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Differential Susceptibility of Naive and Memory CD4$^+$ T Cells to the Cytopathic Effects of Infection with Human Immunodeficiency Virus Type 1 Strain LAI

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CD4$^+$ T lymphocytes of individuals infected with human immunodeficiency virus type 1 (HIV-1) exhibit a qualitative defect in their ability to mount memory responses to previously encountered antigens although their responses to mitogens remain normal. T cells responsible for memory responses can be distinguished from naive T cells based on differential expression of isoforms of the tyrosine phosphatase CD45. It has been suggested that memory CD4$^+$ T cells from infected individuals have a greater virus burden than naive CD4$^+$ T cells and that this accounts for the loss of recall responses in infected individuals. However, it has been unclear whether naive and memory T cells are equally susceptible to infection and to the cytopathic effects of the virus. We therefore infected highly purified resting naive and memory CD4$^+$ T cells from HIV-1-seronegative individuals with HIV-1$_{LAI}$. Infected cells were then stimulated with phytohemagglutinin to render them permissive for viral replication. Cell viability and growth rate were monitored for 8 to 10 days as indicators of cytopathic effects induced by HIV-1$_{LAI}$. Our results indicated that naive and memory CD4$^+$ T cells display marked differences in susceptibility to the cytopathic effects induced by HIV-1$_{LAI}$ infection. The cytopathic effects induced by HIV-1$_{LAI}$ were much more severe in memory CD4$^+$ T cells than in naive CD4$^+$ T cells. Differential cytopathic effects in naive and memory T cells were not due to differences in virus entry into and replication in these cell populations. Rather, memory cells were more susceptible to cytopathic effects. Pronounced cytopathic effects in memory cells were clearly detectable at 7 day postinfection. Cell death occurred at the single-cell level and was not accompanied by syncytium formation. The growth rate of infected memory CD4$^+$ T cells was also severely compromised compared to that of naive CD4$^+$ T cells, whereas the growth rates of both uninfected naive and memory CD4$^+$ T cells were approximately the same. At least a portion of the dying cells exhibited biochemical changes characteristic of apoptosis. These results suggest that the selective functional defects present in the memory CD4$^+$ T-cell subset of HIV-1-infected individuals may in part be the result of the greater susceptibility of memory T cells to cytopathic effects induced by HIV-1.

The depletion of CD4$^+$ T cells is the central and unique pathophysiologic feature of human immunodeficiency virus type 1 (HIV-1) infection and is largely responsible for the profound immunodeficiency that is characteristic of late stages of the disease. One of the central questions in AIDS pathogenesis is whether the direct cytopathic effects of HIV-1 account for the depletion of CD4$^+$ T cells in vivo. Although several potential mechanisms for the destruction of noninfected CD4$^+$ T cells have been described (3, 19, 26, 42, 45, 55), it is also clear that HIV-1 is directly cytopathic for infected cells. Early studies demonstrated that HIV-1 infection is cytopathic for some CD4$^+$ T cells in vitro (49). Two general types of HIV-1-induced cytopathic effects have been observed. In some experimental systems, syncytia form by the fusion of infected cells expressing the HIV-1 envelope (Env) protein and cells expressing CD4 (39, 40, 56). There are also cytopathic effects that operate at the level of individual infected cells; that is, under some conditions, HIV-1-infected T cells appear to die from the infection independent of any cell-cell fusion events (7, 57, 60). Apoptosis has been implicated as one mechanism for the death of HIV-1-infected T cells (37, 62).

Analysis of the role of HIV-1-induced cytopathic effects in CD4 depletion in vivo is complicated by the fact that the CD4$^+$ T-cell compartment is heterogeneous, consisting of both quiescent and activated cells. Among the quiescent CD4$^+$ T-cell population, naive and memory subsets can be defined based on prior exposure to antigen. These subsets can be distinguished based on antigen-induced changes in the patterns of expression of certain cell surface proteins (1, 43, 68). Following initial exposure to antigen, naive T cells undergo blast transformation and enter the cell cycle. Activation results in the upregulation of expression of sets of molecules including transcription factors, effector molecules such as cytokines and perforin, and cell surface proteins such as cytokine receptors and adhesion molecules (12). After several rounds of division, some of the activated cells revert to a resting state in which they persist as memory T cells capable of responding to subsequent exposures to the initiating antigen. Altered patterns of expression of some cell membrane proteins reflect differences between the quiescent and activated states. For example, T-cell activation results in temporary expression of HLA-DR molecules and the α chain of the interleukin-2 (IL-2) receptor. 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 (43). For example, naive and memory T cells express different splice variants of the membrane tyrosine phosphatase CD45 (1, 4, 38, 53). In addition to the quiescent and activated subsets and the naive and memory subsets of CD4$^+$ T cells, functional subsets

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of $CD4^+$ T cells can be defined based on patterns of cytokine secretion (47) and cytolytic activity (46, 55, 58). How infection with HIV-1 affects the various subsets of $CD4^+$ T cells is a matter of great interest.

HIV-1 virions can bind to and fuse with both quiescent and activated $CD4^+$ T cells (21, 70). However, in the case of quiescent T cells, productive infection does not result either because reverse transcription does not proceed to completion in quiescent T cells (69, 71) or because nuclear import of the preintegration complex containing the reverse-transcribed viral DNA does not occur, possibly due to the absence in quiescent T cells of sufficient metabolic energy for transport of the complex through the nuclear pores (5, 6). Because viral replication does not even proceed to the stage of integration in resting T cells, HIV-1-induced cytopathic effects are most certainly operative only in activated T cells, which are fully permissive to productive infection.

It is much less clear whether naive or memory $CD4^+$ T cells are more susceptible to the cytopathic effects of HIV-1 infection following activation. It is expected that viral replication will occur in both types of cells following activation, but differences in the course of infection in the two types of cells remain largely unexplored. The importance of this issue lies in the notion that defects in the memory T-cell response may account for the susceptibility of AIDS patients to infection by frequently encountered and normally nonpathogenic organisms. Defects in the responses of memory $CD4^+$ T cells to previously encountered antigens were noted in early studies of immune responses in AIDS patients (36) and have been attributed to preferential infection of these cells (54). A preferential loss of memory cells from the peripheral blood of seropositive individuals has been observed in some (14) but not other (10, 24, 25, 51) phenotyping studies, although both subsets show profound depletion in AIDS patients. Interpretation of many of the published studies is complicated by the fact that the use of individual phenotypic markers frequently fails to define physiologically relevant subsets. For example, both activated $CD4^+$ T cells and resting memory $CD4^+$ T cells express the RO isoform of CD45, which is widely used as a marker for memory T cells. Improved cell separation technology now permits detailed analysis of the effects of HIV-1 infection on distinct subsets of $CD4^+$ T cells. The purpose of this study was to determine whether naive or memory $CD4^+$ T cells are more susceptible to the HIV-1 infection and to the cytopathic effects of the virus following activation.

