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RESERVOIRS FOR HIV-1: Mechanisms for Viral Persistence in the Presence of Antiviral Immune Responses and Antiretroviral Therapy

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■ **Abstract** The success of combination antiretroviral therapy for HIV-1 infection has generated interest in mechanisms by which the virus can persist in the body despite the presence of drugs that effectively inhibit key steps in the virus life cycle. It is becoming clear that viral reservoirs established early in the infection not only prevent sterilizing immunity but also represent a major obstacle to curing the infection with the potent antiretroviral drugs currently in use. Mechanisms of viral persistence are best considered in the context of the dynamics of viral replication in vivo. Virus production in infected individuals is largely the result of a dynamic process involving continuous rounds of de novo infection of and replication in activated $CD4^+$ T cells with rapid turnover of both free virus and virus-producing cells. This process is largely, but not completely, interrupted by effective antiretroviral therapy. After a few months of therapy, plasma virus levels become undetectable in many patients. Analysis of viral decay rates initially suggested that eradication of the infection might be possible. However, there are several potential cellular and anatomical reservoirs for HIV-1 that may contribute to long-term persistence of HIV-1. These include infected cell in the central nervous system and the male urogenital tract. However, the most worrisome reservoir consists of latently infected resting memory $CD4^+$ T cells carrying integrated HIV-1 DNA. Definitive demonstration of the presence of this form of latency required development of methods for isolating extremely pure populations of resting $CD4^+$ T cells and for demonstrating that a small fraction of these cells contain integrated HIV-1 DNA that is competent for replication if the cells undergo antigen-driven activation. Most of the latent virus in resting $CD4^+$ T cells is found in cells of the memory phenotype. The half-life of this latent reservoir is extremely long (44 months). At this rate, eradication of this reservoir would require over 60 years of treatment. Thus, latently infected resting $CD4^+$ T cells provide a mechanism for life-long persistence of replication-competent forms of HIV-1, rendering unrealistic hopes of virus eradication with current antiretroviral regimens. The extraordinary stability of the reservoir may reflect gradual reseeding by a very low level of ongoing viral replication and/or mechanisms that contribute to the intrinsic stability of the

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memory T cell compartment. Given the substantial long-term toxicities of current combination therapy regimens, novel approaches to eradicating this latent reservoir are urgently needed.

INTRODUCTION

Many viruses have evolved mechanisms for establishing persistence in the host. Typically, persistence involves viral reservoirs, cellular or anatomical sites in which the virus can persist despite antiviral immune responses. Some viruses establish a reversibly nonproductive (or latent) state of infection that allows escape from host immune mechanisms. For HIV-1, mechanisms of persistence and latency have been the subject of much recent interest. This interest reflects the fact that successes in antiretroviral therapy have raised, for the first time since the beginning of the AIDS epidemic, the possibility that the infection might be curable (1). Inhibitors of HIV-1 reverse transcriptase and protease, when used in multiple drug combinations, cause dramatic reductions in viremia in many patients. Plasma virus levels often fall to below the limit of detection of current assays, around 20 copies of genomic viral RNA/ml (equivalent to 10 diploid virus particles/ml) (1–3). Striking decreases in morbidity and mortality have been documented since the introduction of the protease inhibitors (4). However, the goal of curing HIV-1 infection has not yet been achieved, and it is becoming clear that the viral reservoirs established early in the infection not only prevent sterilizing immunity but also represent a major obstacle to curing the infection with the potent antiretroviral drugs currently in use. This review describes recent studies of reservoirs for HIV-1. For a discussion of earlier work on viral reservoirs and latency in HIV-1 infection, the reader is referred to several comprehensive reviews (5–12).

THE NATURAL HISTORY OF HIV-1 INFECTION

As a prelude to the discussion of viral reservoirs, the natural history of HIV-1 infection and the dynamics of infection at the cellular level are briefly reviewed. Clinically, HIV-1 infection may be divided into three phases. During the initial phase, known as primary HIV-1 infection, virus present in the infecting inoculum replicates in the host, infecting cells that express both CD4 and the appropriate coreceptor, usually the chemokine receptor CCR5 (see 13 for a review of HIV-1 entry). Even early in infection, $CD4^+$ T lymphocytes appear to be the major target cells (11). Viremia develops within the first few weeks of exposure, accompanied by infectious mononucleosis-like symptoms in some patients (14). Studies of viral dynamics during primary HIV-1 and SIV infection suggest that virus populations double every 6–10 h in the initial stages of the infection, with each

infected cell giving rise to approximately 20 new infected cells (229, 230). During symptomatic primary infection, levels of infectious virus and of infected cells in the circulation are both very high (15). The concentration of virus particles in the blood is often $>10^6$/ml and can be as high as 10^8/ml. The initial systemic seeding of the peripheral lymphoid organs with HIV-1 occurs as a result of the high levels of viremia that develop during primary HIV-1 infection. Interestingly, studies in the simian immunodeficiency virus (SIV) and simian/human immunodeficiency virus (SHIV) models of HIV-1 infection suggest that the large pool of $CD4^+$ T cells in the gut-associated lymphoid tissue (GALT) is an important site for viral replication in primary infection as well as during subsequent phases of the disease (16, 17). This likely reflects the fact that a relatively high fraction of $CD4^+$ T cells in the GALT are in an activated state and are therefore permissive for HIV-1 replication.

Within a few weeks, the level of virus in the blood declines. This decline coincides with the development of an immune response to HIV-1. Virus-specific cytolytic T lymphocytes (CTL) appear early and may represent a critical host factor in the control of primary HIV-1 infection (18–21). Recent studies in the SIV model have convincingly demonstrated the importance of $CD8^+$ CTL in the control of the viremia of primary infection. Monkeys depleted of $CD8^+$ T cells with monoclonal antibodies are unable to control primary infection (21). The combined effects of CTL and other elements of the immune response cause the amount of virus in the blood to decrease to a lower plateau level or set point. However, as is discussed below, sterilizing immunity is never achieved.

The second phase of HIV-1 infection is the long asymptomatic period between primary infection and the development of clinical immunodeficiency (AIDS). There are two related pathophysiologic characteristics of the asymptomatic phase: ongoing viral replication in the peripheral lymphoid tissues and gradual loss of $CD4^+$ T cells. Although the asymptomatic phase may represent a period of clinical latency, the virus replicates continuously during this time. Free virus can be detected readily in the circulation by appropriate methods (22), although the levels are lower than those observed during symptomatic primary HIV-1 infection or, in patients with AIDS, the third and final stage of the infection. Infected cells are readily detectable in the peripheral lymphoid tissues, and their number correlates directly with plasma virus levels, suggesting that the lymphoid tissues are a major source of plasma virus (23–27). The rate of decline in $CD4^+$ T cells appears to be determined by the level of ongoing viral replication, as patients with higher plasma virus set points progress to AIDS more rapidly (28). The mechanisms underlying CD4 depletion are incompletely understood but probably include a decrease in T cell production by the thymus and an increase in the rate of destruction of T cells in the periphery (29). For a discussion of T cell dynamics in HIV-1 infection, the reader is referred to several excellent reviews (11, 30).

The final phase of the infection is characterized by the emergence of clinical immunodeficiency. In the year or two before AIDS develops, there is often a more rapid decline in $CD4^+$ T cells. This decline may be preceded by an increase in

viral load (31), with viral replication occurring in many sites in addition to the lymphoid tissue (32). In some cases disease progression is associated with the evolution of more pathogenic viral species that utilize the chemokine receptor CXCR4 instead of CCR5 (33–36). As the CD4 count falls below 200 cells/ul, opportunistic infections begin to occur. The degree of CD4 decline is an excellent predictor of the risk for particular infections, providing strong support for the notion that the loss of $CD4^+$ T cells is the central cause of immunodeficiency in this disease.

VIRAL DYNAMICS

The pathophysiology of HIV-1 infection and the mechanisms of viral persistence are best considered in the context of the dynamics of viral replication in vivo. Understanding viral dynamics requires a steady-state analysis of the amount of free virus and the number of virally infected cells present in infected individuals (the viral load) and a dynamic analysis of the rates at which virus particles and virally infected cells are generated and cleared. Substantial progress has been made in understanding key elements of HIV-1 dynamics, and the paradigms developed have already proven useful in understanding the pathogenesis of other infectious diseases (37).

Extent of Infection of $CD4^+$ T Cells

Different experimental approaches have been used to measure infected cells, including limiting dilution or focal analysis of the frequency of cells in the peripheral blood capable of producing infectious virus (38–40), PCR analysis of viral DNA in cells in peripheral blood (41–48), lymph nodes (23–25, 48) and the central nervous system (49), and analysis of viral mRNA species in productively infected cells (27, 32, 50–53). Unfortunately, there is still tremendous uncertainty over the fundamental question of what fraction of $CD4^+$ T cells are infected, with widely divergent answers coming from different experimental approaches. Part of the confusion results from the fact that many of the experimental approaches used to study this issue do not provide information about the distribution of virus between latent and active states, between defective and replication-competent forms, between $CD4^+$ T cell and macrophage compartments, and between circulating and tissue sites. All of these factors are important in understanding viral load.

