Memory Responses in Human Immunodeficiency Virus Type 1-Infected Individuals with Long-Term Viral Load Suppression Are Independent of CD4 Cell Nadir

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Highly active antiretroviral therapy (HAART) has significantly modified the interaction between host immunity and the virus. The response to HAART has been dramatic even in those individuals with low CD4 counts. The excitement associated with this immunologic recovery has been tempered by an inability to eradicate the infection and prevent disease progression in many cases. One area of interest is the role of an individual's CD4 cell nadir in predicting disease recovery or progression. It has been shown that individuals started on HAART with a low CD4 cell nadir do not recover CD4 cell levels to those of uninfected individuals (e.g., see reference 16). Furthermore, the CD4 cell nadir may negatively predict disease progression for individuals with comparable CD4 cell counts (12, 18). Since the ability to respond to immunologic challenge is dependent on memory cells and the ability to develop these cell populations from an existing or replenished naive cell pool, it is possible that peripheral blood lymphocytes (PBL) from individuals with recovering CD4 counts from low CD4 cell nadirs are not as responsive as PBL from those with higher nadirs. There have been many studies evaluating proliferative responses to human immunodeficiency virus (HIV) antigens after long periods on suppressive HAART, but the results have been equivocal (1, 2, 5, 8, 22, 24, 25, 27). In an earlier study we demonstrated that memory cell proliferative responses to p24 antigen could be measured in PBL from patients with plasma viral load suppression. No differences could be found in proliferative responses from PBL between individuals with a low and those with a high CD4 cell nadir. PBL that did not respond to either Casta antigen or p24 were found to have a higher percentage of naive cells than did PBL that responded well to antigen. These data support the contention that, after long-term viral load suppression, PBL from infected individuals have memory cell populations that can respond to antigenic stimulation under inducible conditions.

The persistence of memory responses in suppressive highly active antiretroviral therapy (HAART) has been an area of controversy. By using a previously described proliferation assay that augments specific responses, peripheral blood lymphocytes (PBL) from 61 human immunodeficiency virus type 1-seropositive individuals with CD4 counts of >300/mm³ and suppressed viral burdens were studied for response to p24 antigen as a function of time of viral load suppression on HAART. In the majority of cases, proliferative responses could be measured in PBL from patients with plasma viral load suppression. No differences could be found in proliferative responses from PBL between individuals with a low and those with a high CD4 cell nadir. PBL that did not respond to either Casta antigen or p24 were found to have a higher percentage of naive cells than did PBL that responded well to antigen. These data support the contention that, after long-term viral load suppression, PBL from infected individuals have memory cell populations that can respond to antigenic stimulation under inducible conditions.

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MATERIALS AND METHODS

Human subjects. Sixty-one HIV type 1 (HIV-1)-infected patients who attended the Outpatient HIV Clinics at the University of Miami-Jackson Memorial Hospital Medical Complex were recruited to participate in this study. Prior to enrollment all individuals consented to participation in the study (approved by the institutional human subjects review board of the University of Miami School of Medicine). Criteria for participation were an absolute CD4 count of >300/mm³ and a nondetectable VL (<400 copies/ml). Forty-two of the participants had a VL of <50 c/ml, 14 had a VL of <200 c/ml, and 5 had a VL of >400 c/ml. All participants were on HAART consisting of a protease inhibitor-containing regimen, a nonnucleoside reverse transcriptase inhibitor-containing regimen, or a triple nucleoside reverse transcriptase inhibitor regimen. All participants had asymptomatic HIV disease at the time of enrollment. Medical record abstraction provided information on demographics, time of VL suppression (VL < 400 c/ml), and CD4 cell nadir. The CD4 cell nadir is the lowest documented CD4 count that an individual with HIV disease had ever reached. As such, those individuals with CD4 counts of <200/mm³ at any time in their past were known to have a CD4 nadir that was low (<200/mm³). The exact nadir was not available in all cases. The CD4 nadir for some individuals (8 of 61) could not be discerned because their prior clinic history was unavailable. Since one of the goals of this work was to demonstrate long-term persistence of memory cell responses, data from these individuals were included in Fig. 1 and 2.

Materials. Casta antigen (from Candida albicans) was obtained from Greer Laboratories, Inc. (Lenoir, N.C.). The HIV-1 recombinant viral peptide gag p24 (IIIB) was obtained from Immunodiagnostics, Inc. (Woburn, Mass.). LPS was obtained from Sigma Chemical Co. (St. Louis, Mo.). [³H]thymidine was obtained from New England Nuclear (Boston, Mass.). The fluorescent antibodies anti-CD4–phycoerythrin (PE), anti-CCR7–PE-CY7, and anti-CD45RA–fluorescein isothiocyanate were obtained from Becton Dickinson (San Jose, Calif.). Complete medium consisted of RPMI with antibiotics, l-glutamine, minimal essential medium with nonessential amino acids, minimal essential medium with sodium pyruvate, and 10 mM HEPES plus 10% normal human serum, type AB+ (Atlanta Biologicals, Norcross, Ga.).
FIG. 1. Long-term p24 antigen-specific proliferative responses persist in virally suppressed individuals. The specific proliferation is shown as a function of duration of VL suppression by using the usual LPA (A) and the LPS-adherence assay (B). Closed and open symbols represent significant and nonsignificant responses, respectively, as described in Materials and Methods. PBL from individuals with low, high, and unknown CD4 cell nadirs are represented by squares, circles, and triangles, respectively.