MATERIALS AND METHODS

Preparation of PBLs and resting memory ($CD45RO^+$) and naive ($CD45RA^+$) $CD4^+$ T cells. Blood from HIV-1-seronegative donors was drawn into a heparinized syringe, and peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over discontinuous Ficoll-Hypaque (Pharmacia Biotech) density gradients. Macrophages were depleted by adherence to a plastic culture flask for 4 h at 37°C in culture medium consisting of RPMI 1640 (Mediatech) supplemented with 10% fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine. The nonadherent peripheral blood lymphocyte (PBL) fraction was further processed to isolate resting naive ($CD45RA^+$) and memory ($CD45RO^+$) $CD4^+$ T cells. This was accomplished by using a combination of a negatively selecting magnetic bead depletion step and flow cytometric sorting. PBL were divided into two aliquots and incubated with monoclonal antibodies specific for cell surface proteins expressed on B cells (CD19), macrophages (CD14), $CD8^+$ T cells (CD8), natural killer cells (CD16), and activated T cells (CD69, CD25, and HLA-DR), in addition to negatively selecting antibodies to either CD45RA or CD45RO. Cells were incubated with the cocktail of antibodies for 40 min on ice and washed three times with wash medium (WM) consisting of phosphate-buffered saline (pH 7.2) supplemented with 2% fetal calf serum, 0.1% glucose, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 12 mM HEPES (pH 7.2). Then magnetic beads conjugated with sheep anti-mouse immunoglobulin G antibodies (Dynal) were added to the cells in a 5-ml polystyrene tube, and the suspension was agitated on a bidirectional rocking shaker at 4°C. The bead-to-cell ratio was 15:1. After a 40-min incubation, a magnet was applied to remove the cells attached to the antibody-conjugated beads, and nonattached cells were collected. Magnetic bead depletion was repeated twice to remove any residual beads present in the nonattached cell population. The purified nonattached cells were then incubated with phycoerythrin (PE)-conjugated anti-CD4 (Ortho Diagnostics), Tri-Color-conjugated anti-HLA-DR (Caltag), and complementary positively selecting fluorescein isothiocyanate (FITC)-conjugated anti-CD45RO or anti-CD45RA antibodies (Coulter). After a 40-min incubation on ice, the cells were washed twice with cold WM and sorted by using an EPICS ELITE cell sorter (Coulter) to obtain $CD4^+$ $HLA\text{-}DR^-$ $CD45RO^+$ and $CD4^+$ $HLA\text{-}DR^-$ $CD45RA^+$ cells. Throughout the cell purification procedure, cells were kept cold to prevent any possible T-cell activation. Aliquots of unfractionated PBMC and purified cells were analyzed by flow cytometry to determine the purity of the sorted cells. The anti-CD4 antibody used in the purification reacts with a CD4 epitope that does not overlap with the gp120 binding site on CD4 and as a result does not block infection.

CD4 and CXCR4 expression on purified naive and memory $CD4^+$ T cells was measured by flow cytometry. For CD4 expression, 2×10^5 cells from each population were incubated with PE-conjugated anti-CD4 antibody (Becton Dickinson) for 40 min on ice. Cells were washed twice with cold WM and examined by using an EPICS ELITE fluorescence-activated cell sorter (Coulter). For CXCR4 expression, cells were incubated with antifusin antibody 12G5 (kindly provided by James Hoxie, University of Pennsylvania, Philadelphia) for 40 min on ice. Following two washes, cells were further incubated with Tri-Color-conjugated goat anti-mouse immunoglobulin G (Caltag) for fluorescence-activated cell sorting FACS analysis.

In vitro infections with HIV-1_{LAI}. Naive ($CD4^+$ $HLA\text{-}DR^-$ $CD45RA^+$) and memory ($CD4^+$ $HLA\text{-}DR^-$ $CD45RO^+$) T cells (1×10^6 to 2×10^6) were incubated with pelleted HIV-1_{LAI} (Advanced Biotechnology Inc.) at a multiplicity of infection (MOI) of 1 for 2 h at 37°C in a humidified incubator. After the infection, cells were incubated with 1× Trypsin-EDTA (Gibco-BRL) for 15 min at 37°C to remove any CD4-bound HIV-1 virions. Cells were then washed twice in cold WM. Cells from each population (1×10^6 to 2×10^6) were then incubated with phytohemagglutinin (PHA) and irradiated PBMC from HIV-1-seronegative donors to induce activation of the infected resting cells. After 3 days of incubation, cells were subjected to Ficoll-Hypaque centrifugation to remove any residual irradiated feeder cells. Then infected naive and memory T cells were enumerated, and 1×10^6 to 2×10^6 cells from each population were incubated in culture medium supplemented with 200 U of IL-2 per ml at a concentration of 10^6 cells/ml for further observation.

Analysis of the entry of HIV-1_{LAI} into resting naive and memory $CD4^+$ T cells. To measure the efficiency of HIV-1_{LAI} entry into naive and memory $CD4^+$ T cells, cells were infected with HIV-1_{LAI} at an MOI of 1 for 2 h followed by incubation with 1× Trypsin-EDTA for 15 min at 37° and two washes with cold WM. Infected cells from each population (1×10^6 to 2×10^6) were then activated to induce completion of reverse transcription. Activation was carried out by incubation with PHA and irradiated PBMC from HIV-1-seronegative donors (3×10^6). The following day, PHA was removed, and cells were further incubated in the presence of antiretroviral drugs to prevent further rounds of infection (67). The drugs used were zidovudine (1 μM), ddI (10 μM), and L697,661 pyridinone (0.65 μM). After an additional 24 h, the lymphoblasts were isolated and subjected to Ficoll-Hypaque centrifugation to remove any residual irradiated feeder cells. DNA was isolated from 10^6 cells from each population, using a DNA isolation kit (Gentra). Following DNA isolation, HIV-1 Gag-specific PCRs were carried out with primers 5′-GTIATACCCATGTTTTCAG CATTA-3′ (5′ primer) and 5′-AGCTTCCTCATTGATGGTITCTTT-3′ (3′ primer) to amplify unintegrated and integrated HIV-1 DNA. The PCR product was detected by the probe 5′-AACACAGTGGGGGGACAT-3′ by performing Southern hybridization. The presence of β-globin was monitored by additional PCRs in parallel to control for the quantity and integrity of the DNA used. Serially diluted ACH-2 genomic DNA was subject to PCRs in parallel.

