Peripheral S-Phase T Cells in HIV Disease Have a Central Memory Phenotype and Rarely Have Evidence of Recent T Cell Receptor Engagement

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Heightened proliferation and death of T lymphocytes may play a key role in human immunodeficiency virus (HIV) pathogenesis; however, the mechanism that mediates this effect and the phenotype of the proliferating T cells have not been clearly determined. We assessed S-phase cell frequencies and phenotype by ex vivo bromodeoxyuridine incorporation and flow-cytometric analysis in a group of 35 HIV-infected individuals. Frequencies of S-phase T cells were increased in HIV disease and were related to plasma HIV RNA levels but not to CD4 cell, total T cell, or total lymphocyte counts. S-phase cells were phenotypically defined as “central memory” cells (CD45RO+CD62L+CCR7+). Although activated (CD38+), S-phase cells lacked CD69 expression, rarely expressed CD25, and were not overrepresented among HIV-specific cells, as might have been expected if these cells had recently been activated by HIV antigens. Thus, in HIV infection, central memory T cells may be highly susceptible to bystander mechanisms of immune activation, leading to S-phase entry.

Chronic immune activation may play a major role in HIV pathogenesis. T cell activation and accompanying cellular turnover (proliferation and death) are elevated in HIV-1–infected individuals [1–4], and the degree of activation or proliferation predicts disease progression [5, 6]. In addition, the natural hosts of simian immunodeficiency virus (SIV), sooty mangabeys, resist SIV pathogenesis despite high-level viremia, and a key feature of this infection is that the animals maintain relatively low levels of T cell proliferation [7–9]. Thus, understanding the mechanism that leads to increased T cell turnover in HIV disease may be critical to understanding pathological abnormalities.

One issue surrounding cellular proliferation in HIV disease is whether it is a consequence of immune activation driven by HIV replication or a consequence of homeostatic responses induced by lymphopenia. Previous studies have shown that the frequencies of peripheral S-phase T cells in HIV disease are directly related to plasma HIV RNA levels [10] and that these frequencies diminish with administration of highly active antiretroviral therapy (HAART) [10, 11]. Importantly, levels of T cell proliferation are reduced by administration of HAART, even in individuals who have low CD4 cell counts [11], suggesting that T cell proliferation in HIV disease is not driven by a homeostatic mechanism to compensate for low CD4 cell counts. Instead, the observations are consistent with a role for immune activation in driving T cell turnover [10, 11]; however, these studies did not consider the possibility that homeostatic responses may be blind to the type of cell loss and may instead reflect responses to total T cell or lymphocyte losses.

The S-phase (bromodeoxyuridine [BrdU]–incorporating) cells in the peripheral blood of HIV-infected individuals have been only partially characterized. These cells include both CD4+ and CD8+ T cells that commonly express activation markers, such as HLA-DR and CD38 [12]. Although the cells are activated, it is not clear whether this activation is mediated by direct T cell...
Patients were either receiving treatment with antiretrovirals with controlling HIV replication while receiving HAART. All other patients were lost to follow-up or died of AIDS-related complications. Only 3 of the 35 patients were successfully retained for the study, including 2 from the University Hospitals of Cleveland. Additional blood samples were obtained through the Center for AIDS Research at Case Western Reserve University and University Hospitals of Cleveland. Peripheral blood lymphocytes were isolated by a Ficoll-Hypaque gradient and tested for HIV-1 RNA and CD4+ cell count. CD3+ lymphocytes were sorted for BrdU labeling and flow cytometry. One-milliliter aliquots of whole blood (from heparin tubes) were placed in 15-mL conical tubes and incubated for 48 hours in a 37°C water bath with or without BrdU (10 μmol/L; BD Pharmingen). Control stains included the goat anti-mouse secondary stain without incubation of cells with primary antibody. Staining of CCR7 was performed by use of a primary IgM mouse anti-human CCR7 antibody (BD Pharmingen), followed by a goat anti-mouse IgM phycoerythrin-conjugated antibody. Control staining included the goat anti-mouse secondary stain without incubation of cells with primary antibody. Other surface stains included those for CD38, CD25, and CD69 expression (antibodies from BD Pharmingen). Cells were then treated with a membrane-permeabilizing buffer (BrdU kit), treated with DNase, and stained with anti-BrdU fluorescein-conjugated antibody (BD Pharmingen). For some assays, cells were additionally stained with antibodies against Ki67 (BD Pharmingen). Total events collected ranged from 50,000 to 250,000/sample, and this generally included at least 10,000 CD4+ or CD8+ lymphocytes. Some studies included surface staining with HIV gag tetramer reagent (Beckman Coulter). This tetramer structure binds to CD8+ T cells with specificity for SLYNTVATL peptide in HLA-A 0201–positive individuals.

