Association between Cytomegalovirus-Specific Reactivity of T cell Subsets and Development of Cytomegalovirus Retinitis in Patients with Acquired Immunodeficiency Syndrome

Szu-Min Hsieh,* Sung-Ching Pan,† Chien-Ching Hung, Hsing-Chun Tsai, Mao-Yuan Chen, and Shan-Chwen Chang

The association between cytomegalovirus (CMV)–specific reactivity of T cell subsets and development of CMV retinitis (CMV-R) was prospectively studied in 50 CMV-seropositive AIDS patients. The frequency of CMV-specific CD69 expression on CD8 T cells was similar in patients with and patients without CMV-R (median, 1.0% vs. 1.2%; P = .14). However, the frequency of CMV-specific CD69 expression on CD4 T cells was significantly lower in patients with CMV-R than in those without CMV-R (median, 0.4% vs. 2.25%; P < .001). CMV-specific CD4 T cell reactivity in patients who developed CMV-R shortly after starting highly active antiretroviral therapy (HAART) remained low, although the CD4 cell counts increased markedly. Therefore, development of CMV-R is associated with a poor CMV-specific reactivity of CD4 T cells but not with poor reactivity of CD8 T cells. Development of CMV-R after initiation of HAART is associated with a poor reconstitution of CMV-specific immune response, rather than with immune rebound.

Although the incidence of cytomegalovirus retinitis (CMV-R) in persons infected with human immunodeficiency virus (HIV)–type 1 has declined globally since the introduction of highly active antiretroviral therapy (HAART) [1, 2], CMV-R remains an important cause of morbidity in AIDS patients [3, 4]. A clinically relevant method for identifying AIDS patients at high risk for CMV-R is needed. Many studies have shown that use of the pp65 antigenemia assay [5, 6] or quantitative CMV polymerase chain reaction (PCR) [7, 8] to evaluate the systemic CMV load may help predict the development of CMV diseases. However, the feasibility of using these assays to predict the occurrence of CMV-R is questionable, because a substantial proportion of patients develop CMV-R but have a low or undetectable CMV load or no changes in the CMV load [9–13].

Because the development of CMV diseases is related to insufficient CMV-specific cellular immunity and persistent CMV infection [14, 15], assessment of CMV-specific T cell reactivity may help to identify high-risk patients. Activity of CD8 cytotoxic T lymphocytes (CTL) is essential for control of CMV infection [16, 17], and CD4 T helper lymphocytes are required for the generation of CTL in vivo [18, 19]; however, the roles of T cell subsets in the host defense mechanism against the occurrence of CMV-R in AIDS patients have not been clarified, especially the role of CTL [15, 20, 21]. Furthermore, the development of CMV-R shortly after initiation of HAART in patients with advanced HIV infection is not uncommon [3, 4]. The pathogenesis of CMV-R in these cases remains highly controversial [3, 22, 23].

Currently available assays for evaluation of CMV-specific reactivity of T cells are time consuming and often involve the handling of radioactive materials, and therefore these assays are seldom applied in routine clinical settings. In this study, we attempted to develop a time-saving and relatively easy-to-use assay for quantitatively estimating CMV-specific cellular immunity. CD69, a member of the lectin superfamily of the transmembrane signal-transducing receptors, is the earliest activation surface marker on human lymphocytes [24]. Unlike HLA-DR or CD38, CD69 is barely expressed in T cells with HIV–1–induced dysregulated activation [25, 26]. Although expression of CD69 by T cells after incubation with an antigen is not necessarily antigen specific, we show that the frequency of CD69 expression on T cells after antigen stimulation represents the extent of specific antigen–related activation. In this study, we propose that the frequency of CMV-specific CD69 expression on T cell subsets may be a simple surrogate marker for measuring CMV-specific T cell responses in HIV–1–infected patients and may be applied clinically to help identify patients at high risk for development of CMV-R.
Subjects and Methods

Study subjects. From January 1998 to July 2000, 50 antiretroviral-naive, CMV-seropositive, HIV-1–infected patients with <50 CD4 cells/μL and without active opportunistic infections (except CMV diseases) were enrolled in this study. No patient was receiving antiviral treatment with activity against CMV at enrollment. Patients began receiving HAART within 1 week after enrollment. CMV-specific T cell reactivity was assessed at enrollment (baseline) and after 8 weeks of HAART and assessed again if CMV-R developed. These subjects were screened for CMV-R at enrollment and, if any ophthalmologic problem appeared, during follow-up. Screening for CMV-R was done and the diagnosis was made by an experienced ophthalmologist who was blind to the results of immune evaluation. The duration of follow-up was 6–23 months (median, 14.5 months). To maintain a focus on CMV-R, we excluded patients who developed CMV diseases other than CMV-R from data analysis, because the immunopathogenesis of CMV-R may differ from that of other CMV diseases [9, 12].

