without causing cellular proliferation [87]. Prostratin-containing extracts of *Homolanthus nutans* are used by healers in Western Samoa as a traditional remedy for illnesses such as yellow fever, and preclinical testing of the purified compound is underway. Although the preliminary results with prostratin are encouraging, its clinical potential is hampered by low potency, requiring high and potentially toxic concentrations for HIV induction. Recently it was shown that the closely related compound DPP (12-deoxyphorbol 13-phenylacetate), a non-tumor promoting phorbol ester isolated from the West African "candle plant" *Euphorbia poissonii* [49] and the Moroccan succulent *Euphorbia resinifera* Berg. [74], induces HIV-1 gene expression in latently infected ACH-2 T cells at concentrations 20- to 40-fold lower than prostratin, probably due to its more lipophilic side chain structure [14].

Despite the appeal of using naturally occurring medicinal compounds such as prostratin and DPP as human therapeutics, they have a number of undesirable side effects including downregulation of CD4 and CXCR4, local irritation, tumor promotion, platelet aggregation, and release of inflammatory cytokines. They also are not easily accessed synthetically because of their complex structures. In an attempt to develop more therapeutically useful HIV inducers, Hamer et al [69] explored the use of synthetic analogues of DAG that were synthesized on a five-member ring platform that reduces the entropy of binding to the C1 domain of PKC [108]. By varying the alkyl side-chains of these synthetic DAG lactones, it was possible to maximize their potency for induction of HIV replication while minimizing some of their side effects such as CD4 and CXCR4 downregulation and TNF-α upregulation, leading to significantly higher therapeutic ratios than for naturally occurring compounds such as prostratin and DPP. The two lead compounds were shown to regulate a series of PKC-sensitive genes involved in T cell activation and to be capable of inducing viral gene expression in total and purified resting PBMC from HIV-infected individuals. They also rendered latently infected T cells sensitive to killing by an anti-HIV immunotoxin [69].

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 – a phenotype they have in common with AIDS. Of particular concern is the ability of these compounds to release inflammatory cytokines, potentially leading to respiratory distress syndrome, hypotension and other toxicities [142]. 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. If the latter is true, as is suggested by the differential effects of the DAG lactones, it may yet be possible through combinatorial chemistry to discover compounds with sufficient specificity for clinical use.

The ideal inducing agent would be completely specific for HIV and have no effects on cellular gene transcription. The logical targets are the virally encoded factors Rev, which mediates the nuclear export of intron-containing viral mRNA, and Tat, which enhances the efficiency of transcriptional elongation by binding to the transactivation response RNA stem-loop (TAR) and recruiting the positive transcription elongation factor P-TEFb, which in turn phosphorylates the carboxyl-terminal domain of RNA polymerase II. Although a threshold level of Rev is required for HIV replication *in vitro* [141], there is little evidence that Rev plays a rate-limiting *in vivo*. On the other hand, mutations in Tat are known to be present in several cell line models of latency [47,55,56], and an excess of promoter-proximal transcripts is a hallmark of latency *in vivo* [2]. These observations led Lin et al [99] to test the effects of a purified Tat fusion protein on HIV replication in PBMC cultures from subjects on HAART. They observed activation of full length transcript production by the Tat-containing found protein but not by a control protein. This suggests that Tat, which has the ability to enter cells and translocate to the nucleus, could be used as an inducer of the latent reservoir. It remains to be seen, however, whether this is a general phenomenon, and if so what proportion of latently infected cells are responsive.

Cis Activation

HIV transcription is strongly influenced by the accessibility of the viral promoter and enhancer sequences to specific activators, general transcription factors and RNA polymerase, which in turn depends on nucleosome positioning, higher order chromatin structure, and chromosomal location. The possibility that such *cis*-dominant effects play a role in viral latency is an active area of research.

