An anti-CD45RO immunotoxin eliminates T cells latently infected with HIV-1 in vitro

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ABSTRACT  Despite the success of highly active antiretroviral therapy (HAART) in lowering circulating HIV-1 to undetectable levels in most infected individuals, several studies have documented the presence of a small reservoir of latently infected cells in HAART patients, the majority of which are CD45RO+ memory T cells. We previously have demonstrated that latently infected, replication-competent cells can be generated in vitro after eliminating CD25+ cells with an immunotoxin (IT). The present study was designed to determine whether these latent cells could be eliminated by an anti-CD45RO IT. Our results indicate that the anti-CD45RO IT eliminates >99%, of either M-tropic or T-tropic virus produced by the latently infected cells after mitogen stimulation. This IT also appears to be as effective as the anti-CD25 IT in eliminating the activated, HIV-1-producing cells. In contrast, the anti-CD45RO IT does not kill CD45RA+ naïve cells. Further studies using cells from HIV-1-infected individuals on HAART will be necessary to determine the potential clinical utility of this IT.

The advent of highly active antiretroviral therapy (HAART) has had a significant impact on HIV-1-infected individuals, lowering circulating virus to undetectable levels (1–3). Despite this, latently infected cells can remain in these individuals for significant periods of time (4–6); if HAART is withdrawn, these cells can produce virus (7). Considering the postulated long half-life of latent viral reservoirs (6, 8) and the side effects and cost of chronic HAART (9, 10), it is important to develop new strategies to eliminate the latent reservoir. One strategy is to drive the latent cells into cycle with IL-2 in the presence of HAART until the viral reservoir is exhausted (11–13). Another strategy, not yet explored, is to specifically kill the latently infected cells.

To develop approaches to kill the latently infected cells, we have developed an in vitro model of HIV-1 latency that mimics many (but not all) of the physiologic characteristics of these cells in vivo (14–16). Because there is mounting evidence that the long-term, latently infected CD4+ T cells from patients on HAART are memory cells (4, 17–19), in this report we determined whether it was possible to eliminate our population of in vitro HIV-1-infected latent cells with an anti-CD45RO immunotoxin (IT). Our results demonstrate that the anti-CD45RO IT efficiently eliminates CD45RO+ T cells, but spares naïve T cells and a small number of CD45RA+ memory T cells. Importantly, this IT virtually eliminates latent cells that can produce virus after mitogen stimulation. These results could have potential clinical implications for HIV-1-infected individuals, if the latent cells in patients on HAART are also CD45RO+.

MATERIALS AND METHODS

Generation of Virus Stocks. HIV-1SAN is a primary isolate obtained from T. Folks (Centers for Disease Control and Prevention, Atlanta) (20). HIV-1JR-CSF is a primary isolate, and HIV-189.6 is a dual-tropic primary isolate; both were obtained from the National Institutes of Health AIDS Reagent Repository. Stocks of HIV-1JR-CSF, HIV-1SAN, and HIV-189.6 were grown in phytohemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMCs). HIV-1NL4–3 is a T cell line-adapted isolate obtained as a molecular clone in the plasmid pNL4–3 from J. Zack (Univ. of California, Los Angeles). The plasmid was transfected into HeLa CD4 tat cells obtained from Richard Gaynor (Univ. of Texas Southwestern, Dallas) by using Lipofectamine (GIBCO/BRL). Production of ITs. ITs were prepared by coupling mAbs to deglycosylated ricin A chain (dgA) by using the N-succinimidyl carbonyl α-methyl-(2-pyridyldithio) tolueno crosslinker (21). The following mAbs were used: UCHL1 (22) (a gift from Peter Beverley), a murine IgG2a reactive with human CD45RA; 2H4 (23) (ATCC HB 8570), a murine IgG1 reactive with human CD45RA. RFT5 is a murine IgG1 reactive with human CD25 (24), and RFB4 (25) is a murine IgG1 reactive with human CD22.

Sequential Treatment Protocols. Fresh PBMCs obtained from normal, healthy donors were exposed to HIV-1 for 2 hr and then treated as described in Fig. 1. Complete medium (CM) consisted of RPMI 1640, 15% FCS, L-glutamine, 10% IL-2, and antibiotics, and the ITs were used at 10 nM. A similar experimental design was used in experiments in which HIV-1-infected PBMCs were treated sequentially with the anti-CD25 IT followed by the anti-CD45RA IT or the anti-CD25 IT followed by the anti-CD45RO mAb.

Combination Treatment Protocols. After infection of fresh PBMCs, cells were cultured in 24-well plates for 6 days at a concentration of 10⁶ cells/ml with: (i) CM, (ii) 5 nM of the anti-CD25 IT + 5 nM of the anti-CD45RO IT, (iii) 5 nM of the anti-CD25 IT + 5 nM of the irrelevant control IT (RFB4-dgA), (iv) 5 nM of the anti-CD45RO IT + 5 nM of the irrelevant control IT (RFB4-dgA), and (v) 10 nM of the irrelevant control IT (RFB4-dgA). Cells were washed and activated, supernatants were collected, and concentrations of p24 were determined.

p24 Assays. The concentrations of p24 antigen in cell-free supernatants were measured by using a commercially available ELISA kit (NEN).