Fifteen CMV-seropositive and 10 CMV-seronegative healthy, HIV-seronegative volunteers (age range, 20–35 years) also were enrolled. The serologic tests used to detect anti-CMV antibodies for identification of CMV-seropositive subjects were complement fixation and ELISA (Dade Behring).

Frequency of CMV-specific CD69 expression (%CD69 to CMV) on T cell subsets. Peripheral blood mononuclear cells (PBMC) were prepared from whole blood and isolated by differential centrifugation over Ficoll-Paque (Pharmacia Biotech). PBMC (10⁶ cells/mL) were suspended in complete RPMI medium (Gibco) supplemented with penicillin/streptomycin and 10% fetal calf serum (Gibco) on 24-well plates (1 mL/well). PBMC were incubated with CMV antigen (CMVAg; 2 μL), CMV control antigen (cAg; 2 μL of a commercial solution; BioWhittaker), or phytohemagglutinin (PHA; 0.2%; Gibco) in 24-well plates. After incubation for 24 h at 37°C, PBMC were stained with fluorescein-conjugated monoclonal antibodies (MAbs) to CD4 (CyChrome), CD8 (phycoerythrin), and CD69 (fluorescein isothiocyanate [FITC]; Pharmingen) and analyzed by flow cytometry (Becton Dickinson). PBMC were gated into the lymphocyte population (by forward and side scatter), sequentially gated into CD4 or CD8 cells, and analyzed for CD69 expression. We analyzed 20,000 events in each sample. %CD69 to CMV was calculated by subtracting the frequency of CD69 expression after incubation with cAg from the frequency of CD69 expression after incubation with CMVAg. We arbitrarily defined a positive response of CD69 to CMV on CD4 T cells as >0.5% (because the 95% confidence interval [CI] for the response in 10 CMV-seronegative healthy volunteers was 0.1%–0.4%) and that of CD69 to CMV on CD8 T cells as >1.0% (95% CI for CMV-seronegative healthy volunteers, 0.3%–0.8%).

Isolation of T cell subsets and monocytes from PBMC. Immunomagnetic beads coated with anti-human CD2 MAbs (CD2 CELLlection kit; Dynal) and anti-CD7, -CD16, -CD19, and -CD56 MAbs (Monocyte Negative Isolation; Dynal) were used as recommended by the manufacturers. Monocytes were obtained from the supernatant and suspended in complete RPMI medium for culture, and then CD4 and CD8 T cells were separately isolated from CD2 cells (CD4 and CD8 Positive Isolation; Dynal). The resulting purity of both CD4 and CD8 T cells was >99%.

CMV-specific interferon (IFN)–γ production from CD4 T cells. After CD4 T cells (3 x 10⁵ cells) were stimulated by incubation with monocytes (in a ratio of 5:1) with CMVAg or cAg in 1 mL of complete RPMI medium in a 24-well plate for 48 h, culture supernatant was harvested for measurement of IFN-γ levels by ELISA (Quantikine; R&D Systems). We show levels of CMV-specific IFN-γ production from CD4 T cells as (levels in supernatant of cells incubated with CMVAg) – (levels of cells incubated with cAg).

CMV-specific CD8 CTL activity. The effector and target cells used in cytotoxicity assay were prepared as described elsewhere [26]. In brief, isolated CD8 T cells were prepared as effector cells by incubation with autologous monocytes (as antigen-presenting cells in a ratio of 5:1) with CMVAg, recombinant interleukin (rIL)–2 (50 U/mL; Pharmingen), rIL-4 (100 U/mL; Pharmingen), and granulocyte-macrophage colony-stimulating factor (50 ng/mL; Pharmingen) in 2 mL of complete RPMI medium in 12-well plates for 7 days. Autologous monocytes were pulsed with CMVAg or cAg in 6-well plates (1 mL/well) and prepared as target cells (10⁶ cells/mL). In this study, a nonradioactive lactate dehydrogenase release cytotoxicity assay kit (CytoTox 96 Nonradioactive Cytotoxicity Assay; Promega), which yielded results that were nearly identical to those of a parallel chromium 51 release assay [27, 28], was used according to the manufacturer’s instructions with a ratio of effector to target of 10:1. The percentage of CMV-specific cytosis was calculated by subtracting the percentage of cytosis of cAg-pulsed monocytes from the percentage of cytosis of CMVAg-pulsed monocytes.