Expression of gp41 by infected naive and memory $CD4^+$ T cells. To examine the degree and maintenance of HIV-1_{LAI} infection in naive and memory $CD4^+$ T cells, expression of gp41 was monitored periodically by using the HIV-1_{LAI}-specific anti-gp41 antibody T32 (kindly provided by Pat Earl, National Institutes of Health). This antibody reacts with a determinant in the extracellular portion of the molecule. Cells (0.2×10^5) were pelleted and incubated with anti-gp41 antibody for 40 min on ice. After two washes with cold WM, cells were further incubated with secondary antibody conjugated with Tri-Color (Caltag) for 40 min on ice. After two washes with cold WM, antibody stained cells were analyzed by flow cytometry.

Cytopathic effects and virus production in HIV-1_{LAI}-infected naive and memory $CD4^+$ T cells. To measure the differential cytopathic effects induced by HIV-1_{LAI} in naive and memory $CD4^+$ T cells, the degree of cell death and the cell growth rate were monitored daily for 8 to 10 days. To monitor the cell death, aliquots of cells were stained with trypan blue (Gibco-BRL), and dead and live cells were counted. This procedure was repeated four times for accurate measurement of dead and live cells. To measure the virus production in each population, a supernatants were collected every day and analyzed by using an HIV-1 p24 antigen assay kit (Coulter).

Apoptosis in HIV-1_{LAI}-infected naive and memory $CD4^+$ T cells. To determine whether naive and memory $CD4^+$ T cells show differential susceptibility to

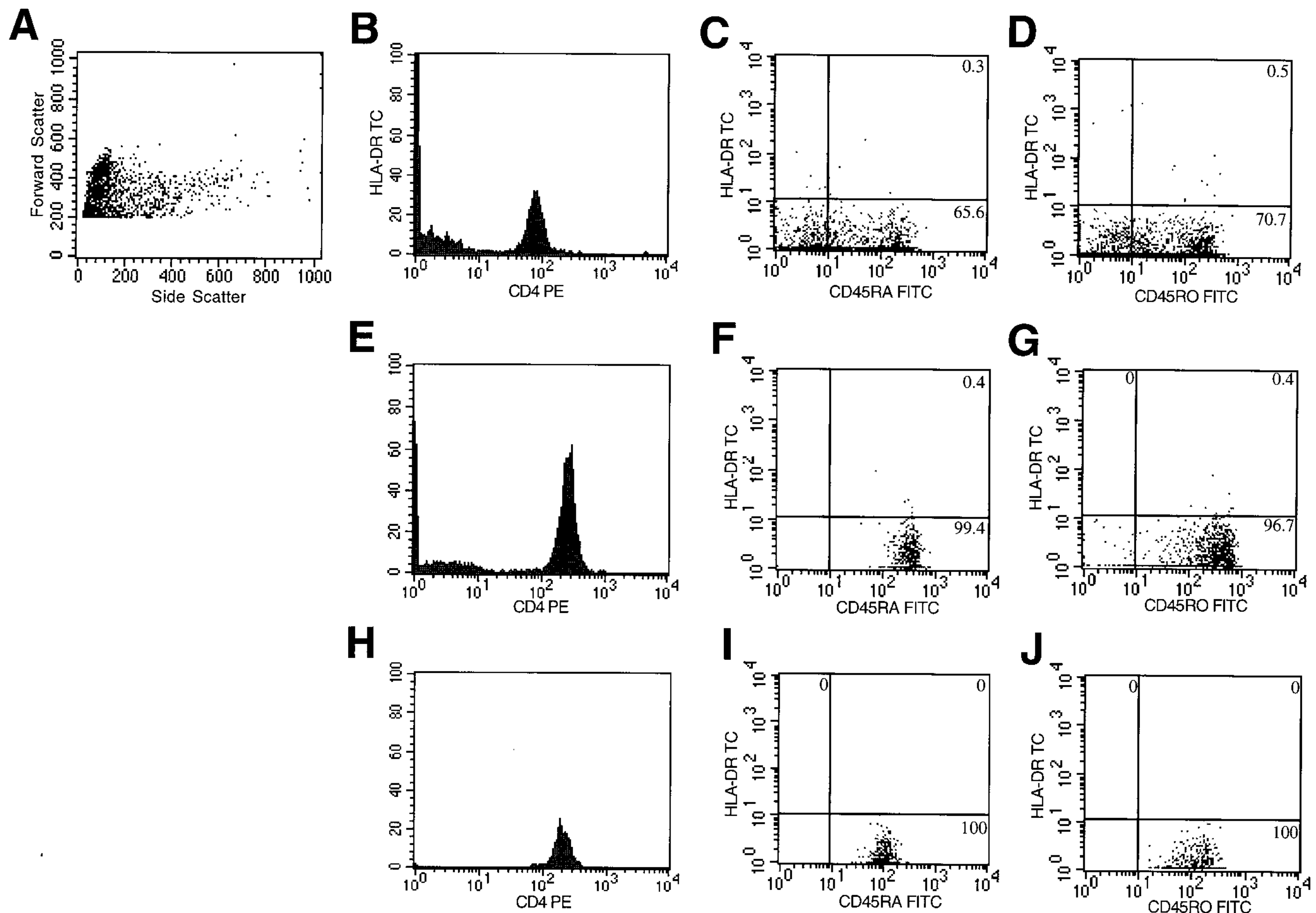

FIG. 1. Isolation of resting naive and memory CD4$^+$ T cells from PBMC of HIV-1-seronegative donors. Resting naive and memory CD4$^+$ T cells from HIV-1-seronegative donors were isolated by using a combination of magnetic bead depletion and flow cytometric sorting techniques. Flow cytometric analyses of populations obtained after each step of a representative purification are shown. Panels A to D are FACS profiles of unfractionated PBL. All fluorescence profiles are gated on small lymphocytes as shown in panel A. Panels C and D are plots of HLA-DR versus CD45RO or RA expression and are gated on the CD4$^+$ population shown in panel B. Panels E to G show fluorescence profiles of cells after magnetic bead depletion of B cells, CD8$^+$ T cells, macrophages, natural killer cells, activated CD4$^+$ T cells, and either CD45RO$^+$ or CD45RA$^+$ cells. Panels F and G are plots of HLA-DR versus CD45RO or RA expression and are gated on the CD4$^+$ population shown in panel E. The resting CD4$^+$ CD45RA$^+$ and CD4$^+$ CD45RO$^+$ T-cell populations were only minimally contaminated with the other population or with activated cells at this stage of purification (99.4 and 96.7% purity, respectively). These bead-depleted cells were then further purified by staining the cells with PE-conjugated anti-CD4, Tri-Color (TC)-conjugated anti-HLA-DR, and FITC-conjugated anti-CD45RA or anti-CD45RO antibodies. Stained cells were subjected to three-color FACS to collect CD4$^+$ HLA-DR$^-$ CD45RA$^+$ and CD4$^+$ HLA-DR$^-$ CD45RO$^+$ cells (H to J). Panels I and J are plots of HLA-DR versus CD45RA or CD45RO expression and are gated on the CD4$^+$ population shown in panel H. As shown in panel H, virtually all of the cells are CD4$^+$ at this stage of the purification. After the sorting process, the resulting cells were virtually free of activated CD4$^+$ T cells as judged by the expression of HLA-DR, and the purity of both the CD45RA$^+$ and CD45RO$^+$ populations was greater than 99.9%.