Abbreviations: HAART, highly active antiretroviral therapy; IT, immunotoxin; PHA, phytohemagglutinin; PBMC, peripheral blood mononuclear cell; dgA, deglycosylated ricin A chain; CM, complete medium.
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**Flow Cytometric Analysis.** Six-parameter flow cytometric analysis was performed on a modified FACSort flow cytometer equipped with a second 632-line diode laser (Becton Dickinson Immunocytometry Systems) by using FITC, phycoerythrin (PE), peridinin chlorophyl protein (PerCP), and allophycocyanin (AP) as the four fluorescent parameters. PBMCs were stained with combinations of FITC-anti-CD45RO (or -anti-CD45RA), PE-CD27, PerCP-CD4, and APC-CD95. In some experiments, PBMCs were stimulated for 4 hr with phorbol myristate acetate (PMA) and ionomycin in the presence of brefeldin A, fixed and permeabilized, and then stained for intracellular cytokines, as described (26). For each cytokine examined (IFN-γ, tumor necrosis factor α, IL-2, and IL-4), the stimulated cells also were stained for CD3, CD8, and the γδ T cell receptor such that cytokine-producing CD4+ T cells could be identified as CD3+, CD8+, and -TCR-γδ (PMA down-regulates CD4 expression). For each analysis, at least 20,000 events were acquired, gated on T cell subset-defining mAbs, and a light scatter gate was designed to include only small viable lymphocytes. List mode multiparameter data files (each file with forward scatter, orthogonal scatter, and four fluorescent parameters) were analyzed by using the PAINT-A-GATEPlus software program (Becton Dickinson Immunocytometry Systems).

The criteria for delineating and quantifying memory and naive T cells have been described. (27). Naive cells are CD95(lo), CD45RO(lo), and CD45RA(+), and memory cells are CD95(lo), CD27(lo), CD45RO(predominantly)+, and CD45RA(predominantly)−. Small numbers of CD4+ memory T cells and up to 50–80% of CD8+ memory T cells are CD45RO(lo)CD45RA(lo), and unlike naive cells, they are CD27(lo) and CD95(lo) (L.J.P., unpublished observations).

**RESULTS**

Treatment of in Vitro HIV-1-Infected PBMCs with the Anti-CD25 IT Produces Latently Infected Cells Containing Incomplete HIV-1 DNA Provirus. PBMCs were infected with HIV-1JR-CSF, and the activated cells were eliminated with the anti-CD25 IT (Fig. 1). As shown in Fig. 2, and in accord with our previous report (16), CD25+ latently infected cells contain only unintegrated, incomplete HIV-1 DNA provirus. After PHA activation, these cells contain full-length reverse transcripts and produce p24 antigen (45 ± 3 pg/ml, mean ± SEM).

The Anti-CD45RO IT Specifically Kills CD45RO+ Lymphocytes. We determined whether the anti-CD45RO IT specifically killed only cells expressing CD45RO. As shown in Fig. 3, treatment of normal PBMCs with the anti-CD45RO IT was highly effective in eliminating CD4+ CD45RO+ T cells; all but 1.48 ± 0.4% of these cells were eliminated. As defined by multiparameter criteria, CD4+ naive cells (red-colored events in Fig. 3) were not eliminated, and variable numbers of CD45RO(lo) memory cells also survived (blue-colored events in Fig. 3) (2.3–15.2% in four experiments). Importantly, stimulation of the surviving cells with phorbol myristate acetate and ionomycin resulted in synthesis of tumor necrosis factor α, IL-2, IFN-γ, and IL-4, indicating that these cells remain functional (data not shown).

Sequential Treatment with the Anti-CD25 and Anti-CD45RO ITs Eliminates Cells That Can Produce HIV. To determine whether the anti-CD45RO IT eliminated the latently infected cells, HIV-1-infected PBMCs were treated as described in Fig. 1. As shown in Fig. 4, as compared with untreated controls, cells incubated in CM followed by a 3-day treatment with the anti-CD45RO IT and a 3-day PHA activation showed a 90 ± 2% reduction in levels of p24. CD25+ cells treated with the anti-CD45RO IT for 3 days and then activated showed a 94 ± 2% mean reduction in the levels of p24.
Cellular Activation.

Anti-CD45RO IT Treatment of PBMCs Latently Infected with Different HIV-1 Strains Decreases p24 Production After Cellular Activation. A series of experiments were carried out to determine whether the ability of the anti-CD45RO IT to eliminate the latently infected cells occurred when cells were infected with different M-tropic, T-tropic, or dual-tropic isolates of HIV-1 and then treated as described in Fig. 1. In six experiments, this population of cells showed no consistent decreases in p24 levels (131 ± 31%) when compared with untreated controls (Fig. 4B).

