Clinical Infectious Diseases

MAJOR ARTICLE

Cytomegalovirus Viremia Associated With Increased Mortality in Cryptococcal Meningitis in Sub-Saharan Africa

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Background. Cryptococcal meningitis and tuberculosis are both important causes of death in persons with advanced human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS). Cytomegalovirus (CMV) viremia may be associated with increased mortality in persons living with HIV who have tuberculosis. It is unknown whether concurrent CMV viremia is associated with mortality in other AIDS-related opportunistic infections.

Methods. We prospectively enrolled Ugandans living with HIV who had cryptococcal meningitis from 2010–2012. Subsequently, we analyzed stored baseline plasma samples from 111 subjects for CMV DNA. We compared 10-week survival rates among those with and without CMV viremia.

Results. Of 111 participants, 52% (58/111) had detectable CMV DNA (median plasma viral load 498 IU/mL, interquartile range [IQR] 259–2390). All samples tested were positive on immunoglobin G serology. The median $CD4^+$ T cell count was 19 cells/μL (IQR 9–70) and did not differ by the presence of CMV viremia ($P = .47$). The 10-week mortality rates were 40% (23/58) in those with CMV viremia and 21% (11/53) in those without CMV viremia (hazard ratio 2.19, 95% confidence interval [CI] 1.07–4.49; $P = .03$), which remained significant after a multivariate adjustment for known risk factors of mortality (adjusted hazard ratio 3.25, 95% CI 1.49–7.10; $P = .003$). Serum and cerebrospinal fluid cytokine levels were generally similar and cryptococcal antigen-specific immune stimulation responses did not differ between groups.

Conclusions. Half of persons with advanced AIDS and cryptococcal meningitis had detectable CMV viremia. CMV viremia was associated with an over 2-fold higher mortality rate. It remains unclear whether CMV viremia in severely immunocompromised persons with cryptococcal meningitis contributes directly to this mortality or may reflect an underlying immune dysfunction (ie, cause vs effect).

Clinical Trials Registration. NCT01075152.

Keywords. cryptococcus; cytomegalovirus; HIV; CMV; cryptococcal meningitis.

Cryptococcal meningitis is the most common form of adult meningitis in people with human immunodeficiency virus (HIV), with a large burden in sub-Saharan Africa [1]. In Uganda, cryptococcal meningitis is more common than all other causes of meningitis combined [2]. Despite increased access to antiretroviral therapy (ART) and an increased emphasis on early ART initiation, the World Health Organization acknowledges that up to half of people presenting to care with HIV have advanced disease [3]. Cryptococcal meningitis most often occurs in the setting of acquired immunodeficiency syndrome (AIDS), and such persons are at risk for having multiple, concurrent opportunistic infections, including cytomegalovirus (CMV) [4].

CMV is a human herpesvirus that typically causes asymptomatic infections in immunocompetent hosts but may cause end-organ disease among immunocompromised persons, in sites such as the gastrointestinal tract, retina, or central nervous system [4, 5]. CMV seroprevalence, as defined by immunoglobin G (IgG) antibody positivity, is typically >90% in African populations living with HIV [6–8]. CMV viremia, likely a better indicator of active disease, has a prevalence of 1.8% to 22.6% in African cohorts living with HIV [6, 9, 10]. The CMV viremia prevalence may be higher in those with advanced disease; for example, Micol et al [11] reported that 55% of Cambodians with CD4 counts <100 cells/μL had detectable CMV viremia. Active CMV replication is associated with increased levels of plasma HIV DNA [12]. Moreover, those with

Received 17 April 2019; editorial decision 24 August 2019; accepted 30 August 2019; published online September 1, 2019.

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Clinical Infectious Diseases® 2020;71(3):525–31

DOI: 10.1093/cid/ciz864

detectable plasma CMV DNA have a 2.2-fold increased hazard of developing an AIDS-defining diagnosis, even when controlling for CD4 count and plasma HIV RNA concentration [13]. Fielding et al [10] found that subclinical CMV viremia was an independent risk factor for mortality among adult males living with HIV in South Africa, while other studies have noted that higher CMV viral loads were associated with greater hazards toward mortality in the pre-ART and early-ART eras [13–15].