apoptosis following infection and activation, aliquots of cells from each population were stained with FITC-conjugated Annexin V (BRAND Applications) and propidium iodide (PI; Sigma) and then analyzed by two-color flow cytometry. Cells (2×10^5) from each infected population were pelleted and resuspended in 450 μl of 1× Annexin V binding buffer supplied by the manufacturer. Then 5 μl of 1× Annexin V-FITC was added to each sample along with 50 μl of PI solution (100 μg/ml). Cells were incubated on ice for 10 min followed by one wash with cold 1× Annexin binding buffer and analyzed immediately by flow cytometry.

RESULTS

Isolation of resting naive and memory CD4$^+$ T cells from HIV-1-seronegative donors. To compare the susceptibility of naive and memory CD4$^+$ T cells to HIV-1 infection and to HIV-induced cytopathic effects following activation, we developed a method for isolating quiescent cells free from any activated T cells and for separating the quiescent cells into naive and memory populations. These populations were then infected with HIV-1 at high multiplicity and exposed to activating stimuli in order to render the cells permissive for viral replication. Cytopathic effects were then assessed in the two populations of cells.

Resting naive and memory CD4$^+$ T cells were isolated by using a combination of magnetic bead depletion and flow cytometric sorting techniques. Figure 1 shows the flow cytometric analysis of populations obtained after each step of a representative purification. Parallel starting aliquots of PBL were subjected to monoclonal antibody-magnetic bead depletion of B cells, CD8$^+$ T cells, monocytes, natural killer cells, and activated T cells and either CD45RA$^+$ or CD45RO$^+$ cells. This negative selection step gave rise to populations of CD4$^+$ DR$^-$ cells highly enriched in either memory or naive cells. The aliquots were each stained with fluorochrome-conjugated monoclonal antibodies to CD4 and to HLA-DR and were stained separately with antibodies to CD45RO or CD45RA. The cells were then subjected to three-color FACS to collect CD4$^+$ HLA-DR$^-$ CD45RO$^+$ and CD4$^+$ HLA-DR$^-$ CD45RA$^+$ populations. After the sorting process, the resulting cells were

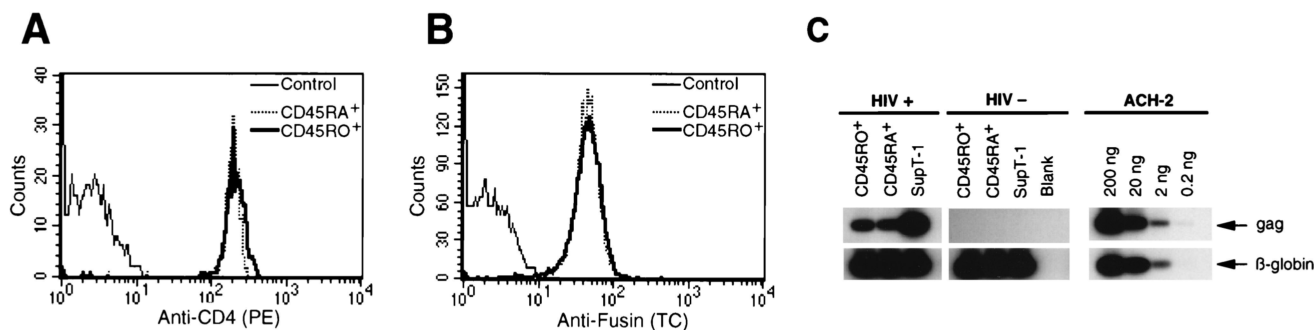

FIG. 2. Entry of HIV-1_{LAI} into resting naive and memory $CD4^+$ T cells. (A) CD4 expression on highly purified resting naive ($CD45RA^+$) and memory ($CD45RO^+$) $CD4^+$ T cells. (B) CXCR4 (fusin) expression on highly purified resting naive ($CD45RA^+$) and memory ($CD45RO^+$) $CD4^+$ T cells. TC, Tri-Color. (C) Analysis of the synthesis of proviral DNA following infection of highly purified resting $CD4^+$ T-cell naive ($CD45RA^+$) and memory ($CD45RO^+$) subsets. Naive and memory cell populations were purified as described in the text and then infected at high multiplicity (MOI = 1) with HIV-1_{LAI} for 2 h followed by trypsinization and extensive washing to remove any bound extracellular virus. Cells were then activated with PHA and allogeneic PBMC and cultured for 24 h. Further rounds of infection were inhibited by the addition of reverse transcriptase inhibitors, and after another 24 h of culture, the lymphoblasts were isolated and DNA was prepared. HIV-1 proviral DNA was quantitated by PCR using Gag primers. Uninfected cells were processed in parallel. Serial dilutions of ACH-2 DNA were also analyzed to demonstrate the sensitivity of the assay to substrate concentration under the conditions used.

virtually free of activated $CD4^+$ T cells as judged by the expression of HLA-DR. The purity of resulting each resting $CD4^+$ T-cell population as determined by expression of naive (CD45RA) and memory (CD45RO) markers was greater than 99.9%. Functional analysis of the naive and memory populations by using [^{3}H]thymidine incorporation assays revealed that in the absence of any in vitro stimulus, neither the naive nor the memory $CD4^+$ T cell subsets showed evidence of cell proliferation. Both proliferated equally well upon activation with PHA for 3 days (data not shown).

