Inconsistent Reconstitution of Cytomegalovirus-Specific Cell-Mediated Immunity in Human Immunodeficiency Virus–Infected Patients Receiving Highly Active Antiretroviral Therapy

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Cytomegalovirus (CMV)–immune recovery was characterized in human immunodeficiency virus (HIV)–infected patients receiving highly active antiretroviral therapy. CMV lymphocyte proliferation (LP), responder-cell frequency (RCF), and interferon (IFN)–γ and interleukin (IL)–2 secretion were studied in CMV-seropositive HIV-infected patients and in CMV-seropositive HIV-uninfected control subjects. HIV-infected patients and control subjects had similar proportions of IL-2 and IFN-γ, but levels were lower in HIV-infected patients. LP and RCF were significantly less frequent and of lower magnitude in HIV-infected patients. The measures of CMV cell–mediated immunity were correlated in HIV-uninfected but not in HIV-infected subjects. To investigate this, IL-2, IL-12, anti-CD28 plus anti-CD49d, or anti–IL-10 was added in vitro, with no effect on LP. However, CD8 cell depletion of mononuclear cells from HIV-infected patients increased LP responses to levels similar to those of uninfected control subjects; before depletion, only RCF correlated with CD4 cell counts, but after depletion, LP also correlated with CD4 cell counts.

The impact of cytomegalovirus (CMV) end-organ disease in human immunodeficiency virus (HIV)–infected patients has dramatically declined since the introduction of highly active antiretroviral therapy (HAART), as evidenced by a decrease in new cases, delayed progression of existing retinitis lesions, reduction in new lesions, and a longer time interval between relapses [1–5]. Maintenance anti-CMV therapy can be safely discontinued in patients who achieve CD4 counts of ≥100 cells/μL after ≥3 months of HAART [6–8]. However, CMV eye lesions occasionally occur in HIV-infected patients receiving HAART, despite high levels of CD4 cells, usually in association with abnormal CMV-specific cell-mediated immunity (CMI) [9]. CMV immune vitritis has been described in association with vigorous CMV-specific CMI [10–12], whereas classic CMV retinitis has been observed in its absence [13–15]. Although CMV retinitis is usually associated with failure to recover CMV-specific CMI, the converse is not always true, that is, HIV-infected patients who do not receive anti-CMV prophylaxis and who do not develop recurrent CMV retinitis have variable levels of CMV-specific lymphocyte proliferation (LP) responses and intracellular cytokine secretions [16]. This conundrum underscores the need for a better understanding of the CMV-specific immune recovery in HIV-infected patients on HAART, to provide useful tools for predicting CMV-associated complications. The goal of this study was to further characterize CMV-specific immune recovery in HIV-infected patients who are receiving HAART and who are at low risk of developing CMV end-organ disease.

Subjects and Methods

Subjects. A total of 60 CMV-seropositive individuals (43 HIV-infected patients and 17 HIV-uninfected control subjects) was enrolled in the study. To participate in the study, HIV-infected patients had to have been receiving HAART (defined as a 3-drug regimen containing ≥1 protease inhibitor or a nonnucleoside analog) for ≥3 months and had to have a CD4 cell count of <100 cells/μL before beginning HAART and ≥100 cells/μL when entering the study. The median CD4 cell count nadir before HAART was 17 cells/μL (range, <10–98 cells/μL). One patient had CMV retinitis before initiating HAART. During the study, CD4 cells counts varied from 110 to 1075 cells/μL (median, 259 cells/μL), and HIV load varied between <104 and 105 copies/mL (median, 103 copies/mL). One patient had CMV retinitis before initiating HAART. During the study, CD4 cells counts varied from 110 to 1075 cells/μL (median, 259 cells/μL), and HIV load varied between <104 and 105 copies/mL (median, 103 copies/mL).

Monoclonal antibodies (MAbs) and recombinant proteins. The following MAbs and recombinant proteins were used in this study: anti–interleukin (IL)–10 (R&D Systems), anti-CD28 (Becton Dickinson), anti-CD49d (Becton Dickinson), recombinant human IL-2 (Boehringer Mannheim), and recombinant human IL-12 (Genetics Institute).

Lymphocyte proliferation assay (LPA). Peripheral blood
Table 1. Cytomegalovirus (CMV)-specific responses in human immunodeficiency virus (HIV)-infected patients responding to highly active antiretroviral therapy (HAART) and in uninfected control subjects.