Lymphocyte proliferation assay. The lymphoproliferative response was assessed by determining the frequency of CD4 T cells with bromodeoxyuridine (BrdU) incorporation after antigen stimulation (BrdU Flow Kit; BD Pharmingen). PBMC (10⁶ cells/well of a 96-well round-bottom plate) were incubated for 6 days with 5 μL of CMVAg or cAg solution in a final volume of 200 μL of RPMI medium. BrdU (final concentration, 10 μM) was added 18 h before harvest. The cells with BrdU incorporation were detected by flow cytometry after staining with anti-BrdU–FITC according to the manufacturer’s instructions. CMV-specific lymphoproliferative response was calculated by subtracting the frequency of CD4 T cells with BrdU incorporation after incubation with cAg from the frequency after incubation with CMVAg. We arbitrarily defined the positive response as >0.5% (95% CI for the response in 10 CMV-seronegative healthy volunteers, 0%–0.4%).

Statistical analysis. Statistical analysis was done with SPSS software (standard version 6.1.3). For analysis of continuous data, we used the nonparametric Mann-Whitney U test. Linear correlation was evaluated by the Pearson correlation coefficient. Comparison of proportions was tested by Fisher’s exact test. All tests were 2-tailed, and P < .05 was considered to be significant.

Results

%CD69 to CMV. To determine whether the %CD69 to CMV on T cell subsets could be used as a surrogate marker for CMV-specific T cell reactivity, we correlated the %CD69 to CMV on T cell subsets with the results of conventional assays. We found that the %CD69 to CMV on CD4 T cells correlated...
with the level of CMV-specific IFN-γ production from CD4 T cells (figure 1), and the relationship between the %CD69 to CMV on CD8 T cells and CMV-specific CD8 CTL activity was also significant (figure 1). CMV-specific lymphoproliferative responses, determined by assessment of the frequency of CD4 T cells with BrdU incorporation, also correlated well with %CD69 to CMV on CD4 T cells ($r = 0.81; P = .03$).

**CMV-specific reactivity of T cell subsets in patients with and patients without CMV-R at enrollment.** Fifty patients were enrolled in the study, but 5 were excluded because they developed CMV diseases other than CMV-R or were lost to follow-up. Of the remaining 45 patients, 9 were diagnosed with CMV-R at initial screening. Among the 36 patients who did not have CMV-R at enrollment, 3 developed CMV-R after initiation of HAART. The other 33 patients remained free of CMV-R or other CMV diseases during follow-up.

The baseline %CD69 to CMV on both CD4 and CD8 T cells in the 45 patients who were included in the study was significantly lower than that in CMV-seropositive healthy volunteers (median, 1.3% vs. 3.1% on CD4 cells; $P = .019$ and 1.4% vs. 3.2% on CD8 cells; $P = .001$). The age, sex, baseline CD4 T cell count, and plasma HIV-1 RNA for patients with CMV-R at enrollment ($n = 9$) and for patients without CMV-R ($n = 33$) were similar (table 1). The baseline %CD69 to CMV on CD8 T cells and CMV-specific CD8 CTL activity were also similar in patients with CMV-R at enrollment and patients without CMV-R. However, the baseline %CD69 to CMV on CD4 T cells in patients with CMV-R at enrollment was much lower than that in patients without CMV-R, although the frequency of CD69 expression on CD4 T cells after mitogen (PHA) stimulation was similar (median, 42.3% vs. 40.8%; $P = .76$). The CMV-specific lymphoproliferative responses of CD4 T cells in patients with CMV-R at enrollment were also significantly lower than those in patients without CMV-R. After 8 weeks of HAART, 9 patients had received ganciclovir therapy, and the %CD69 to CMV on CD4 T cells of those patients improved and became similar to that of patients without CMV-R (median, 2.5% vs. 2.7%; $P = .54$).