Determining the fraction of $CD4^+$ T cells infected in vivo is important because it bears on the critical question of what causes the loss of $CD4^+$ T cells that leads to immunodeficiency. Because HIV-1 is cytopathic for T cells in vitro, it was originally presumed that direct viral cytopathic effects were responsible for CD4 depletion. However, subsequent studies of viral load in HIV-1 infection have raised significant questions about whether the fraction of cells infected is high enough to account for the depletion of the entire $CD4^+$ T cell compartment. An

early in situ hybridization study suggested that even in patients with AIDS, only in a small fraction (< 0.01%) of the total pool of lymphocytes was productively infected, as judged by expression of high levels of viral mRNA (50). Subsequent PCR studies detected HIV-1 DNA in a larger fraction of $CD4^+$ T cells and were interpreted as indicating that most infected cells were in a state of latent infection. Nevertheless, in most conventional DNA PCR studies, cells carrying HIV-1 DNA have been detected only at low frequency (0.01–0.1%) among peripheral blood mononuclear cells (PBMC) of patients in the asymptomatic phase of the infection (42, 43). Studies using in situ PCR have detected HIV-1 DNA in a much higher fraction of cells (as high as 1–40% of PBMC or lymph node cells) (24, 45, 47). The reasons for the discrepancy are unclear. Both conventional and in situ methods may detect both cells with integrated HIV-1 provirus and recently infected cells in which the HIV-1 genome has been reverse transcribed but is not yet integrated (see below). The in situ PCR results are at odds not only with conventional PCR assays but also with virus culture studies in which a much lower frequency of virally infected cells is typically detected (38). Careful analysis of the fraction of PBMC from which virus can actually be cultured has shown that cells harboring replication-competent provirus are actually rare (<0.01%) (38, 40). Since productive infection requires integration of reverse transcribed viral DNA into the host genome (54, 55), the extent of infection can also be addressed by the analysis of the frequency of $CD4^+$ T cells carrying integrated provirus. Studies using inverse PCR to detect the junction between integrated HIV-1 proviruses and host chromosomal DNA have shown that in individuals in the asymptomatic phase of infection, integrated virus is found in <0.01% of $CD4^+$ T cells in the peripheral blood and lymph nodes (40, 56).

It is important to keep in mind that there may be differences in viral burden in different tissues. Lymphocytes in the circulation represent only a small fraction (<2%) of the total lymphocyte pool. During the asymptomatic phase of the infection, the proportion of cells carrying HIV-1 DNA is three- to tenfold higher in the lymph node than in the peripheral blood (23). This difference may be due to the fact that activated $CD4^+$ T cells, which are the primary targets for productive HIV-1 infection, tend to remain in the lymphoid tissues due to expression of high levels of adhesion molecules. The proportion of resting $CD4^+$ T cells harboring integrated virus is not dramatically different in the blood and lymph nodes, as is consistent with the continual recirculation of resting $CD4^+$ T cells (40). To summarize, although $CD4^+$ T cells productively infected with HIV-1 can be readily detected in the blood and lymphoid tissues of untreated patients with HIV-1 infection, the fraction of $CD4^+$ T cells that carry integrated HIV-1 DNA at any given time is very small.

The First Phase of Decay

The development of rapid and quantitative RT-PCR methods for detecting genomic viral RNA in virions in the plasma has proven to be of extraordinary value in understanding viral load in HIV-1 infection. The critical initial obser-

vation was that HIV-1 RNA was present in the plasma of virtually all infected individuals, even during the asymptomatic phase (22). During this phase, levels ranging from 10^2 to 10^7 copies/ml are seen in different patients. Over the short term, levels are reasonably constant in individual patients, indicating a quasi-steady state (22). Studies of the drug-induced perturbations of these steady-state levels (1, 57–61) have provided the critical insights upon which current models for viral dynamics are based.

In 1995, pioneering studies by Wei et al (58) and Ho et al (57) showed that virus production in infected individuals is largely the result of a dynamic process involving continuous rounds of de novo infection of and replication in host cells, with rapid turnover of both free virus and virus-producing cells. In these studies, nevirapine and ritonavir, potent inhibitors of HIV-1 reverse transcriptase and protease, respectively, were given as single agents to patients with HIV-1 infection, and plasma virus levels were monitored closely. Both types of drugs act to prevent new rounds of infection without blocking virus production by cells that already carry integrated HIV-1 DNA. Both agents produced a dramatic (2 log) drop in plasma virus levels in the first two weeks of therapy (Figure 1). This result indicates that the half-lives of plasma virus and of the cells that produce the vast majority of the plasma virus are both very short.

Perelson and colleagues then attempted to measure separately the two processes that contribute to the rapid initial decay of plasma virus: the clearance of free virions and the loss of the productively infected cells (60). They examined plasma viral levels early after the initiation of therapy and fit the experimental data with a model consisting of a set of differential equations describing the dynamics of infected cells and free virus particles. Because the logarithm of the plasma virus concentration fell linearly with time during the initial treatment period, the decay of plasma virus and of productively infected cells was described in terms of exponential processes. The simplest expression describing the dynamics of HIV-1 production and clearance is:

$$dV/dt = N\delta T^* - cV,$$

where V is the concentration of free virus particles, N is the number of virus particles released by a productively infected cell, δ is the decay rate constant for productively infected cells, T^* is the concentration of productively infected T cells, and c is the decay rate constant for free virus. Using this approach, decay rate constants for free virus (c) and for the cells that produce most of the plasma virus (δ) were calculated. The resulting values for c were strikingly similar in different patients, consistent with the idea that free virus in the plasma has a constant intrinsic decay rate (60). The same was true for δ, the decay rate of productively infected cells. Mean values for c and δ were 3 and 0.5 day^{-1}, respectively. These rate constants are most easily evaluated in the form of a half-lives ($t_{1/2} = \ln 2/c$, $\ln 2/\delta$, respectively). For free virus, the half-life is <6 h. Recent particle infusion studies in the SIV system suggest that virions may last in the plasma for only a matter of minutes before being cleared by unknown mechanisms

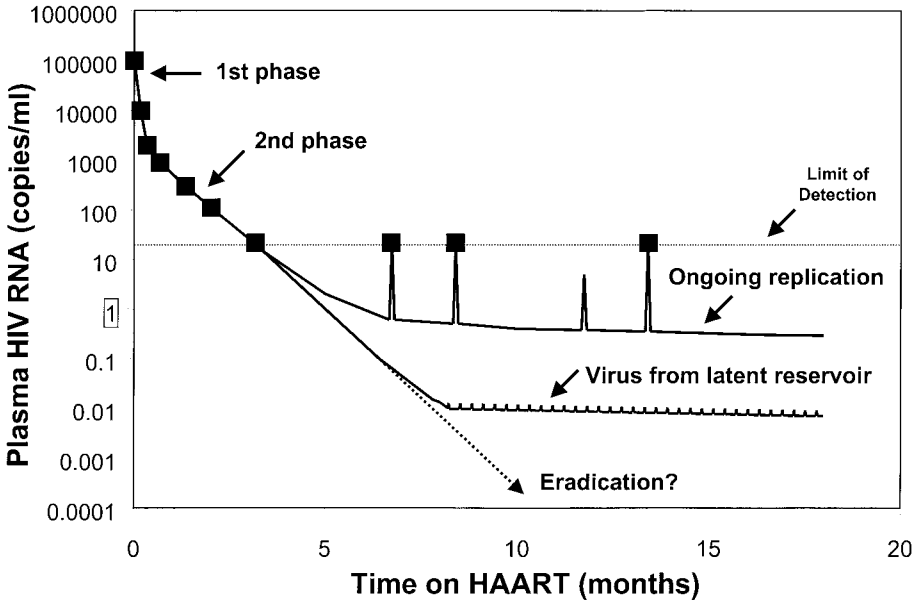

Figure 1 Hypothetical decay curve for plasma virus levels in a patient treated with highly active antiretroviral therapy (HAART). The first phase of decay reflects the short half-life of plasma virus and of the productively infected $CD4^+$ lymphoblasts that produce most of the plasma virus. The second phase of decay reflects the longer half-life of a second population of virus-producing cells. The plasma virus levels fall to a new set point that is generally below the level of detection of current assays. Virus replication continues at a low level, with only very occasional viremic episodes ("blips"). In the hypothetical case of treatment with regimens that completely prevent new infection of susceptible cells by extracellular virus, plasma virus levels would drop further until they reached the extremely low levels associated with activation of cells in the latent reservoir. The intrinsic decay rate of this reservoir remains to be determined.

(62). The most surprising result is that the half-life of the cells that produce most of the plasma virus is only about 1–2 days (60, 61). The lability of both the plasma virus and the cells that produce most of the plasma virus underscores the fact that virus replication is active and ongoing throughout the course of the disease. Direct confirmation of the rapid turnover of productively infected cells has been provided by Haase and colleagues using in situ hybridization analysis of tonsillar biopsies from patients starting antiretroviral therapy (61).

During the asymptomatic phase of the infection, plasma HIV-1 RNA concentrations reach a quasi-steady state, with little change over a time frame of days. Thus, at steady state, $dV/dt = 0$ and $N\delta T^* = cV$, and the plasma virus concentration (V) directly reflects the number of productively infected cells ($V = (N\delta/c)T^*$) (60). Given that the half-life of the cells that produce most of the plasma

virus is 1–2 days, it follows that the steady-state level of viral RNA genomes in the plasma reflects very recent virus production. Thus, the measurement of plasma virus levels provides a real time measure of the rate of replication of the pathogen in the host, something not available in the case of most infectious diseases. As is discussed below, in vivo measurements of virus replication rates have provided important insights into the pathogenesis of the disease.