<table>
<thead>
<tr>
<th>Assay</th>
<th>HIV-infected patients</th>
<th>Uninfected control subjects</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocyte proliferation</td>
<td>4/17</td>
<td>25/29</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SI, median (range)</td>
<td>1.8 (0.5–26)</td>
<td>18.9 (0.5–151)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RCF</td>
<td>11/20</td>
<td>17/18</td>
<td>0.006</td>
</tr>
<tr>
<td>Responders/10^5 PBMC, median (range)</td>
<td>2.8 (&lt;1 to &gt;8)</td>
<td>4.5 (&lt;1 to &gt;8)</td>
<td>0.02</td>
</tr>
<tr>
<td>Interleukin-2</td>
<td>25/30</td>
<td>21/22</td>
<td>0.25</td>
</tr>
<tr>
<td>Level, median pg/mL (range)</td>
<td>9 (&lt;6–127)</td>
<td>120 (&lt;6–1695)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Interferon-γ</td>
<td>28/32</td>
<td>21/22</td>
<td>0.18</td>
</tr>
<tr>
<td>Level, median pg/mL (range)</td>
<td>41 (&lt;2–924)</td>
<td>298 (&lt;2–1171)</td>
<td>0.006</td>
</tr>
</tbody>
</table>

NOTE. Patients were considered to have a response to HAART if they had CD4 cell counts of ≥100 cells/µL 3 months after receiving antiretroviral therapy containing ≥1 protease inhibitor or nonnucleoside reverse-transcriptase inhibitor. PBMC, peripheral blood mononuclear cells; RCF, responder cell frequency; SI, stimulation index.

CD8 cell depletion of PBMC. PBMC were incubated with iron beads coated with anti-CD8 mouse MAb (Dynal) for 30 min at 4°C on a shaker. After the beads were segregated to the tube wall with a magnet, unbound cells were transferred to a clean tube, were washed, and were counted. Cell composition was analyzed by use of a fluorescence-activated cell sorter. CD4 and CD8 cells in whole and depleted PBMC were labeled by use of the Tri-color kit (Becton Dickinson), which was used according to the manufacturer’s instructions.

Results

CMV-specific CMI responses in HIV-infected patients, compared with noninfected control subjects. We assessed CMV-induced LP, RCF, and IFN-γ and IL-2 production in PBMC from HIV-infected patients and uninfected control subjects (table 1). Results were positive for 4 of 17 LPAs performed on stimulation, times previously shown to coincide with maximum secretion of IL-2 and interferon (IFN)-γ, respectively. Supernatants were frozen at −70°C until assayed for IL-2 and IFN-γ, using commercial EIA kits (Endogen), according to the manufacturer’s instructions.
PBMC from HIV-infected patients, compared with 25 of 29 assays in uninfected control subjects. The SIs also were significantly lower in HIV-infected patients versus uninfected control subjects. RCF was positive in 11 of 20 HIV-infected patients (median of 2.8 responders/10^5 PBMC) and in 17 of 18 control subjects (median of 4.5 responders/10^5 PBMC). IFN-γ and IL-2 production was detected in a large proportion of both HIV-infected and control subjects. However, the median levels of IL-2 and IFN-γ in culture supernatants for HIV-infected patients were 9 and 41 pg/mL, respectively, whereas median levels for uninfected control subjects were 120 and 298 pg/mL, respectively. In HIV-infected patients, the likelihood of a positive test was greatest for cytokines and least for LP.

**Correlation of CMV-specific CMI in HIV-infected patients with virologic and immunologic response to HAART.** To examine the interactions between the suppression of HIV replication and CMV-specific CMI in HIV-infected patients receiving HAART, we tested correlations of HIV load with RCF, LP, and cytokine secretions (data not shown). There was a significant inverse correlation of HIV load with RCF ($P = .008$) but not with LP or levels of IFN-γ or IL-2. Furthermore, CD4 cell counts showed a positive correlation with RCF ($P = .013$) but not with other CMI parameters (data not shown). Hence, RCF was the only assay that correlated with control of viral replication and recovery of CD4 cell counts. In addition, these findings also indicated that low levels of CMV-specific LP, IL-2, and IFN-γ could not be explained solely by limited CD4 cell recovery or by persistence of HIV replication.