Little is known regarding whether active CMV replication contributes to an increased risk of death in persons living with HIV who have other high-mortality opportunistic infections, such as cryptococcal meningitis. Interestingly, Ward et al [16] found that CMV viremia was associated with a trend toward increased mortality in persons living with HIV who had tuberculosis, and particularly in older patients. Both tuberculosis and cryptococcosis are caused by intra-cellular pathogens requiring Type 1 T helper (Th1) cell immunity for protection. Viral infections can increase the production of Type 1 interferons (eg, interferon-alpha), which may subsequently impair Th1 cytokine (eg, interferon-gamma) responses [17]. The stimulation of peripheral blood mononuclear cells with CMV antigens results in lower interferon-gamma and tumor necrosis factor alpha levels in CMV-seropositive versus CMV-seronegative kidney transplant patients [18]. Thus, a concomitant infection with CMV could potentially counteract protective Th1 immune responses against *Cryptococcus*. Given that CMV viremia has been associated with mortality in the aforementioned populations, we conducted this retrospective analysis to determine whether CMV viremia was associated with increased mortality in cryptococcal meningitis. We additionally explored the cytokine profile of our participants to assess whether CMV viremia was associated with impaired Th1 immune responses.

METHODS

Study Design

We analyzed samples that had been prospectively collected from participants enrolled in the Cryptococcal Optimal ART Timing (COAT) trial (ClinicalTrials.gov: NCT01075152), a randomized, clinical trial testing early ART initiation at 1–2 weeks versus deferred ART initiation at 4–6 weeks after cryptococcal meningitis [19]. Briefly, ART-naive adults living with HIV who had first-episode cryptococcal meningitis were enrolled at either Mulago Hospital or Mbarara Hospital in Uganda or at GF Jooste Hospital in Cape Town, South Africa, from November 2010 to April 2012. Induction therapy consisted of amphotericin B deoxycholate (0.7–1.0 mg/kg/day) plus fluconazole (800 mg/day) for 2 weeks, followed by "enhanced consolidation," consisting of fluconazole at 800 mg/day therapy for ~3 weeks, until cerebrospinal fluid (CSF) cultures were sterile [20], and then at 400 mg/day through 12 weeks. All participants also received supportive care with therapeutic lumbar punctures, intravenous fluids, electrolyte management, and trimethoprim-sulfamethoxazole prophylaxis. Survival at 26 weeks was the primary endpoint of the parent trial.

All participants enrolled into the COAT trial who had stored plasma available were potentially eligible for this study. We excluded persons screened for the trial who were ineligible (eg, receiving ART) or who died <7 days from diagnosis and prior to trial enrollment. The parent trial received Institutional Review Board approval from United States, Ugandan, and South African institutions. All participants provided written informed consent for the storage and future testing of samples for research purposes.

Laboratory Methods

DNA Extraction and Polymerase Chain Reaction

DNA was extracted from 200 µL of plasma using the QIAcube HT (QIAGEN, Hilden, Germany) and QIAamp 96 Virus DNA QIAcube kit, according to the manufacturer's instructions. All samples were eluted in 100 µL of polymerase chain reaction (PCR)-grade water and were stored at −20°C. Multiplex quantitative PCR was performed using the LightCycler 480 System (Roche, Basel, Switzerland). Detailed PCR methods can be found in the Supplementary Methods. Laboratory staff processed coded samples and were blinded to participant data and outcomes. CMV quantification of copies/mL values were converted to international units (IU) using the World Health Organization International Standard for Human Cytomegalovirus for Nucleic Acid Amplification Techniques (National Institute for Biological Standards and Control code 09/162) [21].

Serological Assessment

CMV IgG serostatuses were assessed using CMV IgG enzyme-linked immunosorbent assay (Diamedix, Miami Lakes, FL), following the manufacturer's instructions. Specimens were run in triplicate at 1:100 dilution, with absorbance measured at 450 nm. Specimens with a measured enzyme-linked immunosorbent assay unit level of ≥10 (IgG index ≥1.0) were considered positive.

Immune Profiling

To assess whether active CMV was associated with differential immune responses, we performed a secondary analysis of previously measured serum and CSF cytokines [22]. First, we analyzed 19 measured cytokines and chemokines (see Supplementary Table 2) using Luminex magnetic bead technology (Bio-Rad Laboratories, Hercules, CA), according to the manufacturers' protocols.