**CMV-specific reactivity of T cell subsets in patients who developed CMV-R shortly after initiation of HAART.** Of the 36 patients who did not have CMV-R at initial screening, only 4 had a baseline %CD69 to CMV on CD4 T cells that was <0.5%, and 3 of those patients developed CMV-R shortly after initiation of HAART that was considered to be successful (table 2). The %CD69 to CMV on CD4 T cells and CMV-specific lymphoproliferative responses of CD4 T cells in those 3 patients were still extremely low when CMV-R was diagnosed (table 2). The %CD69 to CMV on CD4 T cells in those 3 patients at enrollment and after 8 weeks of HAART was significantly lower than that in the 33 patients without CMV-R (median, 0.2% vs. 2.25% at enrollment; $P = .001$ and 0.2% vs. 2.9% after 8 weeks of HAART; $P < .001$). The difference in results from the assessment of lymphoproliferative responses of CD4 T cells was also significant (median, 0.3% vs. 2.4% at enrollment; $P < .001$ and

**Figure 1.** Correlation between frequency of CMV-specific CD69 expression (%CD69 to CMV) on T cell subsets and conventional assays for CMV-specific T cell reactivity. Relationship between %CD69 to CMV on CD4 T cells and CMV-specific interferon (IFN)-γ production from CD4 T cells and relationship between %CD69 to CMV on CD8 T cells and CMV-specific activity of CD8 cytotoxic T lymphocytes are significant, with good correlation ($R^2 = 0.73$ and $R^2 = 0.67$, respectively). Lines, linear regression.
0.2% vs. 3.8% after 8 weeks of HAART; \( P = .001 \). The %CD69 to CMV on CD8 T cells in the 3 patients did not change from enrollment to onset of CMV-R. The patient who had a %CD69 to CMV on CD4 T cells, 0.5% at baseline assessment but did not develop CMV-R had a marked improvement in CMV-specific CD4 T cell reactivity after 8 weeks of HAART (%CD69 to CMV on CD4 T cells, 0.4%–1.8%; lymphoproliferative responses, 0.5%–2.1%). No patient with baseline %CD69 to CMV on CD4 T cells >0.5% (\( n = 32 \)) developed CMV-R after starting HAART (whereas 3 of 4 patients with baseline %CD69 to CMV on CD4 T cells <0.5% developed CMV-R; \( P = .001 \)).

**Discussion**

In this study, we evaluated the CMV-specific reactivity of T cell subsets in CMV-seropositive AIDS patients and tried to discover the association between the results of immunologic assessment and the development of CMV-R. We found that the development of CMV-R, whether it occurred before or after initiation of HAART, was associated with poor CMV-specific reactivity of CD4 T cells. These results imply that the immunologic approach may help to identify patients at high risk for CMV-R and suggest that the CMV-specific CD69 expression is a clinically relevant surrogate marker for T cell reactivity against CMV, because only 24 h of incubation and 2–3 h of handling are needed to obtain results.

Similar to results from other studies that showed that AIDS patients with CMV-R had low or undetectable T cell responses to CMV [15, 21], our data showed that CMV-specific CD4 T cell reactivity in patients with CMV-R is much lower than that in persons without CMV-R. However, we also found that CMV-specific CD8 T cell reactivity was similar in vitro in patients with and patients without CMV-R. Furthermore, no specific

| Table 1. Characteristics and baseline cytomegalovirus (CMV)-specific reactivity of T cell subsets in patients with and patients without CMV retinitis (CMV-R). |
|---------------------------------|-----------------|-----------------|-----------------|
| Variable                        | Patients with   | Patients without |       |
|                                 | CMV-R (\( n = 9 \)) | CMV-R (\( n = 33 \)) |       |
| Age, median years               | 28              | 30              | .54   |
| Sex, male/female                | 10/1            | 26/2            | 1.0   |
| CD4 T cells/\( \mu L \)         | 18              | 25              | .42   |
| Plasma HIV RNA, log_{10} copies/mL | 4.9           | 5.4            | .47   |
| CMV-specific CD69 expression on CD4 T cells, median % (95% CI) | 0.40 (0.18–0.50) | 2.25 (1.72–2.54) | <.001 |
| CMV-specific lymphoproliferative response of CD4 T cells, median % bromodeoxyuridine incorporation (95% CI) | 0.4 (0.1–0.7) | 2.4 (1.1–3.8) | <.001 |
| CMV-specific CD69 expression on CD8 T cells, median % (95% CI) | 1.00 (0.73–1.31) | 1.20 (1.10–1.54) | .14   |
| CMV-specific cytolysis by CD8 T cells, median % (95% CI) | 15 (8–18) | 14 (7–21) | .56   |