**Relationship between components of the CMV CMI in HIV-infected patients and uninfected control subjects.** To explore the hypothesis that CMI regulation differs in HIV-infected patients, compared with uninfected control subjects, we studied the relationship of IFN-γ, the most sensitive CMV-specific CMI indicator in both HIV-infected patients and healthy individuals, with RCF, LP, and IL-2 measured on the same set of PBMC. In uninfected control subjects, the data showed a significant correlation between IFN-γ, IL-2, RCF, and LPA (figure 1A). In contrast, in HIV-infected patients, there was a significant correlation of IFN-γ production with IL-2 but not with LP or RCF (figure 1B). The lack of correlation between cytokine secretion, LP, and RCF, coupled with the observation that cytokines could be detected, albeit at a low level, in most HIV-infected patients suggested that the PBMC from HIV-infected patients recognized the antigen but failed to amplify the cell-mediated response to the same extent as healthy hosts do.

**In vitro interventions that increase CMV-specific LP responses in HIV-infected patients.** Antigen-specific responses are promoted by stimulatory signals delivered by cognate antigen through T cell receptors, Th1 cytokines, and costimulatory molecules and down-regulated by T cell suppressors, Th2 cytokines, and soluble mediators. We attempted to modulate LP in HIV-infected patients by the addition of IL-12 (0.1–10 ng/
mL), IL-2 (0.3–15 U/mL), anti-CD28 and anti-CD49d (3 μg/mL each), or anti–IL-10 (0.02–0.09 μg/mL) or by depletion of CD8 cells. Each of these interventions was tested in ≥4 independent experiments paired with untreated control subjects. Only CD8 cell depletion increased the frequency of LPA-positive results: in 8 paired experiments, 3 LPAs, using whole PBMC, were positive, compared with 7 after CD8 cell depletion (P = .04).

**Effect of CD8 cell depletion on LP in HIV-infected patients and uninfected control subjects.** To determine whether the LPA-enhancing effect of CD8 cell depletion was specific for HIV-infected patients, we tested PBMC obtained from HIV-seronegative volunteers and HIV-infected patients. The CD8 cell depletion did not change either qualitative or quantitative LPA results in healthy control subjects (P > .99). In contrast, in HIV-infected patients, CD8 cell depletion significantly increased the SIs (P = .005). Furthermore, the SIs measured after CD8 cell depletion of PBMC from 8 HIV-infected patients (median SI, 10.8; range, 2.8–139) were similar to SIs for whole PBMC from 29 uninfected control subjects (median, 18.9; range, 0.5–151; P = .22).

CD8 cell depletion of PBMC derived from 8 HIV-infected patients established a significant correlation between CMV LP and IFN-γ production (P = .02), which is similar to that described in healthy control subjects (figure 1A) but contrasts with the results of whole PBMC from HIV-infected patients (figure 1B).

Because the CD8 cell depletion enriched the number of CD4 cells per culture, we examined the relationship of the SI increase with that of CD4 cell percentage in 4 independent experiments. CD8 cell depletion led to a median increase in CD4 cell concentration of 18.5% (range, 12%–30%). The median SI increase in matched experiments was 6.7 (range, 1.7–18). However, the increase in CD4 cell percentage did not correlate with the SI increase (P = .49, Spearman’s rank correlation). This finding indicated that CD8 cell depletion increased the proliferation in response to CMV antigen stimulation by mechanisms unrelated to the increase in CD4 cell culture concentration.

Correlation analyses showed that CD8 cell depletion resulted in a significant association of in vitro LP with peripheral blood circulating CD4 cell numbers (P = .05), whereas such an association was absent in whole PBMC cultures (figure 2).

**Discussion**

We performed a cross-sectional analysis of CMV-specific CMI in HIV-infected patients who, in response to HAART, changed from high to low risk for CMV end-organ disease. We studied cytokine production to assess Th1 responses to CMV, an early event in the stimulation process; LP, which measures antigen recognition and proliferation of responding cells; and RCF, which estimates the number of CMV-specific CD4 cells, using a proliferation-based detection system. CMV-specific IL-2 and IFN-γ responses, although secreted in low amounts in HIV-infected patients, were present in a similar proportion of HIV-infected patients and control subjects. This is in accordance with previous observations showing a high frequency of CMV-specific Th1 CD4 cells in HIV-infected patients [21, 22]. In contrast, we found significantly less positive RCF assays among HIV-infected patients, compared with uninfected control subjects, and even less LP responders in HIV-infected patients. Overall, Th1 cytokines appeared to be the best indicator of CMV-specific immunity in HIV-infected patients.