Entry of HIV-1_{LAI} into resting naive and memory $CD4^+$ T cells. To measure the efficiency of HIV-1_{LAI} entry into naive and memory $CD4^+$ T cells, we first examined the expression level of CD4 and of the CXCR4 (fusin) coreceptor (17) on the surfaces of purified resting naive and memory $CD4^+$ T-cell populations from HIV-1-seronegative donors. As shown in Fig. 2A, the levels expression of CD4 by all populations were equivalent. In addition, naive $CD4^+$ T cells expressed at least as much CXCR4 as memory T cells (Fig. 2B). Therefore, analysis of surface expression of the two proteins directly involved in the entry of HIV_{LAI} does not provide any indication that the memory cells should be preferentially infected. To measure the entry of virus into cells from each population, we assayed for the synthesis of proviral DNA in acutely infected naive and memory $CD4^+$ T cells. Cells were infected at high multiplicity for 2 h and then washed and trysinized to inactivate any residual extracellular virus (61). Because rates of reverse transcription are low in resting cells, the resting naive and memory cells were then activated by addition of PHA and irradiated allogeneic PBMC. After 24 h, antiretroviral agents were added to prevent additional rounds of infection, and after an additional 24 h, lymphoblasts were isolated and genomic DNA from each infected cell population was isolated for PCR analysis of the copy number of HIV-1 DNA. As shown in Fig. 2C, Gag bands were equivalent in naive and memory $CD4^+$ T cells. Parallel experiments on serially diluted ACH-2 DNA demonstrated the sensitivity to the assay to template concentration under the conditions used. Taken together, these data indicate that synthesis of proviral DNA occurs to approximately the same extent in both naive and memory $CD4^+$ T cells following infection and cellular activation.

Expression of gp41 and virus production in infected naive and memory $CD4^+$ T cells. To examine the degree and maintenance of infection of HIV-1_{LAI} in infected naive and memory $CD4^+$ T cells, expression of extracellular gp41 and virus production were monitored periodically following infection and activation. Figure 3A shows representative FACS profiles of gp41 expression in infected naive and memory $CD4^+$ T cells along with uninfected cells. As the FACS data indicate, infected cell populations were uniformly positive for gp41 ex-

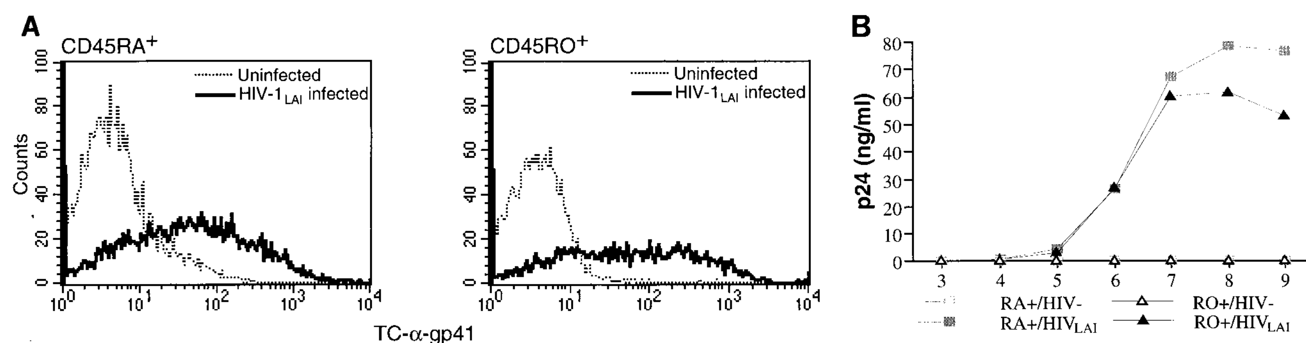

FIG. 3. Expression of gp41 and virus production in HIV-1_{LAI}-infected naive and memory $CD4^+$ T cells. (A) Flow cytometric analysis of gp41 expression by infected naive and memory $CD4^+$ T cells. Resting naive ($CD45RA^+$) and memory ($CD45RO^+$) $CD4^+$ T-cell populations were isolated as described in the legend to Fig. 1, infected with HIV-1_{LAI} for 2 h, washed, and then activated with PHA and allogeneic feeder cells to render the cells permissive for viral replication. After 6 days of incubation, expression of gp41 was monitored by flow cytometry. TC-α-gp41, Tri-Color-conjugated anti-gp41 antibody. (B) Production of p24 antigen by cultures of infected and uninfected naive and memory T cells prepared as described for panel A.