Second, we assessed whether cryptococcal antigen–specific immune responses differed among participants by their CMV status. We created a novel, whole-blood interferon-gamma release assay stimulating against: (1) cryptococcal

glucuronoxylomannan polysaccharide (ie, "cryptococcal antigen"); (2) cryptococcal mannoprotein; and (3) phosphate-buffered saline negative control. For antigen-stimulation assays, we calculated the net cytokine production (antigen to phosphate-buffered saline negative control stimulation). Refer to the Supplementary Methods for assay details. Assessing the immune response was an exploratory, post hoc analysis to determine whether CMV was potentially affecting cryptococcal-specific immune responses.

Statistical Analysis

For the purposes of this study, someone with CMV viremia was defined as anyone with detectable CMV DNA in plasma. A subset of participants had paired samples from different time points tested for the longitudinal concordance of CMV DNA in plasma. Persons with multiple samples were counted as a single negative result if both samples were negative for CMV DNA. Conversely, if both were positive, the CMV viral load was averaged between the 2 samples and counted as a single positive result. There were 4 subjects missing baseline CSF white cell count values and 1 missing a baseline hemoglobin value, so the next available value was used (within ~3 days of baseline).

Baseline characteristics were compared by CMV viremia using the Chi-square test for proportions, the *t*-test for normally distributed data, or the Mann-Whitney U test for non-normally distributed data. The primary outcome in our analysis was 10-week survival. Survival was calculated in days from the date of diagnosis, until either death or 10 weeks. No participants were right-hand censored. A Kaplan-Meier plot with a log rank test compared the 10-week mortality rates by CMV status. Subjects were additionally stratified by CMV viral load as either low (<1000 IU/mL), high (≥1000 IU/mL), or no CMV, to assess whether the magnitude of viremia impacted mortality. Univariate Cox proportional hazards models evaluated the risk factors for 10-week mortality. A fully adjusted Cox model was used to control for confounders of age, sex, CD4 count, Glasgow coma scale score, hemoglobin, CSF white cells, and trial randomization arm. Hazard ratios (HRs), 95% confidence intervals (CIs), and *P* values were reported for each model. Serum, CSF, and antigen-stimulation cytokines were compared by CMV viremia using Wilcoxon rank sum tests. The analysis was performed in SPSS version 23 (IBM, Inc., Armonk, NY) and SAS version 9.4 (SAS Institute, Cary, NC). A *P* value of ≤.05 was considered statistically significant. The cytokines were studied as post hoc analyses that were exploratory for an immune signature as an explanation for differential clinical outcomes in participants with CMV viremia. No formal adjustments were made for multiple testing in the analyses of serum or CSF biomarkers or of antigen-stimulation assays; thus, readers may consider a more extreme *P* value of <.01 to be statistically significant.

RESULTS

We enrolled 177 participants with cryptococcal meningitis in the COAT trial, and we performed CMV DNA quantification on available stored plasma in 111 participants. This study population was comprised of 54% men and 46% women. The mean age was 35 years (standard deviation ±7.8). The median $CD4^+$ T cell count was 19 cells/μL (interquartile range [IQR] 9–70). The mean HIV RNA plasma level was 5.38 $\log_{10}$ copies/mL (standard deviation ±0.55). The plasma samples tested for CMV DNA were taken a median of 6 days (IQR 0–11) from cryptococcal meningitis diagnosis. Baseline characteristics stratified by CMV status are shown in Table 1. CMV viremia was present in 52% (58/111) of subjects. CMV IgG serology was positive on all 109 participants who had sufficient plasma for testing. The median plasma CMV viral load was 498 IU/mL (IQR 259–2390) in those with viremia.

The mortality rates at 10 weeks were 40% (23/58) in those with CMV viremia and 21% (11/53) in those without CMV viremia (P = .03). Persons with CMV viremia had an approximately 2-fold higher risk of death (HR 2.19, 95% CI 1.07–4.49; P = .033; Figure 1). The hazard increased to over 3-fold and remained significant after adjusting for age, sex, CD4 count, Glasgow coma scale score, hemoglobin, CSF white cells, and trial randomization arm (adjusted HR 3.25, 95% CI 1.49–7.10; P = .003; Table 2). This mortality difference continued through 26 weeks, with mortality rates of 41% (24/58) in those with CMV viremia versus 30% (16/53) in those without CMV viremia (adjusted HR 2.18, 95% CI 1.09–4.37; P = .003). Thus, persons with CMV viremia had a 2-fold higher overall mortality rate and, when adjusted for potential confounders of cryptococcal or HIV severity, those with CMV viremia had a 3.3-fold higher risk of mortality at 10 weeks.