It is generally assumed that activated $CD4^+$ T cells are responsible for producing most of the plasma virus and that the rapid initial decay in plasma virus levels following the initiation of potent antiretroviral therapy reflects the rapid turnover of these cells. Immunohistochemical studies suggest that in the secondary lymphoid tissues, most productively infected cells are T lymphocytes (11). The mechanisms involved in the rapid turnover of productively infected $CD4^+$ T lymphoblasts remain unclear. HIV-1 is cytopathic for $CD4^+$ T cells in vitro, and the presumption has been that cytopathic effects of HIV-1 on productively infected $CD4^+$ T cells are involved. A number of different mechanisms of cell killing have been invoked (63–67). Of particular interest is recent work showing that the product of the HIV-1 *vpr* gene can induce apoptosis (68, 69). Previous studies had shown that vpr induces arrest of the cell cycle in G_2 (70–74). This arrest may increase the amount of virus produced by $CD4^+$ lymphoblasts (73, 75), but ultimately results in death of the infected cell. These findings on the pro-apoptotic effects of vpr add to previous studies that have focused on the HIV-1 env protein as a principal mediator of cytopathic effects (for a review, see reference 76).

The rapid decay of productively infected cells may also reflect the killing of these cells by host cytolytic effector mechanisms, especially CTL (77, 78). Nowak and colleagues have argued that the frequency of activated effector CTL in vivo is high enough to account for this rapid turnover (79). It is now well established that CTL can actually lyse HIV-1 infected cells (80). The evidence for a beneficial role of CTL has been accumulating and is reviewed elsewhere (81, 82). Perhaps the most direct evidence comes from recent studies showing that viral load increases dramatically following experimental depletion of $CD8^+$ T cells in SIV-infected rhesus monkeys (21, 83). In addition, recent studies using new methods to enumerate antigen-specific CTL have confirmed that the frequency of CTL specific for HIV-1 is indeed very high in many infected individuals (84). On the other hand, it has recently been suggested that the nef protein may function to downregulate class I molecules, thereby protecting infected cells from lysis by CTL (85). Additional studies will be needed to determine whether the short half-life of most productively infected cells is due to lysis by CTL (79, 86), death resulting from cytopathic effects of viral proteins, or both.

Measurement of the viral clearance rate c allows calculation of P, the rate of virus production, using the equation $P = cV$. Figures in excess of 10^{10} virions per day have been reported (60). The high levels of virus produced per day are instructive in several regards (see reference 59). The high rate of virus production, when considered in the context of the short half-life of the cells that produce most

of the plasma virus, suggests that the rate of new infection of $CD4^+$ T cells is substantial. Uncertainty over the number of virions produced per infected cell (the burst size, N) makes it difficult to calculate precisely the number of productively infected cells (T^*) from the steady-state relationship $N\delta T^* = cV$. Nevertheless, it is clear that in an untreated patient, large numbers of $CD4^+$ T cells become infected daily. The reverse transcription process that occurs in each of those newly infected cells has a sufficiently high error rate that viral genomes with every possible single point mutation arise daily (59). Perelson and colleagues have estimated that if 10^8 new cells are infected per day, then not only are all possible single point mutations generated daily, but almost 1% of all possible double mutations are produced each day (87). This finding has enormous implications for understanding the evolution of drug resistance mutations and viral escape from immune effector mechanisms.

The high rate of virus production also has implications for the debate over mechanisms of $CD4^+$ T cell depletion. The characteristics of the first phase of decay suggest that productively infected cells die quickly, but it has been unclear whether this effect alone is sufficient to account for $CD4^+$ T cell depletion. The absolute number of $CD4^+$ T cells that die per day from infection is still unknown. Some of the death of $CD4^+$ T cells in vivo may result from indirect effects on uninfected $CD4^+$ T cells (88, 89). CD4 depletion may also be a consequence of decreased thymic production of T cells (90–92). Recent evidence suggests that thymopoesis continues in healthy adults (93), but this residual thymic function may not be enough to counterbalance the destructive mechanisms. An excellent review of these complex issues has recently been published (94).

The Second Phase of Decay

Although resistance developed in a matter of weeks in the initial studies of nevirapine and ritonavir monotherapy (58), further studies showed that combinations of antiretroviral agents could produce a decline in plasma virus to undetectable levels in many patients (1–3). After the rapid initial decay during the first 1–2 weeks of treatment, plasma virus declined at a slower rate (1). This was interpreted as reflecting the turnover of a longer lived viral reservoir or infected cell population. Perelson and colleagues (1) modeled the second phase of decay by incorporating additional terms into the above equation to account for the low level of virus production by a longer lived population of virus producing cells, M^*, which were assumed to produce virus at a constant rate p. Thus,

$$dV/dt = N\delta T^* + pM^* - cV$$

This long-lived cell reservoir accounts for only a small portion of the total virus production in an untreated individual ($N\delta T^* >> pM^*$) and becomes evident only when the cells that produce most of the plasma virus have largely decayed (Figure 1). The half-life of the compartment responsible for this second phase of decay was estimated to be 1–4 weeks (1). A biphasic decay process was also observed directly in in situ hybridization studies of productively infected mononuclear cells

in the lymphoid tissues (61). Recent studies in children with perinatally acquired HIV-1 infection have shown that the same biphasic decay process occurs in pediatric patients (95).

The nature of the cellular or anatomical reservoir responsible for the second phase in the classic viral decay curve is still unclear. These cells may be macrophages, which at least in vitro are less susceptible to the cytopathic effects of the virus than are $CD4^+$ lymphoblasts (96, 97). Because HIV-1 is less cytopathic for macrophages (97), infected macrophages can in principle continue to release virus for their normal life span. In uninfected individuals, macrophage turnover is balanced by continuous production of new monocytes in the bone marrow. Monocytes circulate for less than a day and enter the tissues where they differentiate into macrophages and persist with a $t_{1/2}$ of about 2 weeks (98), consistent with the kinetics of the second phase. It is currently unclear what fraction of macrophages in different tissues are infected. In most published studies, the fraction of infected macrophages is very low (40, 99, 100). Thus a great deal remains to be learned about the potential role of infected macrophages as a long-term reservoir for HIV-1.

It is also possible that the second phase is due to the turnover of $CD4^+$ T cells that are in a different state of activation. As is discussed below, the virus does not replicate in T cells that are in a resting state. However, there may be partially activated states that are permissive for lower levels of replication, levels that do not cause rapid destruction of the cells (232).

The virus that appears during the second phase of decay may result from the remobilization of free virions trapped on follicular dendritic cells (FDC) in the germinal centers of the peripheral lymphoid tissue (24, 101–104). These cells do not appear to be productively infected (105) but can trap virus particles on their surfaces. This pool of trapped extracellular virions declines with a $t_{1/2}$ on the order of two weeks in patients on effective combination therapy (61). While there is evidence that trapped virions bound to FDC can retain infectivity (106), the bound virus is likely to be complexed with antibody and complement, and it is not clear how this trapped virus can serve as a source for virus in the plasma during the second phase.

Although initial viral dynamic models have been extremely useful in understanding pathogenesis and the response to therapy, some questions remain. Models of viral decay have been built upon the assumption that no new cells are infected once combination therapy has started. This notion has recently been challenged by Grossman and colleagues who have suggested that the death of infected $CD4^+$ T cells is more appropriately modeled as an aging process in which cells that have been infected for a longer period of time have a higher probability of dying. They have argued that the use of an exponential decay model is not appropriate because it does not incorporate this notion that the chance an infected cell will die is related to the length of time a cell has been infected (107, 231). In their model, combination therapy does not completely block new infection of cells. Rather it results in decrease in the amplitude of local bursts of HIV-1 rep-

lication. In support of this assertion, these authors have shown that treatment with five drugs results in a more rapid decay in plasma virus levels that does treatment with three drugs, consistent with the idea that current combination therapy is not stopping all new infection of susceptible cells (231). They go on to develop a model for HIV-1 persistence that involves local bursts of viral replication associated with immune activation that are attenuated but not fully suppressed by combination therapy. An increasing body of evidence suggests that there may be some low level of ongoing viral replication even in patients whose HIV-1 plasma RNA levels are below the limit of detection (see below). In another theoretical paper, Bucy has argued that distinct cell populations need not be invoked to explain the first and second phases of decay (86). He proposes that a change in the rate of immune clearance following the initiation of therapy is sufficient to explain the two phases. In his model, declining antigen load following the initiation of therapy leads to a waning of HIV-1-specific immune responses, a notion for which there is increasing experimental evidence (108, 109). This in turn reduces the rate of clearance of infected cells. Whether the decline in HIV-1-specific immune responses is rapid enough to account for the two phases that are routinely observed is unclear. Although it is likely that viral dynamics can be effectively modeled in several different ways, it remains clear that patients starting a combination therapy regimen experience a very characteristic biphasic decay in plasma virus levels, consistent with the idea of distinct compartments with different kinetics.

The Eradication Hypothesis

After two months of highly active antiretroviral therapy (HAART), the level of plasma virus falls to below the limit of detection (20–500 copies/ml, depending on the assay used) in a fraction of patients. This fraction can be as high as 80–90% in some studies, particularly in previously untreated patients (2, 3). In the general patient population, it is more typically 50–60%. In patients whose plasma HIV-1 RNA levels are below the limit of detection, it becomes difficult to culture the virus from the blood. There has been hope that in these patients, prolonged treatment might lead to eradication. Perelson et al were the first to use an analysis of viral decay rates in treated patients to make rational predictions of treatment times required for virus eradication (1). These investigators attempted to extrapolate the second phase of decay to zero residual infected cells. Since the nature and total body number of cells responsible for virus production during the second phase were unknown, they assumed that the initial number of chronically infected cells could be as high as 10^{12}, equivalent to the total number of lymphocytes in the body. Even if the number of long-lived infected cells is this high, the second-phase decay extrapolates to <1 residual infected cell in about three years. However, the prediction of eradication was made with the caveat that there may be undetected compartments or viral reservoirs that are not measurable by standard techniques (1). It is now clear that the potential of prolonged combination therapy

to eradicate the virus depends on the characteristics of these compartments and long-lived reservoirs for HIV-1.

