Association between Immune Recovery Uveitis and a Diverse Intraocular Cytomegalovirus-Specific Cytotoxic T Cell Response

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Cytomegalovirus (CMV) causes serious infection in individuals with deficient T cell immunity. In acquired immunodeficiency syndrome, the retina is a major site of progressive infection, despite the availability of therapy that targets CMV. The administration of highly active antiretroviral therapy to suppress human immunodeficiency virus frequently results in resolution of CMV retinitis, but this may be complicated by ocular inflammation termed “immune recovery uveitis” (IRU). To provide insight into the pathogenesis of IRU, the phenotype and specificity of intraocular T cells in a single patient were analyzed. The T cell infiltrate consisted of a diverse population of CD8+ CMV-specific T cells, but only a minority of these T cells recognized the CMV phosphoprotein 65 and immediate early protein 1, which have been considered major targets of the host response. These results imply that reconstitution of CMV-specific T cells plays a role in IRU and suggest that the specificity of T cells engaged in the control of CMV at local sites of reactivation may be broad.

T cells are crucial for preventing cytomegalovirus (CMV) disease. Before highly active antiretroviral therapy (HAART) became available, individuals with T cell depletion due to human immunodeficiency virus (HIV) commonly developed CMV infection [1]. In such patients, retinitis is the most frequent complication, resulting in vision loss due to cytopathic infection of retinal cells and macular edema. The availability of HAART has reduced the incidence of CMV retinitis, and it has been possible to discontinue anti-CMV drugs in most patients with retinitis who experience an increase in CD4+ T cells [2].

A new syndrome, termed “immune recovery uveitis” (IRU), which is characterized by ocular inflammation and decreased vision without active CMV replication, is observed in HIV-positive individuals who recover from CMV retinitis [3]. The pathogenesis of IRU is unknown, but the association with improvement in T cell numbers suggests that infiltration of T cells in response to residual CMV antigens or exposed autoantigens plays a role. In the present study, T cells cloned from the vitreous humor of a patient with IRU were examined for phenotype, specificity, and T cell receptor (TCR) β-chain variable region (Vβ) use.

Methods

Study participant. Vitreous humor was obtained from an HIV-positive patient with IRU. This individual developed CMV retinitis in 1996 while receiving lamivudine and stavudine for HIV infection and ganciclovir for CMV enteritis. When CMV retinitis developed, the patient’s CD4+ T cell count was 20 cells/mm3, and the HIV load was 73,000 copies/mL. Cidofovir was administered intravenously, and CMV retinitis became inactive. Indinavir was added to lamivudine and stavudine in October 1996. In March 1998, the patient’s plasma HIV load was undetectable, and the CD4+ T cell count increased to 140 cells/mm3. In March 1999, the patient’s CD4+ T cell count increased to 370 cells/mm3, but inflammation developed in the left eye, and visual acuity declined. Pars plana vitrectomy was performed to improve vision, and vitreous humor and blood samples were obtained for analysis.

Cell lines and viruses. Fibroblasts and Epstein-Barr virus-transformed B lymphoblastoid cell lines (LCLs) were generated and propagated as described elsewhere [4]. CMV AD169 was obtained from the American Tissue Culture Collection, and supernatant virus was produced by passage in fibroblasts [4]. The mutant CMV strain RV798, which was constructed with a deletion of the unique short (US) region, was propagated similarly [5]. Vaccinia recombinant viruses encoding the immediate early protein 1 (VacIE1), pp65 (Vac/pp65), and gB (Vac/gB) were provided by William Britt (University of Alabama, Birmingham).
Cells were pelleted from the vitreous humor and resuspended in RPMI 1640 medium containing 10% human serum, 50 U/mL interleukin-2, and 30 ng/mL anti-CD3 monoclonal antibody (MAb). Cells were plated at 0.5 cells/well with 7.5 × 10^4 gamma-irradiated peripheral blood mononuclear cells (PBMC) and 1 × 10^4 gamma-irradiated LCLs as feeder cells. T cells in wells in which growth was evident after 14 days were expanded by restimulation with anti-CD3. CD8⁺ T cell clones specific for pp65, IE-1, or gB were isolated from healthy donors [4, 6]. Expression of CD4 and CD8 was determined by flow cytometry, using anti-CD4-fluorescein isothiocyanate and anti-CD8-phycocerythrin MAb.