FIG. 2. Proliferative response to Casta and p24 antigen. The 7-day specific proliferation is shown for the PBL studied in Fig. 1 to Casta antigen (1.0 μg/ml) and p24 antigen (1.0 μg/ml) in the presence of LPS (0.01 μg/ml) and overnight adherence. The open symbols represent those PBL that respond well to antigen (triangles) or poorly to antigen (circles) with resting samples available for phenotype analysis. The closed squares represent the samples that were intermediate between the poor responders and the good responders. The dashed line defines those PBL that did not respond to p24 in this assay as described in the text.
with 21 in the high-CD4-cell-nadir group. There was no significant difference in racial-ethnic distribution, age, gender, or time of VL suppression between those with a high and those with a low CD4 cell nadir. Those with a high CD4 cell nadir had a significantly higher CD4 count at the time of the study than did those participants with a lower starting CD4 cell nadir, consistent with other reported data (16).

### VL suppression and p24 responses
Numerous groups have looked at proliferative responses to p24 as a function of time after initiation of HAART. For patients with extended viral suppression, some groups are able to demonstrate a proliferative response to p24 (1, 2, 24, 25) whereas others are not (5, 7, 22, 27). In a previous study (15) we described a sensitive assay for specifically measuring proliferative responses to p24 antigen. In that work it was shown that the proliferative dose-response to antigen was shifted to the left, providing a sensitive readout for proliferative responses to p24 antigen concentrations. This assay required PBL to adhere to the tissue culture plate and incubation with LPS to augment the response. In Fig. 1A is shown the specific proliferation of PBL to p24 (1.0 μg/ml) in the usual LPA whereas Fig. 1B represents the specific proliferation of PBL to p24 after overnight incubation followed by LPS (0.01 μg/ml) and p24 (1.0 μg/ml) additions. In Fig. 1A there were 47 nonresponders (open symbols), 45 of whom fell below the dashed line (150 cpm/1,000 CD4 cells), whereas only one responder (closed symbols) fell below the dashed line. In Fig. 1B there were 26 nonresponders, 18 of whom fell below the dashed line. There were no responders who fell below the dashed line. The dashed line (150 cpm/1,000 CD4 cells) effectively represents a line of demarcation for responders in a sensitive manner (>90% for both assays). Only two nonresponders in Fig. 1B were responders in Fig. 1A, whereas 23 of the nonresponders in the usual LPA (Fig. 1A) were responders in Fig. 1B (35 total responders of 61). Individuals with low CD4 cell nadirs (<200 cells/mm³), high CD4 cell nadirs (>200 cells/mm³), or unavailable CD4 cell nadirs are shown by squares, circles, and triangles, respectively. PBL from individuals with either a high or low CD4 cell nadir responded to p24 in the boosted assay and did so even after long-lasting VL suppression (Fig. 1B). Of those PBL from individuals with a low CD4 nadir, 47% were nonresponders (15 of 32), whereas 38% of those with a high CD4 nadir were nonresponsive (8 of 21) and 38% of those with an unknown CD4 cell nadir were nonresponders (3 of 8), all nonsignificant differences (P > 0.05).

### High and low responders have different memory cell distributions
In Fig. 1B there were PBL from infected individuals that did not proliferate to p24 antigen in this assay. This group provided an opportunity to address the question of whether the PBL that did not respond to antigenic stimulation in the LPS-adherence assay shared any common properties that might not be revealed in a less sensitive assay. Since data from non-HIV studies have shown that different memory cell populations proliferate differently to antigen and produce different cytokine profiles (11, 13, 26), we decided to compare memory phenotypes in resting PBL. We looked at two specific subsets of resting PBL defined by their ability to respond well to Casta antigen and p24 (the high responders) or to respond poorly to Casta antigen and p24 (the low responders) in the LPS-adherence assay. We reasoned that, by looking at PBL that could proliferate at the two extremes, we might discern subpopulation differences when the unstimulated CD4⁺ T cells from available stored samples were evaluated by flow cytometry for CCR7 and CD45RA expression. Figure 2 shows proliferation of the PBL to p24 (1.0 μg/ml) and Casta antigen (1.0 μg/ml) for all the samples evaluated in Fig. 1. The closed squares represent the samples that were intermediate between the poor responders and good responders. The open circles show the specific proliferation for available stored PBL that were nonresponsive to antigen, and the open triangles show the specific proliferation for available stored PBL that responded well to antigen. When these two populations were studied by flow cytometry for CD4⁺ memory phenotypes, there was a significantly (P < 0.05) greater percentage of CD4 cells that expressed the naïve phenotype, CD45RA⁺ CCR7⁺, for those donor PBL for the low responders compared to the high-