Although HIV integrates into the host genome in a quasi-random fashion with regard to precise sequence, there is increasing evidence for some degree of selectivity in target selection *in vitro*. Schroder and colleagues [159] productively infected a human lymphoid cell line with HIV or an HIV vector, then cloned and mapped 524 integration sites on the human genome sequence. They found that HIV was preferentially inserted into actively transcribed genes, especially those activated by HIV infection, and that local hotspots contained a disproportionately high percentage of all integration events. In a control experiment, HIV integration into naked genomic DNA showed no such preferential localization, thus demonstrating the role of chromatin structure and transcription in integration selectivity. In the converse of this experiment, Jordan et al [81] infected a lymphocytic cell line with recombinant viruses expressing green fluorescent protein (GFP) and selected for latently infected cells on the basis of being GFP^- in the absence of a transcriptional inducer and GFP^+ in the presence of inducer. These clones frequently contained HIV integrated in or nearby to heterochromatic regions of the genome containing alpha-satellite sequences, which was precisely the opposite result observed in a productive infection. Randomly selected integrants did not show such preferential targeting.

These *in vitro* results suggest that HIV can establish a latent infection as a consequence of integrating into an inactive, heterochromatic region of the genome. It is not yet

clear, however, whether this mechanism operates *in vivo*, and if so the proportion of the latent reservoir that it accounts for. This question could in principle be addressed by analyzing the relationship between integration sites and HIV transcription rates in infected cells from HAART-controlled individuals, but this would be a technically demanding experiment due to the necessity of analyzing RNA levels and DNA integration sites at the single cell level. Simply isolating bulk resting T cells and analyzing their HIV integration sites would not give meaningful data since there is likely to be a combination of *cis* and *trans*-dominant mechanisms involved in latency.

The most extensively studied mechanism of *cis*-acting repression of HIV transcription is histone deacetylation. 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 [83,95,96,143]. Margolis and colleagues have shown that the HIV LTR contains binding sites for the ubiquitous host factor LSF, which, via another host factor called YY1, recruits histone deacetylase to integrated LTR sequences and remodels chromatin [35,73,152]. In an elegant application of this finding, pyrrole-imidazole polyamides that specifically target the LSF-binding sequences in the LTR were synthesized and shown to activate HIV expression both in cell lines [34] and in purified resting T cells from infected individuals (David Margolis, personal communication).

In summary, a good deal is now known about how HIV establishes latency and thus avoids conventional therapeutics and the immune system. However, this knowledge has not yet resulted in a clear strategy to flush out the virus from its hiding places or any clinically useful compounds. Attacking *trans*-dominant latency is inherently difficult because of the many side-effects of activating cellular factors involved in immune responses and other important physiological processes. Attacking *cis*-dominant latency is questionable since this mechanism probably accounts for only a small portion of the latent reservoir. Urgently needed are pharmacologically accessible compounds that can (i) specifically bind to viral but not host transcriptional regulatory sequences, perhaps through genetically engineered sequence-specific recognition domains; (ii) recognize HIV even when the provirus is integrated in heterochromatin, perhaps through a high binding affinity or chromatin-modifying domain; and (iii) potently activate transcription, perhaps through a domain that interacts with ubiquitous activator proteins or general transcription factors. Such compounds can be designed on paper, but making them in real life is so far elusive.

STRATEGIES TO KILL INFECTED CELLS

Once viral replication is effectively blocked and the latent reservoir is activated, the next step is to destroy all remaining infected cells. Simply waiting for viral-induced apoptosis to destroy the infected cells will not be sufficient since many important targets for HIV, such as macrophage, are resistant to viral cytopathology. Although Magnani and colleagues have presented evidence that HIV-infected macrophage can be selectively eliminated by brief exposure to Fludarabine, a potent antilekemic nucleoside analog that inhibits the STAT1 pathway [105], there is no similar agent available for T cells. Nor is the immune system up to the task of eliminating the reactivated cells, especially given the decline in HIV-specific T cells that occurs during HAART [3,43,130]. Instead, a more directed "shock and kill" strategy is required (Fig. 1). This strategy requires agents that that specifically recognize and potentiate the killing of cells that express HIV proteins, peptides or RNAs.

Targeted toxins, also known as immunotoxins, represent a conceptually straightforward approach for killing activated HIV infected cells (reviewed in [10,134,172]). These toxins are bifunctional molecules that consist of two domains: a binding domain that recognizes the HIV envelope glycoprotein Env 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 aeruginoias* 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 and specificity to a region of Env that is highly conserved between different clinical HIV isolates. In addition, they must display suitable pharmacokinetic properties including minimal nonspecific toxicity to uninfected cells and stability in the circulation.