**Chromium release and lymphoproliferation assays.** CD8⁺ T cells were assayed for cytotoxicity by use of Cr³⁺-labeled autologous and major histocompatibility complex (MHC) class I-mismatched fibroblasts that were either mock infected, infected with AD169, or infected with RV798 for 30 h. T cell clones that lysed CMV-infected fibroblasts were tested against Cr³⁺-labeled autologous and MHC-mismatched LCLs infected with Vamp65, Vam/IE-1, or Vam/gB [4]. CD4⁺ T cell clones were assayed for proliferation by plating of 1 × 10⁴ T cells in triplicate wells with 2 × 10⁵ autologous gamma-irradiated PBMC as antigen-presenting cells or either medium or CMV antigen [7]. The wells were pulsed with 2.5 μCi of [³H]thymidine for the final 18 h of a 96-h incubation.

**TCR Vβ gene use and Vβ clonotypic-specific polymerase chain reaction (PCR).** RNA was isolated from T cell clones by use of RNeasy (Qiagen) and then reverse-transcribed into cDNA by use of oligonucleotide (dT)15 primers and Moloney murine leukemia virus reverse transcriptase (Gibco BRL). The TCR Vβ gene of each T cell clone was determined by use of a TCR variable region–specific reverse-transcription (RT) PCR [8]. The complementarity-determining region 3 sequence was determined from the PCR product. To detect cytotoxic T lymphocytes (CTL) in peripheral blood, a Vβ family–specific sense primer and a clonotype-specific antisense primer for a variable-diversity-joining sequence of the Vβ gene were used for PCR. The primer sequences for VH-1 CTL were 5'-TGCAGGTGTCGGAGGATA-3' (TCRBV-13SI) (sense) and 5'-CCCCCTGGAGACTTGGA-3' (TCRB-BSNNN) (antisense); the primer sequences for VH-5 CTL were 5'-TAAATTTACTTCCAAGGCAC-3' (TCRBV-6SI) (sense) and 5'-CGTATCTGCCGCCCCAAT-3' (TCRB-BSNNN) (antisense). Total RNA was isolated from stimulation standards prepared by serial dilution of the T cell clone into 10⁶ peripheral blood and from 10⁶ PBMC obtained from the patient. cDNA was synthesized, and one-tenth of the cDNA (corresponding to 10⁶ PBMC) was used for PCR. Quantitation of each T cell clone in PBMC was estimated by comparing the intensity of the PCR product to that of control standards. To ensure the quality of each cDNA sample, RT-PCR was performed on control samples, using primers (5'-TACGCAGTACGGACGAG-3' and 5'-ATTCGATCCATCGGCTG-3') for β₂-microglobulin. PCR for the TCR Vβ gene expressed in clone VH-1 was also performed on genomic DNA isolated from 10⁶ PBMC. One-tenth of eluted DNA was amplified, and products were visualized with ethidium bromide.

**CMV detection using PCR.** PCR analysis was done on 100 mL of vitreous humor. Fluorescent-probe PCR was used to amplify an 86-bp fragment of the CMV UL123 gene [9].

**Results**

**Composition of the T cell infiltrate in IRU.** Twenty-nine T cell clones were isolated from the vitreous humor. Of the 29 clones, 21 were CD3⁺ CD4⁺ CD8⁺, and 8 were CD3⁺ CD4⁻ CD8⁺. The CD8⁺ T cell clones were tested for recognition of fibroblasts infected with the AD169 strain of CMV. Only 3 (VH-2, VH-8, and VH-11) of the 21 lysed AD169-infected fibroblasts (figure 1A). These results suggested that CD8⁺ CMV-specific CTL made up a minor component (14%) of the T cell infiltrate.

It was possible that the frequency of CMV-specific CTL in the eye was underestimated, because newly synthesized viral proteins are not efficiently presented by AD169-infected fibroblasts [4, 6]. Thus, the 21 CD8⁺ T cell clones were tested for lysis of fibroblasts infected with CMV RV798, which has a deletion of the US2, US3, US6, and US11 genes that interfere with MHC-I presentation [5, 10]. Of the 21 CD8⁺ T cell clones, 12 lysed RV798-infected fibroblasts, including the 3 that lysed AD169-infected fibroblasts (figure 1A). Recognition by all 12 clones was MHC-I restricted, because MHC-I-mismatched RV798-infected fibroblasts were not lysed (data not shown). Thus, most of the CD8⁺ T cells (57%) infiltrating the vitreous humor were CMV-specific CTL.