**Statistical analyses.** CD4 cell counts, plasma HIV RNA levels, total T cell counts, and total lymphocyte counts were analyzed as continuous variables. CD4 cell counts and plasma HIV RNA levels were also analyzed as ordinal variables, with 3 possible values. For CD4 cell counts, the categories were ≤200, 201–500, and >500 cells/μL; for HIV RNA levels, the categories were ≤10,000, 10,001–100,000, and >100,000 copies/mL. We assessed the relationship between marker expression and continuous plasma HIV RNA levels by use of Spearman’s rank order correlation, as indicated by the distribution of the data. To determine whether plasma HIV RNA level could be predicted by a combination of the studied variables, we used a forward stepwise procedure to obtain a multiple regression model with optimal fitness to the data. We included stratified CD4 cell counts in the form of indicator (“dummy”) variables in the model, to account for the association between HIV RNA level and CD4 cell count. We included quadratic, cubic, and exponential terms in the model when indicated by the simple regression analyses and plots. Because of expected collinearity between several of the markers, we used extensive regression diagnostic procedures and principal-component analysis to ar-
rived at the most parsimonious model with the largest predictive ability and used multiple fitting strategies to confirm the final selection of variables. Analyses were performed by use of SPSS for Windows (version 11.0; SPSS) and Intercooled Stata (version 6.0; Stata). Curve fitting was performed by use of SigmaPlot (version 5.0; SPSS). All significance tests were 2-tailed, and \( P \leq 0.05 \) was considered to be significant.

**RESULTS**

*Relationship between S-phase cell frequencies and plasma HIV RNA levels, CD4 T cell counts, total T cell counts, and total lymphocyte counts.* To assess T cell proliferation and investigate the determinants of T cell proliferation in HIV disease, we used ex vivo BrdU incorporation to label cells in the S phase. As has been described by others [3, 4, 10], the proportions of BrdU+ cells among both CD4 and CD8 cells were elevated in lymphocytes from HIV-infected individuals (figure 1A). Interestingly, the proportions of CD4 cells in the S phase were significantly increased relative to the proportions of CD8 cells in the S phase in HIV-infected individuals (\( n = 35 \); mean, 0.60% and 0.39% for CD4 and CD8 S-phase cells, respectively), whereas the proportions of CD4 and CD8 cells in the S phase were nearly identical in healthy subjects (\( n = 11 \); mean, 0.064% and 0.062% for CD4 and CD8 S-phase cells, respectively). Thus, HIV infection results in greater proportions of peripheral S-phase cells, and CD4 cells are more often in S phase than are CD8 cells.

To confirm that BrdU+ cells were actively in cell cycle, we performed dual-labeling experiments with BrdU incorporation and another marker of cell-cycle progression, Ki67. As expected, all BrdU+ cells also expressed Ki67, which is consistent with the movement of these cells into the cell cycle (figure 1B). Also as expected, BrdU+ cells represented only a fraction of the Ki67+ cells, since Ki67 is expressed in all phases of the cell cycle except G0, whereas BrdU incorporation occurs only in the S phase. To confirm that BrdU+ cells were not dead and taking up BrdU or binding anti-BrdU antibodies nonspecifically, we examined BrdU+ cells for DNA content by use of propidium-iodide staining. BrdU expression was observed primarily in the G0/G1 and S-phase fraction, but there were very few BrdU+ cells in the hypodiploid apoptotic fraction, which is consistent with labeling of live cells (figure 1C). The incorporation of BrdU in cells with DNA content resembling that of G0/G1 cells likely represents an increased sensitivity to detect cells during early stages of the S phase with BrdU incorporation. This interpretation is consistent with our previous studies, which clearly depicted an increased induction of BrdU incorporation in cells with apparent G0/G1 DNA content after stimulation of peripheral blood mononuclear cells with phytohemagglutinin and interleukin-2 [18].

To consider the possibility that either homeostatic T cell proliferation or HIV replication was the predominant mecha-
anism responsible for increased S-phase entry among T cells from HIV-infected individuals, we evaluated the relationships between the percentages of BrdU+ T cells and plasma HIV RNA levels, CD4 cell counts, total T cell counts, and total lymphocyte counts (figure 2). Nonparametric correlation analyses demonstrated a significant, direct association between plasma HIV RNA levels and frequencies of cycling CD4+ and CD8+ T cells (figure 2). We documented only a weak, inverse relationship between BrdU incorporation and CD4 cell counts among CD4+, but not CD8+, T cells; we found no association between frequencies of S-phase cells and total T cell counts (Spearman’s correlation coefficient [CC], −0.024 and −0.276 for CD4+ and CD8+ T cells, respectively) or total lymphocyte counts (CC, −0.058 and −0.321 for CD4+ and CD8+ T cells, respectively).