RESERVOIRS FOR HIV-1

Potential reservoirs for HIV-1 include various types of long-lived infected cells in various locations in the body. For example, replication-competent HIV-1 can be recovered from cells in the seminal fluid of men on combination therapy who had no detectable plasma virus (110). The nature of the cells harboring HIV-1, the extent of infection of these cells, and the half-life of the cells remain unclear. Nevertheless, this result has obvious implications for the possibility of transmission from patients whose plasma HIV-1 RNA levels are undetectable. Previous studies have demonstrated the presence of HIV-1 in T cells and macrophages in the semen (111) and have suggested that there is some degree of compartmentalization of virus in the male urogenital tract (112).

THE CENTRAL NERVOUS SYSTEM AS A RESERVOIR FOR HIV-1

Another potentially important reservoir for HIV-1 is the CNS (for a review, see 113). In the pre-HAART era, neurological problems were common among infected individuals, with a unique dementia syndrome, HIV-associated dementia (HAD), developing in 15–20% of patients (114). This syndrome was the result of direct effects of HIV-1 on the CNS and could be distinguished from CNS diseases caused by opportunistic infections. The capacity of HIV-1 to enter the CNS and cause disease raises important questions about whether the virus can persist there.

HIV-1 probably gains access to the CNS from the blood stream, either by direct infection of capillary endothelial cells (115) or, more likely, by ingress of infected monocytes/macrophages. The triggering mechanisms for initial monocyte/macrophage recruitment to the brain are unknown but may involve the upregulation of chemoattractant β-chemokines such as MCP-1 (116) and the expression of adhesion molecules on endothelial cells. Studies using an artificial blood brain barrier demonstrated that upregulation of adhesion molecules and proinflammatory cytokines are critical for transendothelial migration (117). Heightened trafficking may occur with peripheral activation of monocytes in late stage HIV-1 infection, which is generally when HAD occurs. The current consensus is that the principal cellular target for HIV-1 in the CNS is the macrophage or microglial cell. A large study in clinically well-characterized adults found no convincing evidence for HIV-1 DNA in neurons, endothelial cells, or oligodendrocytes (118).

Progress in the understanding of the extent of infection within the CNS has been hampered by the obvious difficulty in obtaining tissue and by uncertainties

with regard to the relationship between level of virus in the cerebrospinal fluid (CSF) and levels of virus in the brain parenchyma (119). The development of an excellent animal model for AIDS dementia (120) may facilitate analysis of this important problem. In an extensive study in the SIV system, SIV-infected cells were not detected in the CNS in monkeys during the asymptomatic stage of the infection (32). Following progression to AIDS, SIV infection in the CNS was detected by in situ hybridization (32, 120). Certain strains of HIV-1 might have an increased propensity to invade (neurotropism) and cause damage in the nervous system (neurovirulence). This is significant because the development of HAD is not universal in advanced AIDS, suggesting that there may be viral determinants of heightened risk. Indeed distinct strains of HIV-1 isolated from both peripheral blood and the nervous system of the same individual can have different biological characteristics and cellular tropisms (33, 121). Brain isolates tend to be more macrophage-tropic with specifically conserved regions in a portion of the envelope, the V3 domain (122, 123).

One approach to evaluating viral load in the CNS is to measure virus in the CSF. There is a significant correlation between CSF HIV-1 load and the severity of neurological disease (119, 124–126). However, it remains unclear whether CSF HIV-1 RNA levels actually reflect brain tissue levels. Different brain regions tend to have similar levels of HIV-1 RNA, but there is only a weak correspondence between brain and CSF HIV-1 levels (119). Potential sources of CSF HIV-1 RNA include the meninges, choroid plexus, parenchyma, and trafficking lymphocytes and monocytes. Presumably, the parenchymal levels are the most relevant for the study of neurological disease. CSF HIV-1 RNA might derive from different sources at different stages of HIV infection. Price & Staprans (127) have suggested that transitory infection due to trafficking cells should be distinguished from autonomous infection involving parenchymal infection of macrophages and microglia.

Given that HIV-1 can enter the CNS, there is concern that the virus might persist there and produce CNS disease even in treated patients who have no detectable plasma virus. This scenario is plausible because of the limited CNS penetration of certain protease inhibitors (128). Other important factors include the active efflux of antiretroviral drugs through transporters including p-glycoprotein (129). Early case reports alerted clinicians to this possibility, describing patients who had undetectable or low plasma HIV RNA levels yet significantly higher CSF HIV-1 RNA levels (130). However, despite these concerns, there have been relatively few clinical examples of ‘‘CNS escape.’’ In fact, significant reductions in the incidence rates of HAD have been noted since 1996 (131–133). There is now accumulating evidence that HAART regimes can actually improve neuropsychological performance and radiological abnormalities in those with HAD (134–136).

Given these findings, it remains unclear whether the CNS acts as a reservoir capable of reseeding the systemic compartment. Some perivascular macrophages return to the periphery after a sojourn within the brain (W Hickey, personal com-

munication). Theoretically, these could introduce CNS-derived strains of HIV into the systemic compartment. To date, however, there is no evidence that this occurs in vivo. There is still relatively little information about the effects of antiretroviral therapy on viral replication in the CNS. Several studies have reported successful suppression of HIV in CSF with the long-term use of protease inhibitor containing regimens (137–141). The kinetics of virologic suppression may differ in the CSF compartment, lagging behind the more rapid response seen in plasma (142, 143) and sometimes failing to suppress to undetectable levels despite the use of a CNS-penetrant regimen (144). In one study the half-life of HIV-1 RNA in the CSF compartment was significantly longer than in the blood, falling by 1 log over 20 days ($t_{1/2} = 5$ days) (143).

In summary, the CNS clearly represents a relatively poorly understood site with respect to HIV-1 replication. The CNS is actually several different compartments including the brain parenchyma and the CSF in addition to trafficking monocytes. While HAART has had a measurable impact on the incidence rates of HAD, the possibility exists that CNS escape might occur, particularly in individuals with poor medication adherence or using antiretroviral regimes with poor CNS penetration. Whether the CNS can serve as a long-term reservoir for HIV-1 in patients with good suppression of viral replication remains to be determined.

A STABLE RESERVOIR OF LATENTLY INFECTED RESTING CD4$^+$ T CELLS

Physiologic Basis of Latent Infection of CD4$^+$ T Cells

At the present time, the reservoir that appears to be the major barrier to achieving eradication of HIV-1 is the extremely stable reservoir composed of latently infected resting CD4$^+$ T cells with integrated provirus (40, 56). These cells are not found exclusively in any particular tissue location, but rather populate the peripheral lymphoid tissues and recirculate continuously between them. The existence of a latent reservoir for HIV-1 in resting CD4$^+$ T cells can be best explained by considering the infection of CD4$^+$ T cells in the context of the normal physiology of T cell activation (Figure 2). Naive CD4$^+$ T cells exit the thymus and enter the peripheral lymphoid tissues in a process that continues throughout life (93). These newly generated T cells persist in a resting state until they encounter the relevant antigen. Following initial exposure to antigen, naive T cells undergo blast transformation and enter the cell cycle. Activation results in metabolic changes including increases in nucleotide pools and in the upregulation of expression of sets of proteins that allow the cell to carry out its functions. These include transcription factors, effector molecules such as cytokines, and cell surface proteins such as cytokine receptors and adhesion molecules (145). Many of the activated T cells die within a few weeks after activation, either as a result of additional

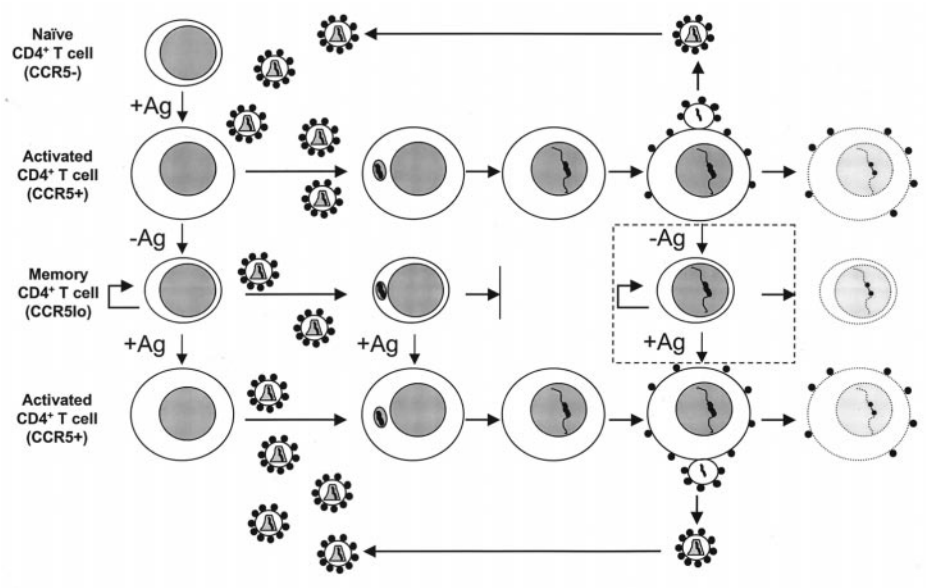

Figure 2 Cellular dynamics of HIV-1 infection of $CD4^+$ T cells. Transitions between resting (small) and activated (large) $CD4^+$ T cells are illustrated by vertical arrows. The normal generation of memory $CD4^+$ T cells is illustrated on the left. These cells are derived from antigen (Ag)-activated $CD4^+$ T cells that revert back to a resting memory state. These memory cells survive for long periods of time, allowing responses to the same antigen Ag in the future. The memory cell compartment may be maintained by a process of proliferative renewal. Successive steps in the life cycle of the virus are indicated by horizontal arrows. R5 isolates can infect activated $CD4^+$ T cells but may infect only the subset of resting memory $CD4^+$ T cells that express sufficient amounts of CCR5. Following infection of resting memory $CD4^+$ T cells, there is a block in the virus life cycle, probably at the level of nuclear import of the preintegration complex containing the viral genome. Resting cells with unintegrated HIV-1 DNA are likely to represent a relatively labile reservoir for the virus (preintegration latency). Productive infection requires Ag-driven activation of recently infected resting $CD4^+$ cells or, more commonly, direct infection of Ag-activated $CD4^+$ T cells. Productively infected cells generally die within a few days from cytopathic effects of the infection or host cytolytic effector mechanisms, but some infected lymphoblasts survive long enough to go back to a resting state (boxed), thereby establishing a stable latent reservoir of resting memory $CD4^+$ T cells with integrated HIV-1 DNA (postintegration latency).