Eight CD4⁺ T cell clones were isolated, but none proliferated in response to CMV antigen (data not shown). A CD4⁺ T helper (Th) response to CMV antigen was detected in the blood at the time IRU was diagnosed, indicating that the patient had circulating CMV-specific Th cells. The absence of a CD4⁺ CMV-specific Th response in the eye should be interpreted cautiously, because a small number of clones were obtained for analysis. Despite the absence of CD4⁺ CMV-specific Th cells in the eye, CMV DNA was not detected in the vitreous humor by PCR.

**Specificity of intraocular CD8⁺ CMV-specific CTL.** Studies examining CD8⁺ CMV-specific CTL responses in the blood of healthy CMV-positive individuals have found a high frequency of CTL specific for the virion protein pp65 and for IE-1 [11–13]. Sufficient pp65 and a related virion protein, pp150, are introduced with the virion to sensitize cells for T cell recognition, even when viral gene expression is blocked with actinomycin D [4, 11]. The requirement for recognition by the CTL clones isolated from the eye was determined by pretreatment of RV798-infected fibroblast target cells with actinomycin D. All 12 CTL clones failed to lyse targets infected in the presence of actinomycin D, demonstrating that de novo viral gene expression was required for recognition by these CTL (figure 1B). All 12 CTL clones also failed to lyse cells infected with Vamp65 and Vam/pp150 (data not shown).

In contrast to pp65-specific CTL, IE-1– and gB-specific CTL do not recognize AD169-infected cells or actinomycin D–blocked RV798-infected cells, but they do lyse RV798-infected cells in which viral gene expression is not impeded (data not shown). Thus, the 12 CTL clones obtained from the patient’s eye were tested for recognition of LCLs infected with Vam/IE-
Figure 1. A, CD8$^+$ T cell clones isolated from the vitreous humor of a patient with immune recovery uveitis lyse cytomegalovirus (CMV)-infected target cells. T cell clones were assayed for cytotoxic activity against Cr$^{51}$-labeled autologous fibroblasts that were either mock infected (white bars), infected with CMV AD169 (black bars), or infected with CMV RV798 (gray bars). Data are shown at a ratio of effector cells to target cells of 10:1. B, CD8$^+$ T cell clones from the vitreous humor require viral gene expression for recognition of target cells. The 12 T cell clones isolated from the vitreous humor and 2 cytotoxic T lymphocyte (CTL) clones (FAF-3C11 [pp150 specific; pp150-CTL] and FAF-3E2 [pp65 specific; pp65-CTL]) isolated from a healthy CMV-seropositive donor were assayed for cytotoxic activity against autologous Cr$^{51}$-labeled fibroblast target cells that were either mock infected (white bars), infected with CMV RV798 (gray bars), or infected with CMV RV798 in the presence of actinomycin D (black bars). Data are shown at a ratio of effector cells to target cells of 10:1. C, Immediate early protein 1 (IE-1)–specific CTL are present in T cells infiltrating the vitreous humor. Twelve CD8$^+$ CMV-specific T cell clones isolated from the vitreous humor of a patient with immune recovery uveitis were assayed for recognition of autologous Cr$^{51}$-labeled Epstein-Barr virus–transformed B lymphoblastoid cell line target cells infected with vaccinia recombinant viruses encoding IE-1 (Vac/IE-1) or glycoprotein B (Vac/gB) and for cytotoxic activity against autologous Cr$^{51}$-labeled fibroblast target cells that were either mock infected, infected with CMV RV798, or infected with CMV AD169. Data shown are for the 2 clones, VH-1 (black bars) and VH-4 (gray bars), that exhibited lytic activity against Vac/IE-1–infected target cells. The ratio of effector cells to target cells was 10:1.
Quantitation of cytotoxic T lymphocyte (CTL) clones isolated from the vitreous humor in patient peripheral blood by polymerase chain reaction (PCR) for the T cell receptor β-chain variable region (Vβ) gene. A. Quantitation of VH-1 CTL by reverse-transcription (RT) PCR. Top panel, Standards (lanes 2–7) were prepared by spiking VH-1 CTL in concentrations from 0.03% to 10% into 10^6 irrelevant T cells from a cytomegalovirus (CMV)-negative donor, synthesizing cDNA, and performing PCR on one-tenth of the cDNA (corresponding to 10^5 peripheral blood mononuclear cells [PBMC]). Lane 8 is a negative control and shows the result of PCR on cDNA from 10^5 PBMC obtained from the CMV-negative donor (CMV^-10^5). Lane 9 shows the result of PCR on cDNA prepared from 10^5 CD8^+ T cells obtained from the patient (Pt CMV^-10^5). The PCR products were electrophoresed on a 2% agarose gel, and the intensity of the bands was visualized by staining with ethidium bromide and then compared with the standards. Bottom panel, RT-PCR was used to assay the cDNA samples for β-2-microglobulin (β2M) as a control. B. Quantitation of VH-5 CTL by RT-PCR. Top panel, Standards (lanes 2–7) were prepared by spiking VH-5 CTL in concentrations from 0.003% to 1% into 10^6 irrelevant T cells from a CMV-negative donor, synthesizing cDNA, and performing PCR on one-tenth of the cDNA. Lane 8 is a negative control and shows the result of PCR on cDNA from 10^5 PBMC obtained from the CMV-negative donor. Lane 9 shows the result of PCR on cDNA prepared from 10^5 CD8^+ T cells obtained from the patient. The PCR products were electrophoresed on a 2% agarose gel, and the intensity of the bands was visualized by staining with ethidium bromide and then compared with the standards. Bottom panel, RT-PCR was used to assay the cDNA samples for β2M as a control. C. Quantitation of VH-1 CTL by genomic PCR. Standards (lanes 2–5) were prepared by spiking VH-1 CTL in concentrations from 0.3% to 10% into 10^6 irrelevant T cells from a CMV-negative donor, extracting DNA, and performing PCR on one-tenth of the DNA. Lane 6 shows the results of PCR on DNA from 10^5 PBMC obtained from the CMV-negative donor. Lane 7 shows the result of PCR on DNA prepared from 10^5 CD8^+ T cells obtained from the patient. The PCR products were electrophoresed on a 2% agarose gel, and the intensity of the bands was visualized by staining with ethidium bromide and then compared with the standards.