Three different types of Env-binding domains have been explored. The first consisted of the soluble amino-terminal region of CD4, the cell surface receptor for HIV, which binds to a pocket in the external subunit of Env, gp120 [92]. Initial *in vitro* testing of a CD4-PE toxin, which was produced in bacteria by recombinant DNA technology, was encouraging. The molecule was found to kill both lymphocytes and monocyte/macrophages expressing Env, to be capable of stopping a spreading HIV infection in an acutely infected T cell line, to have activity against primary as well as laboratory adapted HIV isolates, and to act synergistically with reverse transcriptase inhibitors [4,5,7,8,26,84]. Nevertheless, clinical trails of CD4-PE on chronically infected individuals were unsuccessful due to a dose-limiting hepatotoxicity at approximately 10 :g/kg and a short half-life of 2 to 4 hours. This made it impossible to achieve therapeutic concentrations of the agent in serum, and no reductions in viral load were observed [37,144]. Subsequently the CD4-PE clinical program was terminated and there was a decline in interest in this approach to therapy. Berger and Pastan have speculated that the observed toxicity of CD4-PE was due to reaction with shed, glycosylated gp120 followed by non-specific internalization by a hepatocyte asialoglycoprotein receptor, a mechanism that should not occur in HAART-controlled individuals [10]. An alternative possibility is that CD4, a ubiquitous receptor expressed on many different cell types, recognizes a subset of normal liver cells.

Antibodies are the most common source of Env-binding domains for anti-HIV immunotoxins. *In vitro* testing has been performed on molecules recognizing a variety of epitopes on both gp120 and gp41 including the CD4 binding site, CD4-inducible coreceptor binding site, and different variable loops. Both single-chain (scFv) and disulfide-linked (dsFv) variable regions and intact

immunoglobulins have been employed; the former allow recombinant DNA production but have a short half-life *in vivo* whereas the latter are more stable but must be chemically linked to the toxin [4,6,9,111,114,134,135, 137,172].

Because immunotoxins have an instrinsic non-specific toxicity against uninfected cells, it is crucial to develop binding domains with the highest possible affinity and specificity for Env, an endeavor that can be assisted by detailed structural information about the parental antibody. For example, McHugh et al. [114] used three-dimensional structural information and phage selection data to design a series of single and multiple point mutations in the antibody variable region sequences of an immunotoxin based on 3B3/b12, one of the few human antibodies that neutralizes a wide variety of primary HIV-1 isolates. They found that altering residues in the first and third complementarity-determining regions of the heavy chain increased the potency of the immunotoxin by approximately 10-fold due to both a higher affinity for monomeric and cell surface Env and increased stability against aggregation. Conversion to a disulfide-linked two-chain format further stabilized the protein while retaining its ability to bind to Env from multiple viral isolates, to inhibit Env-mediated cell fusion, and to limit spreading viral infection in peripheral blood mononuclear cells. Recently it was shown that altering the carboxyterminal sequence of the PE moiety of this immunotoxin to the consensus endoplasmic reticulum consensus sequences KDEL further increases its activity against spreading infection of HIV in multiple different blood cell types [104].

The targeted toxins of the future may utilize rationally designed proteins rather than antibodies to target infected cells. In the first example of this approach, Root and Hamer [153] constructed a chimeric protein in which PE is joined to 5-Helix, a designed protein in which five of the six helices that constitute the gp41 trimer-of-hairpins are covalently linked into a single polypeptide [154]. Because 5-Helix lacks a third C-peptide segment, it has a high affinity for the C-terminal region of the gp41 ectodomain – a region well conserved across HIV isolates. The resulting toxin recognized cells expressing Env from a broad spectrum of HIV-1 strains including primary isolates from clades B, D, E, G, and H, and blocked spreading infection while still maintaining potent inhibitory activity against membrane fusion. This type of rational protein design has many potential advantages including the possibility of systematically reducing non-specific binding to uninfected cells and of tailor making recombinant toxins specific for the latent virus present in a particular infected individual [153].