**NOTE.** CI, confidence interval.

| Table 2. Change in immunologic reactivity from enrollment to onset of cytomegalovirus retinitis (CMV-R) in patients who developed CMV-R shortly after starting highly active antiretroviral therapy (HAART). |
|---------------------------------|-----------------|-----------------|-----------------|
| Variable                        | Patient 1       | Patient 2       | Patient 3       |
| Age, years/sex                  | 30/M            | 28/M            | 35/M            |
| Onset of CMV-R after initiation of HAART, weeks | 6               | 12              | 15              |
| Immunologic response            |                 |                 |                 |
| CD4 cells/\( \mu L \)           | 6—57            | 10—167          | 25—136          |
| CD8 cells/\( \mu L \)           | 345—1292        | 168—237         | 420—1574        |
| Plasma HIV RNA, log_{10} copies/mL | 5.1—UD       | 5.5—UD          | 5.3—UD          |
| CMV-specific CD69 expression on CD4 T cells, % | 0.2—0.3       | 0.1—0.4         | 0.3—0.2         |
| CMV-specific proliferative response of CD4 T cells, % bromodeoxyuridine incorporation | 0.3—0.3       | 0.0—0.0         | 0.5—0.2         |
| CMV-specific CD69 expression on CD8 T cells, % | 1.1—1.4       | 1.5—1.3         | 1.2—1.3         |
| CMV-specific cytolysis by CD8 T cells, % | 6—28           | 20—15           | 16—19           |

**NOTE.** Data are value at enrollment—value at onset of CMV-R, unless otherwise indicated. UD, undetectable.
deficiency in CMV-specific humoral immunity in patients with CMV-R was observed [29]. Thus, we propose that the defective CMV-specific CD4 T cell response may be the key component in the immunopathogenesis of CMV-R in AIDS patients. The relatively poor in vitro reactivity of CD8 T cells in AIDS patients (when compared with that in healthy control subjects) may be only contributory to the development of CMV-R. However, we only evaluated CMV-specific immune responses of T cells obtained from peripheral blood, which potentially limits the study. CMV-specific T cell responses in the eye compartment might reflect the conditions of immune control for CMV retinitis more accurately. However, it is difficult and clinically impractical to obtain these T cells from the eye compartment.

In our patients with CMV-R, CMV-specific CD4 T cell reactivity improved significantly after HAART and ganciclovir treatment, as has been shown in other studies [20, 21]. It is difficult to define the real impact of ganciclovir on CMV-specific immune reconstitution, because HAART and ganciclovir were concurrently used in this and other studies [20, 21]. In our opinion, however, suppression of CMV activity by ganciclovir treatment may be contributory, in part, to CMV-specific immune reconstitution.

The development of CMV-R after initiation of antiretroviral therapy seems not uncommon, even in the era of HAART [3, 4], although T cell reactivity against CMV can be reconstituted by HAART [20, 25, 30]. Why CMV-R can develop during successful HAART remains unclear. At least 2 opposing hypotheses have been proposed. One is that CMV-specific cellular immunity fails to recover, even when CD4 cell count increases, because of HIV-induced exhaustion of CMV-specific T cell clones [3], and the other is that subclinical CMV-R becomes clinically apparent due to rebound of the immune function secondary to rapid increase in CD4 cell count [22, 31]. Our data support the former hypothesis, because CMV-specific CD4 T cell reactivity in patients who developed CMV-R shortly after starting HAART remained at very low levels, even though their CD4 cell counts increased markedly and the CD8 T cell reactivity seemed not to change obviously. Thus, we believe that the development of CMV-R during successful HAART may be due to a delay in restoration of CMV-specific immune function, rather than to a rebound in immune function.

In conclusion, we propose that routine determination of %CD69 to CMV on CD4 T cells (but not on CD8 T cells) by flow cytometry in CMV-seropositive patients with advanced HIV infection may be a clinically practical approach. The approach may help identify patients who are at high risk for CMV-R if the CMV-specific frequency of CD69 expression on CD4 T cells is low (e.g., <0.5%). These patients may need more frequent ophthalmologic examinations, even after initiation of HAART.

References
highly active antiretroviral therapy in individuals infected with HIV-1.