Table 1. Baseline Characteristics by Cytomegalovirus Viremia Status

Variable	No CMV Viremia	CMV Viremia	*P* Value
Number per group	53 (48%)	58 (52%)	…
Deferred HIV therapy	22 (42%)	31 (53%)	.21
Women	28 (53%)	23 (40%)	.16
Age, years	37 [±8]	34 [±7]	.06
CD4 count, cells/μL	18 (10–75)	20 (9–52)	.53
HIV plasma RNA, $\log_{10}$ copies/mL	5.40 (5.13–5.74)	5.45 (5.19–5.78)	.59
CMV plasma DNA, IU/mL	…	498 (259–2390)	…
Glasgow coma scale score <15	14 (26%)	16 (28%)	.89
Hemoglobin, g/dL	11.4 [±2.8]	10.6 [±2.5]	.09
CSF white cells <5 cells/μL	13 (25%)	23 (40%)	.09
C-reactive protein, mg/L	47.0 (8.7–80.2)	35.3 (18.5–76.0)	.79

Data are shown as *n* (%), mean [±SD], and median (IQR), with *P* values derived from the Chi-square test, *t*-test, or Mann-Whitney U test, respectively. Abbreviations: CMV, cytomegalovirus; CSF, cerebrospinal fluid; HIV, human immunodeficiency virus; IQR, interquartile range; SD, standard deviation.