To confirm the relationship between viral replication and cellular turnover, we needed to consider the possibility that the association between plasma HIV RNA level and cell cycling was actually a consequence of the inverse relationship between CD4+ T cell counts and HIV RNA levels. Partial-correlation analysis indicated that the association held for plasma HIV RNA levels and BrdU incorporation, for both CD4+ T cells (partial-correlation coefficient, 0.39; \( P = 0.021 \)) and CD8+ T cells (partial-correlation coefficient, 0.56; \( P = .001 \)), in a manner that was independent of CD4+ T cell counts. Further scrutiny of the correlation scatterplots revealed a consistent outlying subject for virtually all markers examined. That individual had the single highest frequencies of BrdU+CD4+ and BrdU+CD8+ T cells, well beyond 3 SDs of the means of the remaining samples. Interestingly, this individual had a unique clinical history of multiple hospital admissions for sepsis, Kaposi sarcoma treated with chemotheraphy, repeated episodes of bacteremia, and a recent limb amputation due to uncontrolled septic arthritis. This clinical history may explain the extraordinary levels of immune activation in this subject and provides some rationale for exclusion of this subject’s sample from the analysis. Removing the outlier from the analysis strengthened the association between BrdU+CD4+ and BrdU+CD8+ T cells with levels of plasma HIV RNA. Conversely, partial-correlation analysis of cycling cell markers with CD4+ T cell count, with controlling for plasma HIV RNA level, showed that the weak association found in univariate analysis did not hold after taking into account the effect of plasma HIV RNA level. Finally, we fitted
several multiple regression models to the data, in an attempt to establish whether total CD4+ T cell counts, total T cell counts, or total lymphocyte counts had any independent association with cell cycling, and found that, regardless of the modeling strategy used, none of these indices could independently predict proliferation-marker frequencies for either CD4+ or CD8+ lymphocytes. These results are consistent with those of previous studies [11] and favor strongly a model whereby both CD4+ and CD8+ T cell entry into the cell cycle is determined predominantly by viral replication and does not reflect a homeostatic response driven by lymphopenia.

Central memory phenotype among peripheral S-phase cells. Peripheral S-phase cells were examined for cell-surface phenotype. Using 4-color flow-cytometric analysis, we found that virtually all the CD4+ and CD8+ T cells that acquired BrdU label during ex vivo incubation expressed CD45RO, CD62L, and CCR7 (figure 3). This phenotype (CD45RO+CD62L+CCR7+) is consistent with that of central memory cells, indicating that these cells had, at some time, been activated by antigen and likely have an enhanced potential to migrate to lymphoid tissues [14]. Marked enrichment of central memory cell markers was noted among the BrdU+ T cell subsets for all HIV-infected individuals (figure 3B). Thus, central memory cells compose the vast majority of cells actively synthesizing DNA in the peripheral blood of HIV-infected individuals.

This observation raised the issue of S-phase T cell phenotype in healthy donors. Analyses of peripheral blood samples indicated that S-phase T cells from healthy donors also commonly expressed markers of central memory cells. A representative result of 4 experiments is shown in figure 4. Thus, circulating S-phase central memory cells are a component of normal physiology; however, the frequency of these circulating S-phase T cells and the relative involvement of CD4 versus CD8 cells are altered significantly in HIV disease.

Markers for recent TCR activation among peripheral S-phase cells. To ascertain whether the increased frequencies of peripheral S-phase cells in HIV infection were a consequence of TCR stimulation and expanded anti-HIV immune responses, we asked whether S-phase cells expressed activation markers consistent with recent TCR stimulation or whether S-phase cells were overrepresented among HIV tetramer-reactive lymphocytes.

Peripheral S-phase cells from HIV-infected donors (table 1 and figure 5A) expressed CD38, a marker of activated T cells; however, these cells rarely expressed CD69, an activation marker that is transiently expressed after TCR stimulation. Furthermore, CD25, a marker of both activated T cells and regulatory T cells, was not enriched among the S-phase subset of CD4+ T cells (table 1 and figure 5B). Although there was enrichment of CD25-expressing cells within the S-phase CD8+ T cell subset (table 1), the overall proportion of S-phase CD8 cells expressing this marker was low (mean, 5.2%). This finding stands in stark contrast to the marked enrichment of CD25-expressing cells...
within the S-phase subset when CD4+ or CD8+ T cells were activated in vitro by cytomegalovirus antigen or agonistic anti-TCR antibodies (data not shown). These results suggest that most peripheral S-phase cells have not been recently stimulated via TCR activation.