antigen-induced activation, which can trigger expression of pro-apoptotic regulatory molecules such as FasL and TNF-α, or as a result of diminishing concentrations of cytokines needed to promote survival (reviewed in 146). Some of the activated cells escape these death pathways, exit the cell cycle, lose expression of activation markers such as HLA-DR, and revert to a resting state in which they

persist as memory T cells capable of responding to subsequent exposures to the initiating antigen. The return to quiescence may involve expression of the transcription factor LKLF, which functions in an unknown manner to decrease FasL-associated killing and to regulate T cell quiescence and survival (147). The survival of resting $CD4^+$ T cells may be dependent upon cytokines including IL4, IL-6, and IL-7 (148, 149). This linear differentiation model in which memory T cells are derived from activated effector cells that escape programmed cell death has been recently confirmed for $CD8^+$ T lymphocytes (150) and likely holds for $CD4^+$ T cells as well.

The state of activation and history of antigen exposure of a given T cell can be discerned by analysis of the patterns of expression of certain cell surface proteins (151–153). Altered patterns of expression of some cell membrane proteins reflect differences between the resting and activated states. For example, T cell activation results in rapid and temporary expression of CD69 and of the α chain of the IL-2 receptor (CD25) as well as a slower and more prolonged upregulation of HLA-DR that persists while the cell is in an activated state. Antigen-driven activation also leads to an essentially permanent change in the expression of other cell membrane proteins. These changes can be used to distinguish naive and memory T cells (151). For example, naive and memory T cells express different splice variants of the membrane tyrosine phosphatase CD45 (CD45RA and CD45RO, respectively) (153–156), and naive cells express the lymphocyte homing receptor L-selectin (CD62L), which is typically not found on memory T cells (157). Phenotypic analysis of naïve and memory cells is somewhat hampered by the finding that memory cells can revert to a naïve ($CD45RA^+$) phenotype (158–160).

The ability of HIV-1 to bind to and enter resting and activated $CD4^+$ T cells is a function of the expression of sufficient levels of both CD4 and an appropriate chemokine receptor and depends upon the chemokine receptor utilization capacity of the env protein of the viral isolate in question (13). The most commonly transmitted forms of HIV-1 utilize the chemokine receptor CCR5 as a co-receptor. Because CCR5 is upregulated upon T cell activation, these viruses can bind to, infect, and replicate in activated $CD4^+$ T cells. The infection of resting $CD4^+$ T cells by viruses utilizing CCR5 appears both inefficient and restricted to those of the memory phenotype. Infection of resting memory cells by these viruses can be observed only in the presence of a large inoculum and likely represents the infection of the small fraction of cells with continued low level CCR5 expression (T Pierson & RF Siliciano, unpublished data). Even when CCR5-utilizing viruses enter resting memory $CD4^+$ T cells, there is a block in the replication cycle such that no virus is produced (see below). Viruses that utilize the chemokine receptor CXCR4 can bind to and enter resting naïve and memory $CD4^+$ T cells and activated $CD4^+$ T cells because CXCR4 is expressed at sufficiently high levels on all of these cell types to mediate the entry. However, there is again a block in the replication cycle in resting $CD4^+$ T cells. Since at any given time the majority

of lymphocytes are in a resting state, it is important to understand the consequences of infection of resting $CD4^+$ T cells.

Preintegration Latency

Although HIV-1 does not replicate in resting $CD4^+$ T cells, it can establish a state of latent infection in resting cells through two mechanisms, both of which are operative only in cells that are in a quiescent G_0 state (5). A preintegration form of latency is observed immediately following direct infection of resting $CD4^+$ T cells. As discussed above, HIV-1 virions can bind to and fuse with resting $CD4^+$ T cells under some conditions. This is followed by reverse transcription of the genomic RNA, a process that occurs in a large preintegration complex containing the viral genome and virion proteins including reverse transcriptase, integrase, matrix, and vpr. Previous studies suggested that the post-entry block in the virus life cycle in resting $CD4^+$ T cells resulted from either an inability to complete the reverse transcription reaction (161, 162) due to low nucleotide pools (163), or failure to import the ribosome-sized preintegration complex into the nucleus (165). In short-term in vitro infections of resting $CD4^+$ T cells, reverse transcription does not proceed to completion. However, in longer term in vitro cultures and in in vivo studies, complete reverse transcripts can be found in resting $CD4^+$ T cells (40, 44, 164; T Pierson & RF Siliciano, manuscript in preparation). These results suggest that although reverse transcription is slow in resting $CD4^+$ T cells, an additional block exists, probably at the level of nuclear import (165). Interestingly, recent experiments using retroviral vectors to transduce primary $CD4^+$ lymphoblasts have shown that overexpression of the transcription factor NFATc is sufficient to allow previously activated $CD4^+$ T cells to remain completely permissive for productive HIV-1 infection at time points (17 days post activation) when control, nontransduced cells are no longer able to complete reverse transcription (166).

Although infected resting cells do not normally produce infectious virus, pioneering studies by Zack et al (161) and Bukrinski et al (44) demonstrated that recently infected resting $CD4^+$ T cells can function as an inducible latent reservoir for HIV-1. If an infected resting T cell is activated by antigen before the preintegration complex becomes nonfunctional, then the subsequent steps of nuclear import, integration into host chromosomes, virus gene expression, and release of infectious virions can all occur (44, 161, 164). Although there is some controversy in the literature over exactly how long functional preintegration complexes can persist in resting $CD4^+$ T cells, this preintegration form of latency appears to be relatively labile, persisting a matter of days to weeks. It is important to keep in mind that, in untreated patients, resting $CD4^+$ T cells with this unintegrated form of HIV-1 DNA are much more prevalent than cells with stably integrated HIV-1 DNA and that this unstable form of latency is detected in virus culture assays in which the resting cells are subjected to activating stimuli (40, 44, 46).

Postintegration Latency

A more stable form of latency may occur in $CD4^+$ T cells that have undergone integration of proviral DNA (40, 52, 56, 167–169). Because nuclear import is dependent upon T cell activation, postintegration latency can, in principle, result only from the return of an activated T cell with integrated provirus back to a resting state in which there is minimal expression of viral genes (Figure 2). In order for a productively infected $CD4^+$ T lymphoblast to enter a condition of postintegration latency, it must survive the homeostatic mechanisms that kill the majority of activated T cells, the cytopathic effects of the virus, and virus-specific cytolytic host effector mechanisms. The cell must survive for long enough to allow it to revert to a resting memory state in which there is minimal expression of HIV-1 genes. This form of latency may arise when there is infection of activated cells that are in the process of transitioning back to a resting state. In this situation, the cells may be permissive for reverse transcription, nuclear import, and integration, but not fully permissive for virus gene expression (7). The cells therefore escape the rapid destruction that is the usual fate of productively infected cells.

Recent in vitro experiments using pseudotyped HIV-1 vectors have demonstrated that certain cytokines, including IL-2, IL-4, IL-7, and IL-15, can provide signals that render resting $CD4^+$ T cells permissive for nuclear import, integration, and virus gene expression (170). In the absence of cytokine stimulation, transduction was not seen. These results raise the possibility that infected resting cells may transit from a labile preintegration state to stable form of latency through both antigen-dependent and cytokine-dependent mechanisms.

Evidence for Postintegration Latency in Vivo

Although it had been long presumed that the integration of HIV-1 DNA into the genomes of infected $CD4^+$ T lymphocytes would allow viral persistence, until recently little direct evidence suggested that resting $CD4^+$ T cells with integrated provirus function as a latent reservoir for HIV-1 in infected individuals. In important early experiments, transformed cell lines carrying integrated HIV-1 DNA were used to model latent infection (167, 168, 171). Cell lines were infected in vitro, and surviving cells that grew out and contained integrated HIV-1 DNA were analyzed. As is discussed below, studies of these cell lines provided information about the molecular mechanisms involved in the regulation of HIV-1 gene expression.