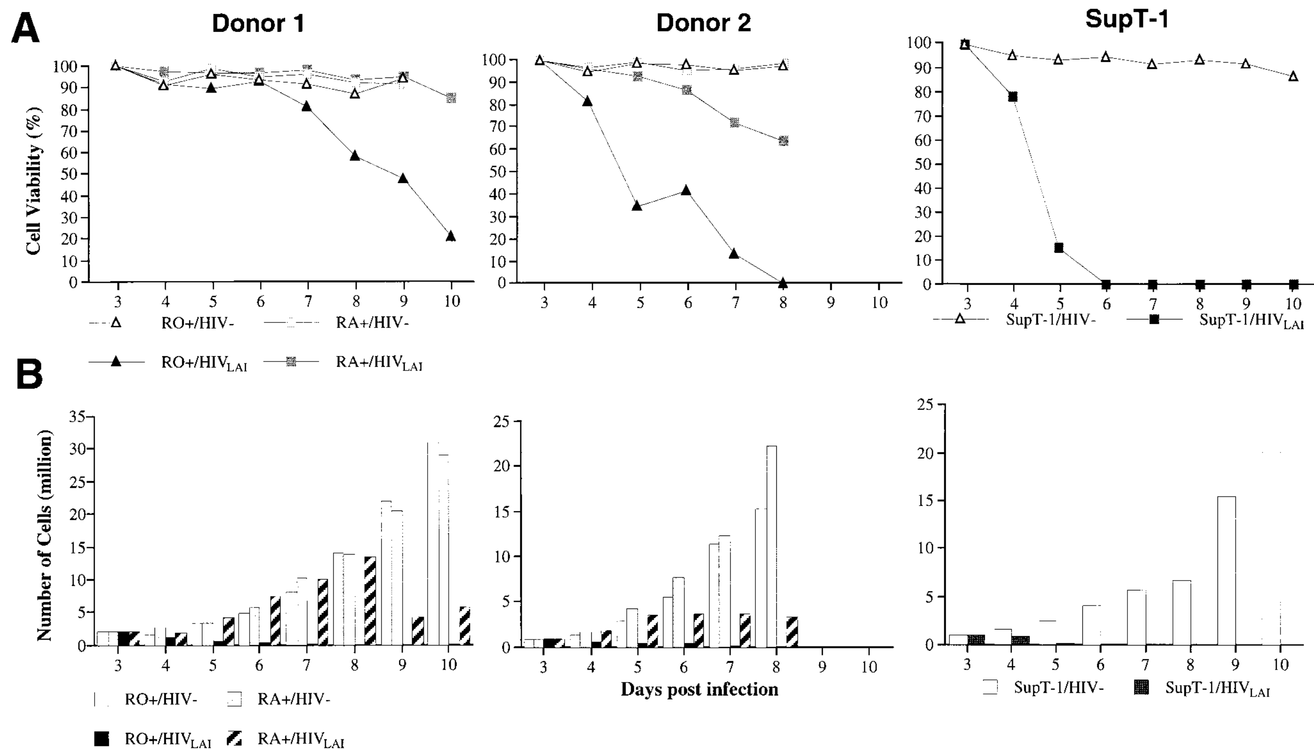

FIG. 4. Differential cytopathic effects in naive and memory CD4$^+$ T cells induced by HIV-1$_{LAI}$. (A) Effect of infection with HIV-1$_{LAI}$ on the viability of naive and memory CD4$^+$ T cells from two different donors and on the viability of SupT1 cells. Resting naive and memory CD4$^+$ T-cell populations were isolated as described in the legend to Fig. 1, infected with HIV-1$_{LAI}$ for 2 h (MOI = 1), washed, and then activated with PHA and allogeneic feeder cells to render the cells permissive for viral replication. The viability of the infected cells and of uninfected control cells from two different seronegative donors was monitored by trypan blue exclusion. The viability of infected and uninfected SupT1 cells was also monitored. (B) Effect of infection with HIV-1$_{LAI}$ on the growth rate of naive and memory CD4$^+$ T cells from two different donors and on the growth rate of SupT1 cells.

pression. The level of gp41 expression on infected memory CD4$^+$ T cells was either slightly higher than or equal to that of infected naive CD4$^+$ T cells. The level of virus production in each infected population was also quantitated by measuring the amount of p24 in culture supernatant (Fig. 3B). The levels of virus production were approximately the same in both infected memory and naive CD4$^+$ T cells. The drop in virus production in memory cells at the later time points was due to the lack of viable memory CD4$^+$ T cells. These results indicated that HIV-1 infection of and replication in naive and memory CD4$^+$ T cells were approximately equivalent.

Differential cytopathic effects in naive and memory CD4$^+$ T cells induced by HIV-1$_{LAI}$. To determine whether naive and memory CD4$^+$ T cells are equally susceptible to the cytopathic effects induced by HIV-1, we infected highly purified resting naive and memory CD4$^+$ T cells with HIV-1$_{LAI}$ and then activated the cells with PHA. Then cells were incubated for 8 to 10 days in culture in the presence of IL-2, and cytopathic effects were assessed by measuring viability and number of each cell population. Infections were carried out at a high multiplicity (MOI = 1) so that most or all of the cells in the culture were infected (Fig. 3A). Therefore, the cytopathic effects observed represent cytopathic effects in infected cells, not HIV-1-induced cytopathic effects in noninfected cells (19). Figure 4A shows representative viability curves for cells from two different donors and for a T-cell line, SupT1. The viability of both infected naive and memory cells was maintained at a high level (above 90%) for up to 5 days postinfection. However, the viability of infected memory T cells started to drop significantly after 7 days of infection and rapidly dropped thereafter. The viability of infected naive CD4$^+$ T cells was maintained at the relatively high level throughout the course of the incubation period. In some experiments, cytopathic effects were observed in infected naive cells, but the viability of infected naive CD4$^+$ T cells was always at least 40% higher than that of infected memory CD4$^+$ T cells. The difference in the susceptibility of naive and memory cells to HIV-1-induced cytopathic effects was noted in each of seven different experiments using cells from five different normal donors (Table 1). The viability of both uninfected naive and memory CD4$^+$ T cells was maintained at a high level (greater than 90%) throughout the cul-

TABLE 1. Cytopathic effects in naive and memory CD4$^+$ T cells

Donor	Cell viability 7–9 days postinfection (%)[a]	
	Naive CD4$^+$ T cells	Memory CD4$^+$ T cells
1	21	85
2A[b]	0	64
2B	52	90
2C	27	67
3	20	62
4	48	85
5	16	50
Avg	26	72

[a] Highly purified resting naive and memory CD4$^+$ T cells were infected with HIV-1$_{LAI}$ and then activated as described in Materials and Methods. Viability was measured by trypan blue exclusion.

[b] Donor 2 was assayed on three separate occasions.

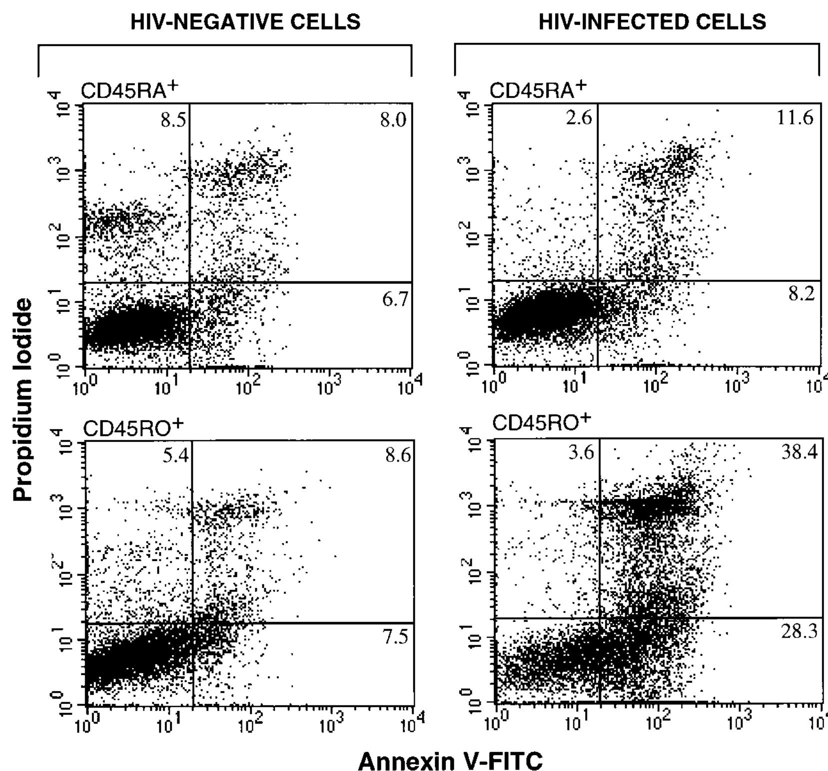

FIG. 5. Apoptosis in HIV-1_{LAI}-infected naive and memory $CD4^+$ T cells. Resting naive ($CD45RA^+$) and memory ($CD45RO^+$) $CD4^+$ T cells were infected and activated as in the previous experiments. At day 5 after infection, cells were stained with PI and with FITC-conjugated Annexin V. Uninfected control cells were also stained. Stained cells were analyzed by two-color flow cytometry.

ture period. In control experiments, SupT1 cells were also infected with HIV-1_{LAI} and viability was monitored. There was rapid drop in cell viability of infected SupT1 cells as early as day 3 postinfection, and all infected cells died by day 6.