To explore further the potential role of TCR engagement in induction of S-phase entry among these circulating T cells, we examined the frequency of circulating S-phase cells among HIV peptide/tetramer–binding cells. Cells with specificity for an immunodominant gag peptide were not enriched among S-phase lymphocytes in the peripheral blood from 3 HIV-infected individuals examined (figure 6). Since, in the lymphoid tissue of infected individuals with high-level HIV replication, HIV antigens are likely the most prevalent antigens, these results suggest that the peripheral S-phase cells had been activated by mechanisms that were independent of recent TCR engagement.

**DISCUSSION**

Mechanisms that drive cellular activation and cell-cycle progression in HIV disease are not well defined. On the basis of only a relationship between the frequency of proliferating cells and plasma HIV RNA levels (and not CD4 cell counts), others have argued that increased cell division in HIV disease does not represent a homeostatic response driven by cell loss [11]. Our results, which also take into consideration total T cell counts and total lymphocyte counts, confirm this interpretation. Thus, S-phase cell frequencies are most likely driven by factors related to viral replication.

How, then, does viral replication drive cellular proliferation? Some insights may be derived from the finer phenotypic characterization of circulating S-phase cells. Earlier work has shown that, during HIV infection, CD45RO+ cells are the predominant
Figure 4. Central memory phenotype in S-phase lymphocytes from healthy donors. Whole blood from a healthy donor was labeled with bromodeoxyuridine (BrdU) for 1 h ex vivo, and the cells were subsequently processed for flow-cytometric analyses of BrdU incorporation and coexpression of CD45RO and CD62L. Isotype control stains were used to set the quadrants, and background staining is shown (top histograms). The nos. in the histograms represent the percentages of cells in each quadrant. The histograms are representative of 4 experiments.

Table 1. Percentages of bromodeoxyuridine (BrdU)+ and BrdU−/H11002 T cell subsets that express activation markers.

<table>
<thead>
<tr>
<th>Cell subsets</th>
<th>BrdU+ subset</th>
<th>BrdU− subset</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
</tr>
<tr>
<td>CD4 cells</td>
<td></td>
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</tr>
<tr>
<td>CD25+ (n = 12)</td>
<td>12.0</td>
<td>10.0</td>
</tr>
<tr>
<td>CD69+ (n = 6)</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>CD38+ (n = 7)</td>
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<td>95</td>
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<tr>
<td>CD8 cells</td>
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</tr>
<tr>
<td>CD25+ (n = 14)</td>
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<td>5</td>
</tr>
<tr>
<td>CD69+ (n = 6)</td>
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</tr>
<tr>
<td>CD38+ (n = 7)</td>
<td>95.9a</td>
<td>97</td>
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* Mean values between BrdU+ and BrdU− subsets were statistically different (P < .05); parametric paired t tests and nonparametric Wilcoxon tests were used for analyses, depending on the distribution of the data.
nodes of mice infected in the footpad with HSV-1 have clearly demonstrated a marked induction of CD25 and a loss of CD62L expression 5 days after challenge [19], and CD25⁺ T cells are enriched in the lungs of mice challenged with influenza virus infection, presumably as a consequence of T cells reencountering antigens [20], where these cells were presumably reencountering antigen. Nevertheless, S-phase T cells in the periphery may be in a unique stage of differentiation that is not entirely reflective of cells activated by antigen in lymph nodes or at other tissue sites. Also, some caution is warranted in interpreting the lack of S-phase enrichment among HIV-specific CD8⁺ T cells, since it is possible that viral escape has occurred in these individuals and that the appropriate antigen may no longer predominate to drive these cells into the S phase. Further studies of TCR diversity and specificity among peripheral S-phase T cells will ultimately help to resolve these issues.

Irrespective of the precise determinants or sites of activation, our observations suggest that central memory cells may be selectively influenced by immune activation in HIV disease. This may be important since these cells are key components of immunological memory. Importantly, central memory cells appear to have proliferation defects in HIV disease when activated by antigens [21]. We speculate that dysregulated activation of central memory T cells could underlie cellular impairments by mechanisms that are not yet clearly defined.

Finally, our previous findings demonstrated that T cells from HIV-infected individuals have a tendency to spontaneously enter into the S phase in vitro without the addition of an exogenous stimulus and that S-phase cells are prone to undergo apoptosis [18]. Thus, cell-cycle entry may result in cell death and act as a mechanism for cellular depletion in HIV disease.

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References