The new generation of HIV-specific targeted toxins are in the early stages of preclinical development. Goldstein and colleagues [64] analyzed the ability of the gp120-specific immunotoxin 3B3-PE [6] to augment suboptimal HAART in the thy/liv SCID-hu mouse model of acute HIV infection. Although the immunotoxin by itself had little effect, mice that were treated with both a low dose of HAART and the immunotoxin suppressed viral production for a full month after the end of treatment. Pincus and collaborators [136] tested several anti-HIV immunotoxins targeted to gp120 or gp41 in a model of spreading HIV infection in which SCID mice are injected with a mixture of HIV-infected and HIV-susceptible human CD4 tumor cells. They found the strongest anti-HIV effects using a combination of a ricin-based anti-gp41 immunotoxin, 41.1-RAC, and a tetrameric CD4-human Ig fusion protein; the later both potentiates the activity of the immunotoxin by making gp41 more accessible and also acts as a fusion inhibitor. In order to model HIV latency, Brooks et al [15] took advantage of the observation that cellular and HIV RNA expression decrease dramatically during human thymocyte maturation in the thy/liv SCID-hu mouse model of HIV infection, thereby generating an abundant and stable source of primary cells with a quiescent phenotype in which inactive virus can be induced by cellular stimulation [16]. They found that purified latently infected cells were killed by a potent variant of the 3B3-PE immunotoxin [114] only after costimulation with antibodies to CD3 and CD8 or following treatment with prostratin or IL-7, which activated the reporter virus without inducing cell division. Importantly, the latter treatment was demonstrated to leave uninfected cells capable of responding to subsequent stimulation, suggesting functional preservation of the immune response [15].

These preclinical results have revived interest in using targeted toxins to reduce the latent reservoir of HIV. The use of anti-CD45RO or anti-CCR5 immunotoxins to reduce the target population for latent HIV infection has also been proposed [17,112,156] but might prove too toxic for practical use. In either case, it is clear from the experience with CD4-PE that extensive safety and efficacy testing in animals, including SHIV- or SIV-infected nonhuman primates, will be absolutely essential before such agents are introduced into humans.

THE NEED FOR NOVEL APPROACHES TO SUPRESS REKINDLING

Suppose that it were possible to induce 99% of all latent virus, to kill 99% of all cells expressing HIV proteins, and to block 99% of new infections. Would this sort of "triple whammy" be sufficient to give stable remission? Probably not. Calculations suggest that there would still be sufficient viral replication and reseeding of the reservoirs to cause viral rebound upon cessation of treatment. Unfortunately, this prediction has been borne out by clinical trails; even in heavily treated patients in whom no replication competent virus could be detected by the most sensitive available methods, viral rebound occurred within a few weeks or months of stopping HAART [36,89]. Urgently need are novel approaches that, without daily antiretroviral medication, suppress rekindling of infection by residual virus sequestered in anatomical and cellular reservoirs.

The most widely discussed and researched tactic is to reinforce the body's own immune defenses through methods such as autovaccination by structured treatment interruption (STI) or therapeutic vaccination with inactivated viral particles, recombinant viruses, or DNA. To date, none of these approaches has been reproducibly successful either in animal studies or controlled human clinical trials, and STI has been abandoned due to the potential to generate drug-resistant virus [1,110]. HIV uses multiple strategies to evade both humoral and cellular immunity (reviewed in

[129,133]). Moreover, recent results suggest that boosting HIV-specific CD4 helper cells, which are considered vital for the development and maintenance of anti-HIV CD8 cytotoxic T lymphocytes (CTLs), may actually help the virus more than the host due to the ability of HIV to preferentially grow and evolve in this compartment ([42]; Dominik Wodarz, personal communication).