Taken together, these experiments demonstrate that the latent cells prepared in vitro are CD45RO+ CD45RA− and that the elimination of these cells requires the toxin portion of the IT.

Treatment with the Anti-CD45RO IT Is As Effective As Treatment with a Combination of the Anti-CD25 and the Anti-CD45RO ITs in Eliminating Cells That Can Produce HIV. We determined whether there was an advantage in targeting both productive and latent cells as well as cells that were transitioning from one state to the other. As shown in Fig. 5, in four experiments, a mixture of the anti-CD25 and anti-CD45RO ITs or the anti-CD45RO IT alone reduced subsequent production of p24 in mitogen-activated cells by 99.9 ± 0.07% and 99.7 ± 0.3%, respectively, demonstrating that the anti-CD45RO IT alone was as effective as the combination of the two ITs. In both cases, this decrease was greater than that observed by using the sequential treatment protocol, probably because the PBMCs were exposed to the ITs for 6 days instead of 3. Despite the >99% reduction in mitogen-induced virus production, treatment of the HIV-1-infected PBMCs with one or both ITs did not eliminate all the memory cells (i.e., those defined as CD45RO+ CD27+ CD95hi) as 3–8% remained (Fig. 5). These experiments indicate that the CD45RO+ cells are the major, if not the sole, reservoir of HIV-1 in the in vitro-infected cells.

DISCUSSION

Over the past several years, we have developed a model of in vitro HIV-1 latency in which large numbers of cells can be studied. These cells share many key features with in vivo latent cells. Thus, they are resting, but replication-competent and do not produce virus unless they are activated with PHA or anti-CD3 or are cocultured with activated cells, at which time they again produce large amounts of virus (14, 15). However, in contrast to in vivo latent cells, our in vitro latent cells contain only incomplete viral genomes (16). Although most investigators using cells from HIV-1-infected individuals have defined latent cells as resting CD4+ DR− CD45RO+ cells containing integrated viral DNA (17–19), they also have documented the coexistence of cells with unintegrated virus (4, 5). Indeed, older (28) as well as more recent studies (6, 29, 30) dispute the concept that the “latent” reservoir corresponds only to long-term memory cells containing integrated virus and demonstrate that there is a low level of ongoing viral replication as well as the presence of resting cells with unintegrated HIV-1 DNA. Regardless of this controversy it was of obvious interest to determine whether our in vitro-generated latent cells were CD45RO+. If this were the case, it is likely that strategies to
kill or inactivate these cells will be applicable to killing CD45RO+ latent cells in vivo whether or not the status of the viral genome is the same or different. We therefore prepared CD25+ latently infected cells and determined whether they could be killed by ITs directed against CD45RO+ or CD45RA+. After treatment, cells were analyzed by flow cytometry or were activated with mitogen to measure concentrations of p24. The major findings to emerge from these studies are: (i) The anti-CD45RO IT effectively eliminated its target cell populations but had no effect on the CD45RA+ cells. The anti-CD45RO IT did not eliminate the few CD4+ CD45RO− memory cells, which remained viable and functional as determined by cytokine expression after activation. (ii) After 6 days of treatment, the anti-CD45RO IT virtually eliminated mitogen-driven virus production. (iii) The anti-CD45RO IT had little or no effect on the latently infected cells. (iv) The anti-CD45RO IT was effective in eliminating the latent reservoirs of both M-tropic and T-tropic HIV-1. (v) Based on comparisons between the anti-CD45RO IT alone and the combination of the anti-CD45RO + the anti-CD25 ITs, it appears that the anti-CD45RO IT is as effective as the anti-CD25 IT in eliminating the activated, HIV-1-producing cells.

Obviously, a key question emerging from our findings is whether this IT also will eliminate the more “conventionally defined” latent cells from patients on HAART. Thus, in vivo, all the latent cells may not express CD45RO or may be CD45RO−. If, however, the anti-CD45RO IT does eliminate all of these cells, a critical question concerning the potential clinical application of an IT directed against CD45RO is whether such an IT will reduce the latent viral reservoir at the expense of significantly compromising the immune system. Arguing against this: (i) Naive T cells are spared by the anti-CD45RO IT. (ii) There is evidence that CD8+ CD45RO+ memory cells can revert to a CD45RA− phenotype (31–33). (iii) There is a small population of anti-CD45RO IT-resistant CD4+ CD45RO− CD27− CD95+ memory cells that do not produce HIV-1 after mitogen activation and these cells are functional. (iv) Based on other reports (34) it appears that new T cells can be generated in the thymus after HAART therapy. Taken together these considerations suggest that it may be feasible to use the anti-CD45RO IT in vivo.

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