The proof that resting $CD4^+$ T cells with latent, integrated provirus are present in vivo required the development of techniques for the isolation of extremely pure populations of resting $CD4^+$ T cells as well as methods for the unambiguous detection of integrated HIV-1 DNA in the presence of excess unintegrated HIV-1 DNA and the isolation of replication-competent virus from the purified resting $CD4^+$ T cells. In 1995, Chun et al used a special inverse PCR method to demonstrate unambiguously the presence of cells with integrated HIV-1 DNA in

extremely pure populations of resting $CD4^+$ T cells isolated from infected donors (56). The purification procedure used yielded cells that were $CD4^+$ but lacked expression of early (CD25, CD69) and late (HLA-DR) activation markers. The PCR method used amplifies the junction between the HIV-1 provirus and the upstream genomic DNA and does not detect unintegrated HIV-1 DNA. In the same study, replication-competent virus was obtained from these cell populations by stimulating the cells with the mitogen PHA. In the absence of mitogenic stimulation, virus cannot be recovered from highly purified resting cell populations, and therefore virus isolated from these cells may be considered to have come from a latent reservoir. The frequencies of cells with integrated HIV-1 DNA among the resting $CD4^+$ T cell populations were similar, and extremely low (0.05%), in the blood and lymph nodes of infected individuals who were not yet on effective therapy consistent with the continual recirculation of resting T lymphocytes (40). The frequencies were not higher in patients with advanced disease, suggesting that a relatively stable steady state is established in which only a minute fraction of the resting $CD4^+$ T cell population carries integrated HIV-1 DNA (40). The total body number of resting $CD4^+$ T cells with integrated HIV-1 DNA was estimated to be approximately 10^7 cells, only a fraction of which carried replication-competent forms of the virus. Among resting $CD4^+$ T cells, integrated HIV-1 DNA was present primarily among cells with a memory phenotype (40). Recent studies using a virus culture technique to detect postintegration latency have also demonstrated that most latently infected cells have a $CD45RO^+$ phenotype (J Siliciano, RF Siliciano, unpublished results), consistent with the mechanism proposed in Figure 2.

It has often been presumed that the proviral DNA in T cells represents mainly defective archival sequences. However, with enhanced virus culture conditions designed to induce uniform activation of resting $CD4^+$ T cells, replication-competent virus could be recovered from highly purified resting $CD4^+$ T cells (40). The frequencies of resting $CD4^+$ T cells with replication-competent provirus are lower than the frequencies of resting $CD4^+$ T cells with integrated HIV-1 DNA (as detected by inverse PCR). This suggests that some of the integrated HIV-1 DNA in resting $CD4^+$ T cells is defective. Nonetheless, the finding that replication-competent virus can persist in a latent form in resting $CD4^+$ T cells raised the possibility that this reservoir might represent a major barrier to virus eradication in patients on combination therapy (see below).

Molecular Mechanisms for Postintegration Latency

What accounts for the ability of HIV-1 to persist in a latent, integrated form in resting $CD4^+$ T cells? Three general mechanisms are described below. It should be pointed out the latency in vivo may involve multiple mechanisms.

1. Inefficient or absent initiation of transcription. The simplest mechanism for latency involves the absence in resting $CD4^+$ T cells of transcription from the

HIV-1 LTR. This could result from proviral integration into chromosomal sites that become inaccessible to the transcription machinery in quiescent cells (172) or from the absence in resting cells of requisite forms of host transcription factors that activate gene expression from the HIV-1 LTR (167, 173–178). The U3 region of the HIV-1 LTR functions as the viral promoter (for reviews, see 179 and 180) and contains binding sites for host transcription factors that function as positive regulators of T cell activation–specific gene expression in normal, uninfected T lymphoblasts; these include Ets, NFAT, and NFκB. The LTR actually contains two tandem, highly conserved binding sites for NFκB. Following the pioneering studies of Nabel & Baltimore (174) showing that this inducible host transcription factor regulated HIV-1 gene expression, Fauci and colleagues explored the notion that latent infection of T cell might involve the absence of the requisite host transcription factors (167, 173, 181). They showed that in transformed T cell lines carrying an integrated copy of the HIV-1 genome, upregulation of HIV-1 gene expression following exposure to TNF-α was mediated through NFκB (173). More recently, it has been shown that reporter viruses with mutations in the NFκB binding sites replicate poorly in activated $CD4^+$ T lymphoblasts, consistent with an important role for NFκB in HIV-1 gene expression (182) . In resting $CD4^+$ T cells, NFκB may be sequestered in the cytoplasm through interaction with IκB, and it has been suggested that the absence of a nuclear NFκB in resting $CD4^+$ T cell with integrated HIV-1 genomes prevents transcription of viral genes. Leiden and colleagues have suggested that HIV-1 gene expression is regulated by inducible T cell enhancons that are composed of Ets, NKκB, NFAT, and AP-1 proteins and that control the expression of a number of genes that are upregulated in activated T cells (183). However, the hypothesis that postintegration latency is regulated at the level of initiation of transcription has not been tested directly in primary $CD4^+$ T cells. Latently infected cells are rare in vivo, and it is therefore hard to carry out this kind of mechanistic study. An additional problem is that no in vitro model exists for a resting G_0 $CD4^+$ T cell with integrated HIV-1 DNA. The transformed cell lines used in many of the published studies on latency may not mimic the transcriptional status of the extremely quiescent cells that constitute the latent reservoir for HIV-1 in vivo.

2. Failure of transcriptional elongation. A more recently identified mechanism for latency involves the absence in resting $CD4^+$ T cells of host factors that interact with the HIV-1 tat protein and allow tat-mediated upregulation of transcriptional elongation (53, 184–187). It is now clear that tat-mediated upregulation of HIV-1 gene expression is dependent upon association of tat with the *tar* sequence in the HIV-1 RNA in a process that involves the host proteins cyclin T1 and CDK9. These proteins are both components of the p-TEFb CTD kinase complex, which phosphorylates the C-terminal domain of RNA polymerase II, a modification that increases the processivity of the enzyme. Expression of both cyclin T1 and CDK9 is upregulated upon T cell

activation (184), and levels in resting $CD4^+$ T cells may be too low to allow the tat-mediated enhancement of transcriptional elongation.

3. Failure to export unspliced viral mRNAs into the cytoplasm due to low levels of the HIV-1 rev protein (52, 168, 169, 188–190). In some cell lines carrying an integrated copy of the HIV-1 genome, upregulation of HIV-1 gene expression is associated with a shift in the predominant class of viral mRNAs made from the small 2-kb mRNAs encoding regulatory proteins tat, rev, and nef to the larger 4-kb singly spliced and 9-kb unspliced mRNAs coding env and gag/pol, respectively. In the absence of sufficient threshold amounts of the rev protein, the 9-kb RNA that serves as a mRNA for gag and pol and as the viral genome may be spliced, thereby preventing the production of virus particles. This threshold effect may reflect a requirement for multimerization of rev on the rev responsive element in the viral RNA (190). Under conditions where only low amounts of viral mRNAs are made, the level of rev may be insufficient to prevent splicing of the full-length viral RNA molecules. According to this model, some multiply spiced transcripts may actually be made in latently infected cells. While numerous studies have detected multiply spliced HIV-1 RNA in unfractionated blood or lymph node cells from infected individuals, it has been unclear whether this RNA is present in latently infected cells since the studies have not been performed on purified resting $CD4^+$ T cells. Thus at the present time, the molecular mechanisms of postintegration latency remain unclear.

LATENTLY INFECTED CELLS AS A BARRIER TO VIRUS ERADICATION

The demonstration that $CD4^+$ T cells in the postintegration state of latency were present in infected individuals (40, 56) raised the concern that these cells might function as a long-term reservoir for HIV-1. The importance of this reservoir derives from two fundamental aspects of the biology of these cells. First, as discussed in the previous section, the level of viral gene expression is likely to be very low in latently infected resting $CD4^+$ T cells, rendering the cells resistant to viral cytopathic effects and host cytolytic effector mechanisms. Second, resting $CD4^+$ T cells can live for long periods of time. The half-life of $CD4^+$ T cells in normal humans has not been extensively studied, but some estimates based on the survival of cells with DNA damage induced by high dose irradiation have been published (158, 159). In these studies, half-life of T cells expressing the RO isoform of CD45, found on memory cells, is in the range of 5–6 months (159). Because the forms of chromosomal damage studied cause death of the cell if it goes through mitosis, these studies actually measure only the intermitotic half-life of the cells, the time required for half of the cells to divide or die. Memory cells and their progeny may actually survive for much longer periods of time

(>20 years) as a result of occasional cell division stimulated by persistent antigen, cross-reacting antigen, or cytokines. The long-term survival of memory cells is a fundamental property of the immune system, essential for protection against previously encountered pathogens. The ability of HIV-1 to establish a state of latent infection in resting memory $CD4^+$ T cells thus provides a potentially important mechanism for viral persistence. Although reactivation of virus from this latent reservoir normally contributes only a minute fraction of the plasma virus in untreated individuals, this reservoir assumes tremendous significance in patients who are on HAART and in whom productively infected cells have decayed to the point where plasma virus is no longer measurable.

The notion that resting $CD4^+$ T cells might represent a barrier to HIV-1 eradication in patients on combination therapy was dramatically illustrated by three studies that examined whether replication-competent virus could persist in the resting $CD4^+$ T cells of patients on HAART (191–193). These studies focused on a subset of patients who responded extremely well to therapy and who had been aviremic for as long as 2.5 years. Although it is generally very difficult to isolate virus by conventional methods from patients on long-term HAART, all three groups found that with enhanced culture techniques, replication-competent virus could be readily isolated from resting $CD4^+$ lymphocytes of these patients. The culture methods used were similar in that, in each case, the patient's T cells were subjected to conditions that would efficiently activate resting $CD4^+$ T cells and thereby allow them to express latent virus. In one study, a cross-sectional analysis suggested that the frequencies of latently infected $CD4^+$ T cells did not decrease during the first two years of therapy (191). This is in marked contrast to other viral reservoirs examined to date, all of which show readily measurable decay rates (Figure 1).