It may therefore be necessary to improve on natural immunity by genetic engineering. One idea is to generate HIV-specific CTLs that recognize a highly conserved HIV epitope and kill infected cells independent of CD4 cell help or MHC. Toward this end, several researchers have generated "universal" chimeric T cell receptors in which the ζ (zeta) subunit of CD3, the cytoplasmic portion of the molecule involved in signal transduction, is joined to a polypeptide that recognizes Env; e.g., a gp120-specific scFv or the transmembrane and extracellular portions of CD4 [148,151]. In preclinical studies, CD8 T cells expressing the universal T cell receptor genes have demonstrated antigen-specific proliferation, cytokine production, and cytolytic activity against HIV-infected cells equivalent to that observed for naturally occurring anti-HIV CTLs [178]. Phase I/II clinical trials of a CD4ζ chimera have generated tantalizing but not definitive results [38,116,175]. Patients infused with large numbers of syngenic or autologous T cells transduced with a retrovirus vector carrying CD4ζ maintained the genetically marked cells for up to one year in PBMC, rectal biopsies, and gut associated lymphocytes. Two studies found decreases from baseline in HIV burden in some reservoir assays, a trend toward fewer patients with recurrent viremia, and reductions in rectal tissue-associated HIV RNA [38,116]. However, the major clinical endpoints of reduced viral load or significant depletion of viral reservoirs were not achieved.

Genetically engineered microbes represent a novel approach to HIV therapy. The idea is to purposefully infect people with bacteria or yeast cells that express anti-HIV proteins, peptides, or drugs, either as secreted products or on the cell surface, with the hope of protecting against initial infection or rebound of existing virus. In an early example of this strategy, Chang and colleagues [24] engineered a natural vaginal isolate of *Lactobacillus jensenii* to secrete soluble CD4, and demonstrated that the resulting bacteria could inhibit HIV infection *in vitro*. This approach could be extended in several useful ways such as the use of potent, broadly neutralizing scFvs, designed protein inhibitors such as 5-Helix, and fusion-inhibiting peptides. The range of microbes could be extended to include natural commensal inhabitants of the gut, rectum, and oral cavity, or organisms that have been deliberately altered to better colonize human beings.

CONCLUSION: BE THERE FOR THE CURE

Although we are still far from a cure for HIV infection, our increasing knowledge of the mechanisms of viral persistence focus attention on the most pressing needs: better HAART, specific inducers of latent viral gene expression, agents that can kill infected cells and prevent new cycles of infection without constant medication, and more sensitive methods to detect the very low levels of virus remaining in drug-treated individuals. Achieving these goals will not be simple. Due to its relatively poor transmission frequency and long latency, HIV has been subject to strong evolutionary selection for persistence in human beings. Given the high replication and mutation rates of HIV, it is not surprising that the virus is so far winning the evolutionary race against its human host. Nevertheless, the outstanding success of HAART gives us some important hints on how to achieve stable remission.

First, many drugs are better than one. Even though monotherapy with one antiretroviral drug doesn't work, a combination of several drugs can dramatically reduce viral loads. The take-home message is to combine treatments rather than rely on any single compound.

Second, it's better to target viral rather than cellular gene products. One reason that HAART is more specific and successful than cancer chemotherapy is that HAART inhibits enzymes such as reverse transcriptase that play no role in normal cellular metabolism whereas chemotherapy targets normal cellular components such as DNA polymerase. The same principle will apply towards drugs for curing HIV infection.

Third, hit the virus with something it has never seen before. Drugs such as the protease inhibitors are successful in part because they have no close analogs in nature and therefore HIV has had little opportunity to evolve countermeasures. By contrast, HIV has been fighting – and winning – against the natural immune system ever since the virus first emerged. It is unlikely that minor tinkering with the immune system by measure such as STI or therapeutic vaccination will overcome the virus's intrinsic advantage in this regard.

The final lesson from HAART is economic rather than scientific. One of the reasons for the rapid development of antiretroviral drugs was financial: there was money to be made in life-long treatment of HIV infection. This is not necessarily true for a cure, which would actually deprive the pharmaceutical industry of a profitable market; this may help to explain the apparent paucity of commercial investment in this field. It is up to government and the non-profit sector to take up the slack in funding of this area, just as it is up to basic and clinical scientists to pursue the knowledge that will be necessary to achieve stable remission of HIV infection. The ultimate benefit of a cure for HIV will be measured not in the dollars that it generates but in the lives that it saves.

ACKNOWLEDGEMENTS

I thank Claude Klee, Daniel Douek, David Margolis, Joseph Kovacs, Nitin Saksena, Roger Pomerantz, and members of the Hamer lab for their useful comments and suggestions on the manuscript.

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Received: 5 December, 2003 Accepted: 29 January, 2004