Discussion

It has been hypothesized that IRU results from infiltration of T cells in response to antigens in the eye [14], and our results provide direct evidence that the infiltrate is composed of CD8^+ CMV-specific CTL. These results require confirmation in additional patients but support the clinical use of steroids to treat IRU.

A surprising finding was that IE-1 and pp65 were recognized by few of the CTL isolated from the eye. Previous studies have suggested that the T cell response to CMV is predominantly directed at pp65, which is a major component of the virion matrix, and IE-1, which is synthesized soon after infection [11–13]. These data were derived from studies in which blood from healthy CMV-positive donors was analyzed using fibroblasts infected with CMV AD169 or recombinant viruses that express selected CMV proteins. However, the specificity of T cells engaged in control of CMV at local sites of infection has not been examined. Analysis of the T cells infiltrating the eye

1 or Vac/gB. Only 2 clones (VH-1 and VH-4) lysed targets infected with Vac/IE-1 (figure 1C), and none lysed autologous LCLs infected with Vac/gB (data not shown). These results demonstrate that most T cell clones isolated from the vitreous humor were not specific for pp65, pp150, IE-1, or gB but recognized distinct newly synthesized viral antigens.

TCR Vβ use of CD8^+ CMV-specific CTL and quantitation of CTL in the blood. A multiplex RT-PCR was used to identify the TCR Vβ genes expressed by 8 of the 12 CD8^+ CTL clones [8]. Seven of the 8 CD8^+ CMV-specific T cell clones expressed a unique TCR Vβ gene, demonstrating that the intraocular response was diverse. Clone-specific primers were designed for 2 clones (VH-1 and VH-5) to estimate the frequency of these CTL in the blood. PCR assays of patient cDNA samples demonstrated that clone VH-1 made up ~3%–10% (figure 2A) of the peripheral CD8^+ T cells, and VH-5 made up ~0.03% (figure 2B). To validate the results obtained by RT-PCR, we performed PCR on genomic DNA prepared from PBMC, using the primers to detect the single copy of the TCR Vβ gene in the VH-1 CTL clone. The frequency of VH-1 in PBMC found by this method was approximately equivalent to that obtained by RT-PCR (figure 2C). These findings demonstrate that the CTL isolated from the eye were not restricted to this site but were present in easily measurable frequencies in the blood.