Reconstituted CMV CMI in HIV-infected patients differed both qualitatively and quantitatively from CMI in healthy control subjects. Significant correlations were found in healthy individuals for CMV-specific LP, RCF, IL-2, and IFN-γ, whereas, in HIV-infected patients, only IL-2 and IFN-γ were significantly correlated. A high proportion of HIV-infected patients produced IL-2 and IFN-γ after CMV stimulation, which indicates that the antigen was recognized and Th1 responses were activated, but the normal amplification of the response failed to occur in most HIV-infected patients. This suggests that HIV-infected patients have a defect in the regulation of CMV CMI, most likely at the level of cell division and effector expansion. Factors that play a major role in the amplification of T cell responses to cognate antigens involve signals delivered...
by Th1 cytokines and costimulatory molecules [23, 24]. Conversely, T cell suppressors and Th2 cytokines down-regulate the generation of antigen-specific T cell effectors [25, 26]. Several defects of the antigen-specific immune response regulation were reported in HIV-infected patients before the introduction of HAART, including insufficient costimulatory signals during antigen presentation, low Th1 cytokine production, and high inhibitory Th2 responses [27–33].

An additional contributor to the decreased CMV-specific LP could be CD8-mediated suppression. This is consistent with RCF yielding a higher proportion of positive results, compared with LP. Both LP and RCF assays rely on measurement of proliferation, but the limiting-dilution step in the RCF procedure might attenuate the adverse effect of suppressive T cell regulators.

Several experimental conditions were designed to probe these potential mechanisms of LP inhibition. The addition of IL-12 or anti-CD28 and anti-CD49d, potent CD4 costimulating molecules, to PBMC cultures did not increase proliferative responses. The addition of IL-2 increased proliferation both in CMV-antigen and mock-infected control wells such that the net effect was no change in the SI. Anti–IL-10 treatment of PBMC cultures did not affect proliferation, which indicates that imbalance between Th1 and Th2 responses did not contribute to the absence of CMV-specific LP. In contrast, CD8 cell depletion significantly increased the number of positive SI results for HIV-infected patients and increased the SI values of CMV-stimulated cultures to levels similar to those measured in healthy control subjects. This effect was specific for HIV-infected patients, and it was not present in uninfected control subjects. CD8 cell depletion of PBMC from HIV-infected patients also established a significant correlation between LP and IFN-γ production that was not present in undepleted cells.

Circulating activated CD8 cells are increased in patients with unsuppressed HIV replication [34–36]. There are ≥4 ways in which CD8 suppressor activity could contribute to the results that we obtained: (1) CMV antigen–stimulated CD4 cells could express HIV-specific antigens and become targets of HIV-specific CD8 cells present in the PBMC culture, (2) gp120 signaling through CXCR4, which induces chemotaxis and activation of T cells, might have enhanced in vitro CD8 regulatory functions [37], (3) preactivated CD8 cells may have nonspecifically lysed bystander CD4 cells in tissue culture, or (4) CD 8 cells might have produced excess IL-4 and thereby inhibited PBMC proliferation [38]. One or more of these mechanisms might be operative in the suppression of CMV-specific proliferation of PBMC.

The clinical relevance of this abnormal CD8-mediated immune response is unclear, because HIV-infected patients similar to our patient population have safely interrupted anti-CMV maintenance therapy [6–8]. None of the subjects of this investigation developed de novo CMV disease, despite the lack of prophylaxis. Hence, the CD8-mediated inhibition of CMV LP does not seem to impair the ability of the host to control CMV reactivation. However, abnormal CD8 cell responses might play a role in the immune vitritis of HIV-infected patients receiving HAART, in whom T cells represent the bulk of the local inflammatory infiltrate [39]. Further immunophenotypic studies of the local infiltrate would define the extent to which CD8 cells contribute to this process.

There was a significant correlation of CD4 cell counts with RCF and CD8-depleted LPA results, which suggests that the increase in circulating CD4 cell counts in HIV-infected patients determined an increase in the number of CMV-specific CD4 cells, which seemed to be fully functional. This finding supports the clinical observation that HIV-infected patients with >100 CD4 cells/µL are protected against CMV disease. HIV load inversely correlated with RCF, but this association might not be independent of CD4 cell counts, which significantly increased inversely to HIV load. Other immune functions failed to correlate with CD4 or HIV load, corroborating the complexity of the immune recovery process in HIV-infected patients.

References

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