The existence of a persistent latent reservoir for HIV-1 required a reevaluation of the eradication hypothesis. Included in the original model developed by Perelson and colleagues was the idea that the dynamics of productively infected $CD4^+$ T cells would be governed by the following equation:

$$dT^*/dt = kVT - \delta T^*$$

in which productively infected cells (T^*) are generated from uninfected T cells (T) with a rate constant k in a process that depends on the plasma virus concentration (V) (60). The cells are cleared with a rate constant δ. Later models (1) incorporated the notion that productively infected T cells could also arise by activation of latently infected cells (L) with a rate constant, a:

$$dT^*/dt = kVT + aL - \delta T^*$$

At high viral loads, the contribution of the latent reservoir is small ($\{kVT^* >> aL$). However, if the half-life of latently infected cells is very long, then this pool could clearly thwart efforts to eradicate the virus even under conditions where the plasma virus level (V) is very low. Accurate measurement of the half-life of the latent reservoir required the development of assays to detect latently infected cells.

Assays for Latently Infected Cells

Both molecular and virologic approaches have been used. Detection of latent integrated provirus by molecular methods presents two serious problems. First, conventional PCR assays for HIV-1 DNA do not distinguish between unintegrated HIV-1 DNA and the stable integrated form. Second, unless the assays are performed on rigorously purified resting $CD4^+$ T cells, there is no way to tell whether the HIV-1 genomes detected are latent. In the original studies defining a latent reservoir for HIV-1, an inverse PCR method was used to detect unambiguously the integrated form of HIV-1 DNA in highly purified resting $CD4^+$ T cells (56). This method amplified the junction between the LTR and upstream host genomic DNA. An *Alu* PCR method has also been used (193). Although these methods allow detection of cells with integrated HIV-1 DNA, they are cumbersome and can only be made quantitative with the use of a limiting dilution format. In addition, these methods suffer from the problem that they detect defective as well as replication competent HIV-1 DNA.

An alternative approach is to attempt to culture virus from purified resting $CD4^+$ T cells by activating them with mitogenic stimuli (40, 191). In conventional virus culture assays (38, 194), the patient's cells are cocultured with PHA-activated lymphoblasts from a normal donor, but no specific step is included to induce uniform activation of resting cells from the patient. Some investigators have suggested that the detection of latently infected cells might be facilitated by the inclusion of the mitogen PHA, which can activate resting T cells (39). Quantitative analysis of latently infected cells in vivo was made possible through the development of enhanced culture techniques in which purified resting $CD4^+$ T cells are first isolated and then subjected to conditions that induce activation with high efficiency (40). Addition of PHA alone to resting $CD4^+$ T cells is insufficient as PHA activation is dependent upon the presence of monocytes/macrophages. Thus, purified resting $CD4^+$ T cells from the patient are mixed with an excess of irradiated PBMC from a normal donor in the presence of PHA. In subsequent steps, $CD4^+$ lymphoblasts from normal donors are added to amplify any virus released from latently infected cells. $CD8^+$ T cells, which can suppress virus replication (195–197), are deleted from both the patient and donor lymphoblast populations. The activation conditions used in these assays actually allow expansion of $CD4^+$ T cells with high cloning efficiency (198), and the approach can be made quantitative through the use of a limiting dilution format.

This enhanced virus culture assay has the advantage that it will only detect replication-competent forms of HIV-1. However, these assays can detect both recently infected cells in the preintegration state of latency and cells in the post-integration state of latency. There are several ways around this problem. First, the preintegration state of latency is labile. If viral replication is stopped or dramatically reduced with antiretroviral drugs, then the number of recently infected resting cells should fall, and culture assays done at long time intervals ($>$3 months) after initiating effective therapy may preferentially detect integrated virus

(J Blankson, D Persaud, RF Siliciano, unpublished data). The number of cells in the preintegration state of latency is correlated with viral load, and analysis of this correlation has shown that as the viral load falls below 20 copies/ml, the number of cells in the preintegration state of latency should represent only a minor fraction of the total amount of latent virus detected in the enhanced culture assays. For this reason, enhanced virus culture assays done on patients with undetectable plasma virus are likely to detect mainly cells in the postintegration state of latency. An additional problem with this assay is that when the frequency of latently infected cells is low, detection requires addition of large numbers of purified resting $CD4^+$ T cells while maintaining appropriate conditions of cell density (194) and ratios of irradiated donor PBMC and of $CD4^+$ lymphoblasts.

Half-Life of the Latent Reservoir for HIV-1

The detection of latently infected cells in patients on HAART raised the question of how long these cells would persist. Definitive measurement of the half-life of the latent reservoir in resting $CD4^+$ T cells required longitudinal studies in individual patients over long periods of time. The first such measurements have recently been reported. It now appears that decay of the latent reservoir cannot be expected in the average patient who is responding well to HAART. In a study of 35 patients who were being treated with HAART according to current guidelines (199) and whose plasma virus levels were undetectable by a standard RT-PCR assay with a sensitivity down to 200 copies/ml, the latent reservoir was found to be extremely stable, with a mean half-life of over 43 months (200). Even with conservative estimates of the total body number of latently infected cells, an average of $>$ 60 years of treatment would be required to eradicate this compartment. In fact, the mean slope was not statistically different than zero, indicating that there may not be decay in the average patient. This study used an enhanced virus culture method to detect latently infected cells, and the viruses obtained from this reservoir were fully replication competent in vitro and therefore likely to be capable of rekindling the infection in patients who stop therapy. The extremely slow decay rate of this reservoir raises the disturbing prospect that in some patients the time required for HIV-1 eradication with current combination regimens may be so great that other intervening problems such as cumulative toxicities of antiretroviral drugs (201) may make eradication difficult if not impossible. The persistence of the virus in latently infected T cells is at least one reason that, with extremely rare exceptions (202), rapid rebound of plasma viremia has been noted in all patients who have interrupted therapy (203).

The slow decay rate measured in the study cited above is best thought of as the *observed* decay of the reservoir in the average patient who has responded well to current standard of care therapy. The true *intrinsic* decay rate of this reservoir may be faster if there is still ongoing viral replication leading to the entry of new cells into the reservoir (see below). Evidence that the intrinsic decay rate is faster has come from several sources. In one patient in the above study, the latent

reservoir declined with $t_{1/2}$ of only 3 months. In addition, more rapid decay had been observed in a recent study of the latent reservoir in patients who have been started on HAART during primary HIV-1 infection (204). The latent reservoir is established early in primary infection, and latently infected cells can be detected in patients who have been started as early as 48 h after presentation with acute retroviral syndrome (191, 200, 205). Zhang et al have used enhanced culture assays to measure the decay of resting $CD4^+$ T cells carrying replication-competent virus in a small highly selected group of patients who were started on therapy during primary HIV-1 infection. The latent reservoir was seen to decay with a mean half-life of about 6 months (204). In further studies by this group in a larger cohort of patients, a slower mean decay rate has been observed ($t_{1/2} >$ 12 months), and slow decay has been associated with the occurrence of intermittent low level viremia (206). Thus, there is agreement that in only a small subset of patients on current standard of care therapy is there measurable decay of the latent reservoir (200, 204, 206). Long-term persistence of HIV-1 is supported by a recent study in which integrated HIV DNA, circular HIV-1 DNA, and various forms of HIV-1 RNA in PBMC were all found after the first year of therapy to reach a very stable plateau with little additional decay (207). Further studies with longer follow-up will be needed to determine whether this reservoir actually undergoes any decay in a clinically meaningful time frame.

Another approach to measuring the half-life of the latent reservoir is to examine the decay of HIV-1 DNA in cells in the peripheral blood. Perelson et al reported that HIV-1 DNA in PBMC decayed with a mean $t_{1/2}$ of about 5 months (1). A $t_{1/2}$ of 10 months was recently reported for a small series of patients who had responded well to antiretroviral therapy (208). It is important to keep in mind that standard PCR assays do not distinguish integrated and unintegrated HIV-1 DNA and that such measurements cannot be used to define the turnover of cells in the postintegration state of latency unless the conditions are such that cells in the preintegration state of latency no longer contribute significantly to the measurement.

Factors Contributing to the Stability of the Latent Reservoir

The remarkable stability of the latent reservoir for HIV-1 can be explained in several ways. The observed stability is consistent with the fact that the reservoir is composed at least in part of memory T cells carrying integrated HIV-1 DNA (40). The biological function of memory T cells is to persist and provide protection against previously encountered microorganisms. The long-term survival of antigen-specific memory T cell is accepted for $CD8^+$ T cells (209) and is becoming increasingly well documented for $CD4^+$ T cells (210). As discussed above, the intermitotic half-life of memory T cells has been estimated to be in the range of 5–6 months (158, 159). The actual life-span of a memory cell and its progeny may be much longer, consistent with the nearly life-long memory associated with many infections. Recent studies in the murine system indicate that T cell memory

may be maintained by occasional proliferation of memory cells. It is possible that latently infected cells may undergo occasional proliferation driven by stimuli that do not fully turn on HIV-1 gene expression. In this situation, the virus would be able to persist by taking advantage of the normal homeostatic mechanisms that maintain immunologic memory.

Another explanation for the stability of the reservoir has been recently put forward by Bucy. Noting the decline in CTL responses in patients on suppressive antiretroviral therapy, he suggests that the rate of clearance of cells activated from the latent reservoir is reduced in treated patients due to the decline of HIV-1-specific immune responses (86).

A third explanation for the stability of the latent reservoir is that the reservoir is being reseeded by a low level of ongoing viral replication in patients on combination therapy. As discussed below, several lines of evidence suggest that a low level of viral replication may be occurring in patients with undetectable viral loads. The issue of ongoing replication is an important one because it is directly related to strategies for therapy. If ongoing replication sustains the latent reservoir, then intensification of therapy may stop residual replication and reveal the intrinsic decay rate, allowing viral eradication if this decay rate is fast enough. If the reservoir is intrinsically stable, then strategies to flush out latently infected cells must be considered.