Figure 2. Quantitation of cytotoxic T lymphocyte (CTL) clones isolated from the vitreous humor in patient peripheral blood by polymerase chain reaction (PCR) for the T cell receptor β-chain variable region (Vβ) gene. A. Quantitation of VH-1 CTL by reverse-transcription (RT) PCR. Top panel, Standards (lanes 2–7) were prepared by spiking VH-1 CTL in concentrations from 0.03% to 10% into 10^6 irrelevant T cells from a cytomegalovirus (CMV)-negative donor, synthesizing cDNA, and performing PCR on one-tenth of the cDNA (corresponding to 10^5 peripheral blood mononuclear cells [PBMC]). Lane 8 is a negative control and shows the result of PCR on cDNA from 10^5 PBMC obtained from the CMV-negative donor (CMV^-10^5). Lane 9 shows the result of PCR on cDNA prepared from 10^5 CD8^+ T cells obtained from the patient (Pt CMV^-10^5). The PCR products were electrophoresed on a 2% agarose gel, and the intensity of the bands was visualized by staining with ethidium bromide and then compared with the standards. Bottom panel, RT-PCR was used to assay the cDNA samples for β-2-microglobulin (β2M) as a control. B. Quantitation of VH-5 CTL by RT-PCR. Top panel, Standards (lanes 2–7) were prepared by spiking VH-5 CTL in concentrations from 0.003% to 1% into 10^6 irrelevant T cells from a CMV-negative donor, synthesizing cDNA, and performing PCR on one-tenth of the cDNA. Lane 8 is a negative control and shows the result of PCR on cDNA from 10^5 PBMC obtained from the CMV-negative donor. Lane 9 shows the result of PCR on cDNA prepared from 10^5 CD8^+ T cells obtained from the patient. The PCR products were electrophoresed on a 2% agarose gel, and the intensity of the bands was visualized by staining with ethidium bromide and then compared with the standards. Bottom panel, RT-PCR was used to assay the cDNA samples for β2M as a control. C. Quantitation of VH-1 CTL by genomic PCR. Standards (lanes 2–5) were prepared by spiking VH-1 CTL in concentrations from 0.3% to 10% into 10^6 irrelevant T cells from a CMV-negative donor, extracting DNA, and performing PCR on one-tenth of the DNA. Lane 6 shows the results of PCR on DNA from 10^5 PBMC obtained from the CMV-negative donor. Lane 7 shows the result of PCR on DNA prepared from 10^5 CD8^+ T cells obtained from the patient. The PCR products were electrophoresed on a 2% agarose gel, and the intensity of the bands was visualized by staining with ethidium bromide and then compared with the standards.

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provided a unique opportunity to evaluate the specificity of T cells involved in clearing CMV. Most of the CMV-specific CTL clones were only detected when target cells infected with CMV RV798 were used, and the clones failed to recognize cells infected with AD169 or with vaccinia recombinants encoding pp65, IE-1, pp150, and gB. Analysis of the TCR gene rearrangements expressed by the CMV-specific CTL in the eye revealed that the intraocular CTL response was highly polyclonal. Moreover, the T cell clones responding to CMV in a patient with IRU were not restricted to the eye and were easily detectable in the blood at frequencies of up to 3% of CD8+ T cells.

The use of a mutant CMV strain that lacked genes that interfere with antigen presentation was critical for identification of the complexity of the CTL response to CMV in this patient. However, many of the CTL elicited in response to RV798 failed to recognize target cells permissively infected with wild-type CMV. This raises the question as to how these CTL limit virus replication in vivo. One possibility is that the viral genes responsible for down-regulating MHC-I may not be equally active in cells of all lineages. A second possibility is that dendritic cells take up and cross-present viral antigens to T cells [15]. CTL elicited by cross-presentation by dendritic cells may be ineffective for direct recognition of permissively infected cells but could mediate antiviral effects by producing cytokines that overcome the defects in antigen presentation in infected cells, by recruiting other effector cells, and/or by interfering with viral replication. Our findings suggest that additional studies of the diversity of the CTL response to CMV are warranted.

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References