Mitogen-driven proliferation was also measured in infected and uninfected naive and memory T-cell populations. Figure 4B shows representative cell growth curves for parallel cultures from the same two donors and for SupT1 cells. The growth rate of infected memory $CD4^+$ T cells was severely compromised; in general, there was no detectable increase in cell number. The infected naive T cells grew in the first 3 to 4 days after infection but eventually stopped proliferating. At any given time point, the degree of cell growth in infected naive T cells exceeded that of infected memory T cells. Uninfected naive and memory cells grew well at comparable rates. And as expected, the growth of infected SupT1 cells was not detected, although that of uninfected cells was normal. These data clearly show that cytopathic effects induced by HIV-1_{LAI} are much more severe in memory $CD4^+$ T cells than in naive $CD4^+$ T cells, judged by the rate of cell death and cell growth.

Apoptosis in HIV-1_{LAI}-infected naive and memory $CD4^+$ T cells. To determine whether apoptosis contributes to the more severe cytopathic effects observed in infected memory $CD4^+$ T cells, aliquots of cells from each population were stained with FITC-conjugated Annexin V (BRAND Applications) and PI and analyzed by flow cytometry. An early biochemical change in cells undergoing apoptosis involves the redistribution of the phospholipid phosphatidylserine to the outer leaflet of the plasma membrane, where it functions as a signal for phagocytosis (34, 44, 64). PS is normally found on the inner leaflet of the plasma membrane of healthy living cells. Annexin V binds to PS tightly in a Ca^{2+}-dependent manner, and therefore Annexin V staining can be used to delineate apoptotic cell populations (Annexin-positive and PI-negative cells). Figure 5 shows a representative profile of the FACS analysis. As expected, the percentage of PI-positive cells (dead cells) in infected memory T-cell population was much greater than that observed in the naive population (48.8% versus 15.2%), whereas that of uninfected cells was around 5 to 6% in both populations. These data confirm that HIV-1-induced cytopathic effects are more severe in infected memory $CD4^+$ T cells than in infected naive $CD4^+$ T cells. In addition, the number of cells undergoing apoptosis (Annexin-positive, PI-negative cells) was greater in the infected memory population than in the infected naive population. The percentages of apoptotic cells in uninfected naive and memory T cells were approximately the same. These results indicate that least a part of the cell death that occurs infected memory T cells is due to apoptosis.

DISCUSSION

We have shown that following infection with HIV-1 and activation, memory $CD4^+$ T cells are more susceptible to the cytopathic effects of HIV-1_{LAI} than naive $CD4^+$ T cells. This was demonstrated by isolating highly purified populations of resting naive and resting memory $CD4^+$ T cells and then exposing both populations to high-multiplicity HIV-1 infection followed by activating stimuli. Given that the majority of $CD4^+$ T cells are in a resting state at any given time in vivo, these experimental conditions may mimic the mode in which many $CD4^+$ T cells become infected in vivo with T-cell-tropic isolates. Following activation, both populations become competent to support virus replication, but memory cells die much

more quickly. Syncytia are not observed in primary $CD4^+$ T cells under our culture conditions. Rather, HIV-1 infection appears to result in pronounced single-cell killing of activated memory $CD4^+$ T cells. At least some of the cells die through a process with the biochemical characteristics of apoptosis. Thus, despite the fact that naive T cells begin to acquire phenotypic characteristics of memory cells following activation, they are more resistant to the cytopathic effects of HIV-1 than are memory T cells. We have recently shown that resting $CD4^+$ T cells with integrated HIV-1 DNA are rare in vivo, probably because productively infected, activated $CD4^+$ T cells rarely survive long enough to revert to a resting, memory state (11). Our results suggest that if the activated cells were in a naive rather than a memory state prior to activation, they are more likely to survive the cytopathic effects of the virus and revert to a resting state carrying integrated provirus. This is important because these surviving cells may represent a long-term reservoir for the virus. Our results also suggest that if resting memory $CD4^+$ T cells are exposed to HIV-1 in vivo and then activated by encounter with the relevant recall antigen, they may not proliferate and carry out effector functions due to the severe cytopathic effects observed in this population. This observation may explain part of the defect in recall responses in infected individuals (36).

The mechanism by which infected memory cells die is unclear. Our experiments were carried out with high-multiplicity infections (MOI = 1) so that all of the cells in the culture were infected. Therefore, although HIV-1 infection may be associated with the killing of noninfected $CD4^+$ T cells in vivo (3, 19, 26, 42, 45, 55), the present studies are relevant to understanding cytopathic effects resulting from direct infection of $CD4^+$ T cells. We showed that some of the infected memory cells were viable as judged by exclusion of PI but stained with Annexin V. This finding suggests a programmed cell death mechanism. However, although Annexin V staining is reasonably specific for cells undergoing programmed cell death, it is also possible that some other membrane remodeling process accounts for the Annexin V staining. In addition, some of the cells were stained with both Annexin V and PI. These cells could have died through either apoptotic or necrotic processes. Therefore, it is not clear that apoptosis is the predominant mechanism for HIV-1-induced cell killing in general or for killing of memory cells in particular.