ONGOING VIRAL REPLICATION IN PATIENTS ON HAART

Although the postintegration state of latency clearly represents one mechanism for the persistence of HIV-1, there is another interrelated mechanism that involves low levels of ongoing replication. The persistence of virus in treated individuals whose plasma HIV-1 RNA levels are below the limit of detection may reflect the fact that therapy is not suppressing all ongoing viral replication. Original models of viral dynamics in HIV-1 infection postulated that potent antiretroviral regimens would stop all new infection of susceptible cells types in vivo. However, several lines of evidence (reviewed below) suggest that the current regimens are not completely effective in preventing any additional cells from being infected. New cycles of infection may be occurring, but at a set point that is generally below the limit of detection (Figure 1). Ongoing viral replication might involve the generation of new infected cells via de novo infection from extracellular virus released from other infected cells. Each new round of infection is of course subject to inhibition by the protease inhibitors at the stage of virus maturation and by RT inhibitors at the stage of reverse transcription. If the drugs do not completely block new infections, then persistence could be explained by low level of replication as long as new cells are successfully infected at a rate that balances the rate at which infected cells die. In this model, the virus might persist even without the aid of latent reservoir (Figure 1). A more likely scenario is that occasional

activation of the latent reservoir feeds this ongoing replication and that conversely this ongoing replication contributes to the stability of the latent reservoir by generating new latently infected cells.

Evidence for ongoing replication in patients on HAART comes from several sources:

1. Many patients who have plasma virus levels below that limit of detection of conventional assays (200–500 copies/ml) have occasional positive determinations (termed ''blips'') when more sensitive assays for viral RNA are used (206, 211). These ''ultrasensitive'' assays currently detect HIV-1 RNA in the plasma down to a level of 10–50 copies/ml. The source of these blips is unclear.
2. When patients are switched from three-drug regimens to simpler two-drug ''maintenance'' regimens, breakthrough viremia is observed in a significant fraction of patients (212–214). The failure of the maintenance therapy trials suggests that current three-drug regimens barely contain viral replication.
3. Several recent studies suggest that productively infected cells can be detected in individuals who are aviremic on HAART. Detection methods include in situ hybridization or RT-PCR assays for cells expressing HIV-1 RNA and immunohistochemical detection of cells expressing HIV-1 proteins (27, 207, 211, 215). In an elegant study by Hockett et al, cells expressing full-length viral RNA were quantitated in lymph node biopsy specimens. Quantitative analysis of the relationship between the number of RNA positive cells and plasma viremia suggested that in patients with a viral load below 50 copies/ml, there could be as many as 100,000 productively infected cells at any given time. Of course, the detection of RNA^+ cells does not in itself prove an ongoing viral replication. It is possible that these cells are cells reactivated from latency. However, 100,000 cells RNA^+ cells would represent a substantial fraction of the latent reservoir (estimated to be $10^6 - 10^7$ cells), inconsistent with the slow turnover of the reservoir. A more plausible idea is that viruses released following the activation of latently infected resting $CD4^+$ T cells occasionally spread and infect other cells, fueling a low level of ongoing replication in patients on HAART.
4. The detection of recently infected cells suggests active replication. Chun et al (205) measured total and integrated HIV-1 DNA in resting $CD4^+$ T cells from patients on HAART. They concluded that the difference between the two measurements was due to the presence of recently infected cells with unintegrated HIV-1 DNA (193). Similarly, 2-LTR circles can be detected in PBMC from patients on HAART (207). These are formed from complete linear reverse transcripts that undergo an aberrant end-to-end ligation reaction instead of integration into the host chromosome. They appear to be unstable and may prove to be an important marker of recent infection.
5. A recent study suggests that in some aviremic patients, viruses with altered *env* sequences arise, consistent with ongoing replication (204). However, *env*

evolution was noted only in a subset of patients (2/8). Other patients on HAART show no *env* evolution, consistent with preservation of earlier viral sequences in the latent reservoir (204, 216). Interestingly, there is only limited evidence for evolution in the *pol* gene despite the stringent selection enforced by antiretroviral drug regimens. Most of the viruses isolated from the latent reservoir show a drug sensitive genotype (191, 217, 218), and direct sequencing of PBMC DNA has shown no evidence for the evolution of drug resistance among patients who were aviremic on therapy (208). It is possible that in patients on HAART, the rate of replication is so low that the ordered accumulation of multiple *pol* mutations needed for high-level resistance rarely occurs unless the patients have persistent low-level viremia (192). *Env* mutations may accumulate as a result of the fact that changes in some regions of the env protein have little negative effect on viral replication.

In summary, several lines of evidence suggest that new cells may become infected in patients on HAART whose plasma HIV-1 RNA measurements are below the limit of detection of current assays. A major unresolved issue is the relationship between the pool of latently infected cells and the low-level ongoing replication that is observed in patients on HAART. Are the productively infected cells in these individuals cells that have been activated from latency? It is likely that there is some communication between the pool of latently infected cells and the cells that are actively replicating, with the latent pool seeding the smoldering ongoing replication and the ongoing replication generating new latently infected cells (see Figure 1).

APPROACHES FOR CLEARING OR CONTAINING THE LATENT RESERVOIR

Given that the latent reservoir for HIV-1 appears to represent a major barrier to virus eradication, several investigators have explored approaches for eliminating this reservoir. One approach involves intensification of combination therapy. It is possible that the stability of the latent reservoir reflects a low level of viral replication that ''reseeds'' the reservoir. It follows that intensification of antiretroviral therapy may completely inhibit viral replication and thus result in a quicker turnover of the reservoir (206).

Given the long half-life of the reservoir and the toxicity associated with HAART, other approaches for the elimination of this reservoir are also being considered. One strategy involves the use of cytokines to activate latently infected cells and ''flush out'' the reservoir (12, 219). The goal is to promote viral transcription and replication in cells that harbor latent virus. These cells are then likely to die either from cytopathic effects of the virus or from recognition and lysis by virus-specific CTL. Virus produced from these cells should be unable to

infect other cells due to the presence of the antiretroviral agents. A recent study has shown that a combination of IL-2, IL-6, and TNF-α can induce activation and viral replication in latently infected resting cells in vitro (220). This same combination of cytokines was shown previously to be able to drive resting T cells to proliferate in the absence of antigen (221). IL-2 is one of the few T cell–activating agents approved for use in humans for the treatment of other diseases. Prior to the widespread availability of the protease inhibitors, several groups demonstrated that IL-2 could produce increases in CD4 counts (222–224). While the CD4 count increases observed in these studies were promising, no decreases in viral load were seen. In the era of HAART, a new approach involving the use of IL-2 to activate HIV-1 gene expression in latently infected cells has been explored by Fauci and colleagues (12, 225). Although latently infected cells do not express the high-affinity IL-2 receptor (40, 56), IL-2 may activate these cells through the low-affinity IL-2 receptor or indirectly by inducing the release of other cytokines. In a recent study, patients on HAART plus subcutaneous IL-2 were tested for the presence of latently infected cells. In a subset of the patients examined (3/14), virus could not be isolated from purified resting $CD4^+$ T cells, suggesting a very low frequency of latently infected cells (225). However, the occurrence of viral rebound observed following interruption of therapy in these patients suggests that levels of latently infected cells below the limit of detection of current assays can persist or that other forms of the virus may persist (12). Therefore, it remains unclear whether IL-2 will have clinical benefit in this situation. While this strategy is potentially promising, its in vivo application is limited by the fact that treatment with IL-2 causes the nonspecific activation of a large number of T cells resulting in significant toxicity. This would also hold true for treatment with the anti-CD3 antibody, although this reagent is sometimes used in the treatment of transplant rejection. The combination of a regimen of HAART with an agent that would specifically activate only those resting $CD4^+$ T cells that are infected would be ideal. However, no such agents are currently available.

An alternative approach is to attempt to eliminate all memory T cells. This is based on the notion that latent virus will be found in the memory $CD4^+$ T cell compartment. In a provocative in vitro study, Vitetta and colleagues have shown that an anti-CD45RO immunotoxin can eliminate latently infected cells (226). In this case, the latently infected cells were generated by in vitro infection of PBMC from normal donors followed by an anti-CD25 treatment to kill activated cells. The latently infected cells under study were most likely cells in the preintegration state of latency. Impressive reductions in the amount of recoverable virus were achieved by treating the cells with an anti-CD45RO-toxin conjugate. The application of such an approach in vivo is complicated by the fact that it would kill activated and memory cells from both the $CD4^+$ and $CD8^+$ T cell subsets. In addition, a small portion of the latent virus may be present in naïve $CD45RO^-$ T cells (227). These latently infected cells may arise through phenotypic reversion of memory cell, infection of developing thymocytes, or through other unknown mechanisms.

Finally, it is possible that strategies to enhance immune responses to HIV-1 may allow immunologic control of the small amount of virus that emerges from the latent reservoir. The capacity of the immune system to control latent HIV-1 is illustrated by the fact that some long-term nonprogressors have no detectable plasma virus, possibly as a result of a strong immune response to HIV-1 (228). Recent studies suggest that latent virus is present in such individuals but is being controlled (202; J Blankson, R Siliciano, unpublished results).

SUMMARY

Recent studies have identified an extraordinarily stable reservoir in which HIV-1 can potentially persist for life even in patients on effective antiretroviral therapy. This reservoir consists of a small pool of latently infected resting $CD4^+$ T cells. These cells carry an integrated copy of the HIV-1 genome and can produce infectious virus when the cells are activated by the appropriate antigen. The stability of the reservoir may reflect the fact that it is continuously reseeded by a low level of ongoing viral replication. Alternatively, it may reflect normal mechanisms that maintain the level of memory T cells relatively constant. In any event, elimination of this reservoir by novel therapeutic approaches will likely be required before eradication can be achieved.

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