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**TABLE 1. Participant demographic and immunologic distribution by CD4 cell nadir**

| Characteristic                  | Low (<200/mm³) | High (>200/mm³) | Unknown |
|---------------------------------|----------------|-----------------|---------|
| Racial-ethnic group (n)         |                |                 |         |
| Hispanic                        | 17             | 9               | 3       |
| African American                 | 10             | 10              | 5       |
| Haitian-Bahamian-Jamaican       | 3              | 2               | 0       |
| White non-Hispanic               | 2              | 0               | 0       |
| Gender (n)                      |                |                 |         |
| Male                             | 21             | 16              | 4       |
| Female                           | 11             | 5               | 4       |
| Median age in yrs (range)       | 46 (30–63)     | 50 (30–63)      | 48 (30–55) |
| Median CD4 count (range)        | 429 (311–1,002) | 580⁶ (333–1,487) | 903⁶ (406–1,711) |
| Mean time of VL suppression (mo)| 18.7           | 25.3            | 26.9    |

* The median CD4 count of the high-nadir group is significantly different from that of the low-nadir group (P < 0.025, Holm t test).
* The median CD4 count of the unknown-nadir group is significantly different from those of both the high- and low-nadir groups (P < 0.0005, Holm t test).

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responder PBL (36 compared to 23%, respectively), as shown in Fig. 3A. There were no differences seen in percentages of central memory or effector memory phenotype (Fig. 3B and C). The differences in proliferative response to antigen were not simply a consequence of fewer cells with the memory phenotype. As shown in Fig. 3E and F there is no difference in the absolute number of cells per well for each subset for individuals who responded poorly to Casta and p24 antigen (low responders) and those who responded well to Casta and p24 antigen (high responders). These PBL were chosen from available resting samples according to their response to antigen as shown in Fig. 2. Gating and staining are described in Materials and Methods. Significance was determined using the Mann-Whitney rank-sum test. NS, nonsignificant.

DISCUSSION

In non-HIV-infected individuals long-term cellular memory may persist even in the absence of antigen and play an important role in vaccination strategies (see, for example, reference 9). In HIV disease the system may be somewhat different since HIV itself impacts host immunity and the recovery from HAART is incomplete. Studies demonstrating long-lived CD4 memory responses in HIV-1-infected individuals on a suppressive HAART regimen have been mixed. Some groups are able to measure memory responses to antigenic stimulation in proliferation assays after long periods of VL suppression whereas others are not (1, 2, 5, 8, 22, 24, 25, 27). Data from intracellular cytokine staining have also suggested that the frequency of memory cells to HIV antigen does diminish over time (21). Therefore, it is important to understand whether HIV-infected individuals have persistent long-term memory responses to recall antigen and to measure them accurately. In an earlier study we described an assay that used LPS and monocyte adherence to augment specific proliferative responses to p24 (15). In the study described here we use this assay to evaluate proliferative responses to p24 in a HAART-suppressed cohort of infected individuals with CD4 counts greater than 300 cells/mm³. It was shown in Fig. 1 that the sensitivity of the usual LPA was not sufficient to reveal proliferative responses to p24 in the majority of cases (Fig. 1A), whereas with the LPS-adherence assay the majority of the PBL did proliferate to p24 (Fig. 1B). This was true for individuals who had a low or high CD4 cell nadir. This is in distinction from other studies that have suggested that those with a high CD4 cell nadir have poorer proliferative responses to antigen than do those with low CD4 cell nadirs (6).

In this study the majority of PBL from patients responded to p24 antigen in the LPS-adherence assay. There is a small group of patients whose PBL did not respond to either p24 or Casta antigens in the LPS and adherence assay (Fig. 2), consistent with a functionally anergic population. Interestingly, resting PBL from these individuals had a greater percentage of naive CD4⁺ T cells than did PBL from infected individuals who responded well to antigenic stimulation (Fig. 3A). When the absolute number of cells was evaluated, there was no difference between high and low responders for both the naive and memory phenotypes (Fig. 3D to F). These data suggest that the poor proliferation in one group compared to those that re-
spond well to antigen is not simply a consequence of a disproportionate amount of naive cells but an inherent difference in the memory subsets that proliferate. It is possible that the recovery of naive cells for these two populations was different even though there was no significant difference in the duration of suppression between groups. However, numerous studies have shown that memory cells account for the early CD4 cell recovery followed by a slow persistent increase in naive CD4 cells. After 1 to 2 years on suppressive HAART the CD4 count stabilizes as dictated by the naive and memory cell populations (4, 17, 19).

The importance of using innate immunity to drive proliferative responses may in part stem from producing an environment in which effector cytokines augment and recruit specific proliferative responses not easily seen without this adjunctive step (14, 20). The fact that by using the LPS-adherence assay it was possible to discern a group of nonresponders who shared the property of more naive cells compared to a set of PBL that proliferated well to antigen suggests that this assay may have use in other systems when the evaluation of immune responsiveness is important. Since immune activation may be predictive of response to HAART (3), it would be interesting to see if there are any correlations between activation parameters and proliferative responses in the LPS-adherence assay.

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