There is considerable evidence that the Env protein is involved in the single-cell killing induced by HIV-1 and that the Env-dependent cytopathic effects involve interactions of the Env protein with CD4 (7, 31, 33, 35, 41, 59). In productively infected $CD4^+$ lymphoblasts, both gp160 and CD4 are synthesized on the rough endoplasmic reticulum. Therefore, the possibility for intracellular interaction of newly synthesized gp160 and CD4 molecules exists (13). Evidence that HIV-1-induced single cell killing involves intracellular interactions between the Env protein and CD4 comes from studies showing that susceptibility to the cytopathic effects of HIV-1 infection is related to levels of CD4 expression. In $CD4^+$ T-cell lines infected by HIV-1, cells that grow out with stably integrated virus tend to have low or absent CD4 expression, perhaps reflecting a selection against coexpression of Env protein and CD4 (29). In addition, cells of the monocyte/macrophage lineage, which express only low levels of CD4, can be infected by HIV-1 but do not show cytopathic effects (23, 28, 48, 52). The level of expression of CD4 by subclones of the monocytic line U937 is correlated with susceptibility of the clones to HIV-1-induced cytopathic effects (2). In an interesting series of studies, Koga et al. have analyzed the cytopathic effects resulting from env gene expression, using a transfection system with an inducible promoter (32, 33). $CD4^+$ and $CD4^-$ sublines of U937 and the T-cell tumor line Jurkat were transfected with a vector carrying the HIV-1 env gene under the control of the metallothionein promoter. Cytopathic effects were observed upon induction of env gene expression only in the $CD4^+$ sublines, suggesting that env gene expression is not in itself toxic to the cell. Rather, it is the coexpression of gp160 and CD4 that produced cytopathic effects at the single-cell level, possibly due to the formation of intracellular complexes of gp160 and CD4 (31, 41). Recent studies by Cao et al. have emphasized the role of internal membrane fusion events triggered by intracellular CD4-Env interactions (7).

One important issue is whether the differences in susceptibility of naive and memory cells observed with HIV-1_{LAI} will reflect differences with other HIV-1 isolates, particularly primary isolates. It is now clear that isolates from different infected individuals show variation in biological properties including rates of replication in vitro, ability to infect various T-cell and monocyte/macrophage cell lines, ability to induce syncytia, and ability to induce cytopathic effects (8, 18, 20). However, in many studies, the capacity of a viral isolate to induce syncytia in certain T-cell lines has been taken as evidence of a cytopathic isolate. Many of the earlier studies in this area are now being reinterpreted in light of recent discovery of the utilization of different coreceptors by different HIV-1 isolates (9, 15–17). At the present time, it is unclear what fraction of primary isolates cause single-cell killing of primary $CD4^+$ lymphoblasts, and it is quite possible that many if not most isolates will have this effect. We have shown that differential cytopathic effects on naive and memory T cells can also be observed if the cells are activated first and then infected (11a). Thus, in principle, the results should apply to T-cell-tropic and macrophage-tropic isolates, both of which can infect activated $CD4^+$ T cells. However, the difference is more pronounced if the cells are infected prior to activation. Another important issue is related to the effects of the HIV-1 Vpr protein, which has been recently shown to induce cell cycle arrest in infected cells (30). It is important to note that the present studies were performed with an isolate of HIV-1 that was initially adapted for growth in immortalized T-cell lines. Whether defects in vpr and possibly other accessory genes in such isolates modulate the differential cytopathic effects of HIV-1 infection on naive and memory T cells is not yet clear.

Previous studies have addressed the issue of the preferential infection and depletion of naive and memory $CD4^+$ T cells in infection by HIV-1 and simian immunodeficiency virus (SIV) (10, 22, 27, 50, 51, 54, 63, 66). Some (63) but not other (10) studies suggest that there is a preferential loss of memory cells from the peripheral blood of seropositive individuals. The overall rate of decline of naive and memory $CD4^+$ T cells in infected individuals is an extremely complex problem influenced by the rates of production of each population, the frequency of infection of each population, the susceptibility of each population to the cytopathic effects of infection, and the susceptibility of each population to indirect killing mechanisms. Our study addresses only one aspect of this complex dynamic situation, the susceptibility of naive and memory cells to the direct cytopathic of the virus, and it does not necessarily follow from our study that memory cell should be depleted more rapidly in vivo than naive cells.

Preferential infection of $CD4^+$ subsets was initially demonstrated in the case of SIV infection of macaques (22, 66). In primates, CD44 shows a bimodal distribution among peripheral blood T cells. Activated T cells are $CD44^{high}$. Following SIV infection of macaques, $CD44^{high}$ $CD4^+$ T cells are preferentially infected and deleted (66). In the human, CD44 has a

unimodal distribution on CD4^{+} T cells, and cells bearing high or low amounts of CD44 appear to be equally susceptible to HIV-1 infection (54). Early studies by Schnittman et al. (54) suggested that the naive and memory CD4^{+} subsets differ in susceptibility to HIV-1 infection both in vivo and in vitro. Following short-term in vitro infection with HIV-1, memory cells contained 4- to 10-fold more HIV-1 DNA than did naive cells. When naive and memory subsets of peripheral blood CD4^{+} T cells were isolated from HIV-1-seropositive individuals, memory CD4^{+} cells again showed 4- to 10-fold-higher levels of HIV-1 DNA by PCR analysis. In these studies, no effort was made to remove activated cells, and so it is likely that the memory population also included recently activated cells in which the virus may replicate efficiently. It is therefore not clear to what extent the observed preferential infection of memory cells observed reflects infection of activated cells. Vyakarnam et al. (65) have suggested that memory cells may die more quickly from the cytopathic effects of HIV-1. However, in this study it was not established whether the rapid killing of memory cells was due to preferential infection of these cells or greater susceptibility to cytopathic effects of infection. Again, interpretation was complicated by the failure to remove activated cells. Our study has focused on the consequences of infection of resting cells and has indicated that HIV-1 infects resting naive and memory cells to roughly the same extent but causes more dramatic cytopathic effects in memory cells following activation. By focusing on resting T cells, we have avoided some of the difficulties inherent in using only CD45 isoforms to define naive and memory subsets. For example, Roederer et al. have argued that naive T cells from the CD8^{+} T-cell subset are depleted in HIV-1 infection, with a similar but less dramatic pattern for CD4^{+} T cells (51). These authors used both CD45 isoforms and CD62 expression to define naive T cells (CD45RA^{+} and CD62^{+}) because they identified a significant fraction of CD45RA^{+} CD62^{-} cells which are not felt to be naive cells based on expression of activation markers (DR and CD38). Our study has focused on resting T cells lacking activation markers, allowing for clear definition of naive and memory subsets based on CD45 isoform expression.

There is considerable reason to believe that antigen plays a critical role in driving CD4^{+} T cells into states in which they are susceptible to infection by HIV-1 and subsequent destruction by viral cytopathic effects or immune mechanisms. It is therefore reasonable that frequently activated cells would be deleted first. The selective loss of memory cells can account for the decreased responsiveness of CD4^{+} T cells from AIDS patients to recall antigens. Our results indicate that at least a portion of this effect may be due to the marked susceptibility of activated memory T cells to the cytopathic effects of HIV-1 infection. The extent to which the accelerated death of infected memory T cells accounts for CD4^{+} T-cell dysfunction and depletion depends on the extent to which the CD4^{+} T-cell population is infected in vivo, a subject which is still not well understood.

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