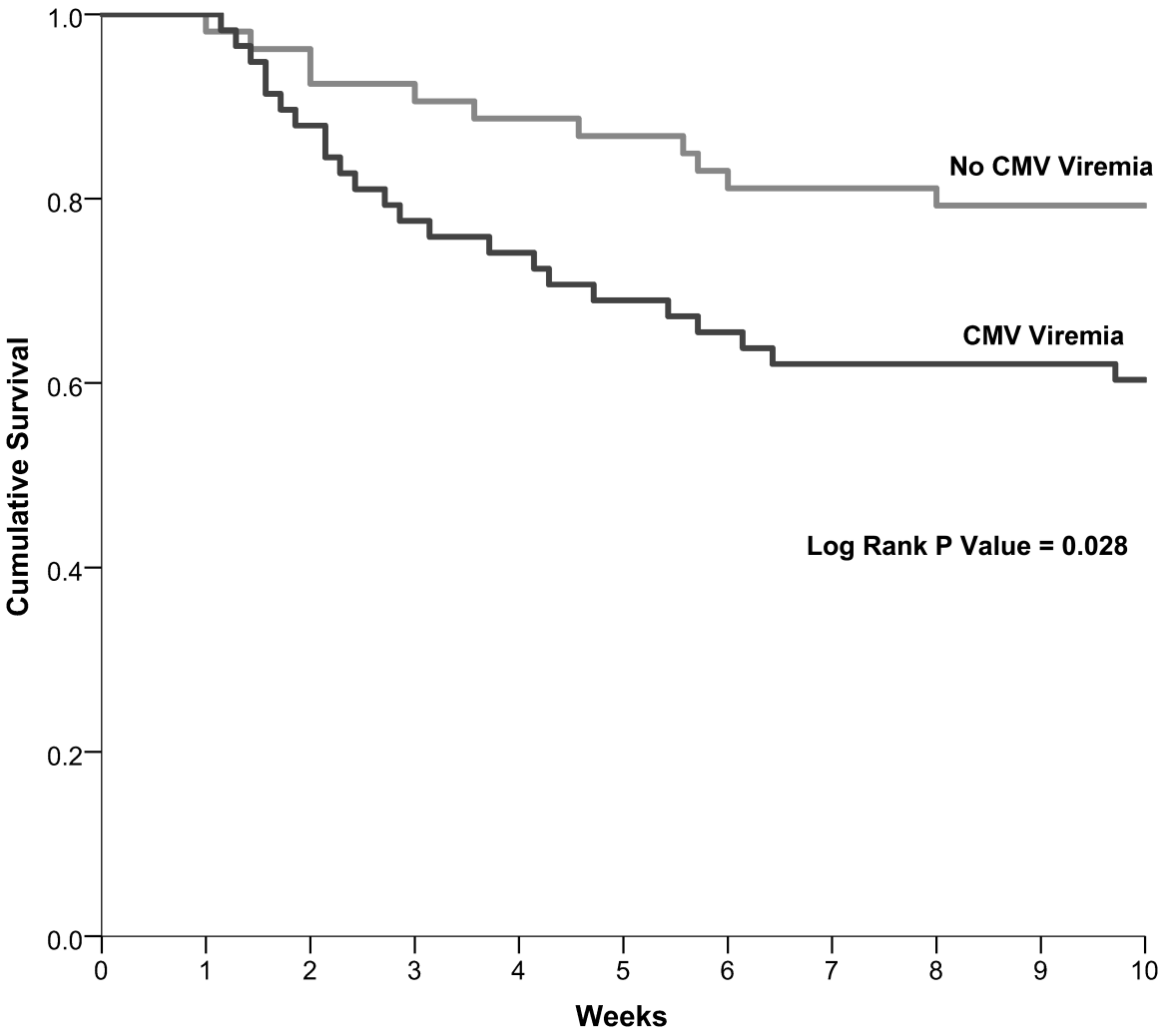

Figure 1. Survival at 10 weeks by cytomegalovirus (CMV) viremia status after human immunodeficiency virus-associated cryptococcal meningitis in Kaplan-Meier model. Mortality by subgroup was as follows: no CMV viremia and early antiretroviral therapy (ART) (23%, 7/31); no CMV viremia and deferred ART (19%, 4/22); CMV viremia and early ART (56%, 15/27); and CMV viremia and deferred ART (26%, 8/31). Abbreviation: CMV, cytomegalovirus.

To test whether mortality was associated with the magnitude of CMV viremia, we stratified CMV viral loads into low (<1000 IU/mL) and high (≥1000 IU/mL) groups. Of the 58 participants with CMV viremia, 37 had low CMV viral loads (median 295 IU/mL, IQR 198–427) and 21 had high CMV viral loads (median 3520 IU/mL, IQR 2210–5240). Baseline characteristics were similar between the groups with low and high viral loads (Supplementary Table 1). While both groups with

Table 2. Hazard Ratios for 10-Week Mortality From Cox Regression Model

	Univariate			Multivariate		
Variable	Hazard Ratio	95% CI	*P* Value	Hazard Ratio	95% CI	*P* Value
CMV viremia	2.19	1.07–4.49	**.033**	3.25	1.49–7.10	**.003**
Women	2.15	1.08–4.29	.03	2.66	1.25–5.62	.01
Age, per year	1.01	.97–1.05	.71	1.04	.99–1.09	.12
CD4 count, per 10 cells/μL	1.00	.97–1.05	.70	1.00	.99–1.01	.97
Glasgow coma scale <15	1.71	.84–3.45	.14	2.07	.97–4.41	.06
Hemoglobin, per g/dL	0.90	.79–1.02	.10	0.93	.80–1.09	.38
CSF white cells <5 cells/μL	0.75	.35–1.62	.47	0.76	.33–1.73	.51
Early HIV therapy trial arm	1.90	.94–3.83	.07	2.50	1.21–5.81	.01

Bold values indicate to highlight the primary variable of interest in context of outcome.
Abbreviations: CI, confidence interval; CMV, cytomegalovirus; CSF, cerebrospinal fluid; HIV, human immunodeficiency virus.

CMV viremia had lower survival rates than the CMV-negative group, the mortality rates did not significantly differ between those with low CMV viral loads (43%, 16/37) and those with high CMV viral loads (33%, 7/21; $P = .46$). CMV-viremic participants developed paradoxical immune reconstitution inflammatory syndrome at similar rates (21%, 11/53) as non-viremic participants (17%, 10/58; $P = .64$). The 10-week mortality rate was higher in the CMV-viremic participants receiving early ART (56%, 15/27), compared to in those who deferred ART (26%, 8/31; $P = .02$), consistent with the overall COAT trial results. Therefore, the randomization arm was included our multivariate analysis.

We assessed for risk factors associated with CMV viremia. The median $CD4^+$ T cell count did not differ between 20 cells/μL (IQR 9–52) in those with CMV viremia versus 18 cells/μL (IQR 10–75) in those without CMV viremia ($P = .53$). Age, sex, HIV RNA plasma level, and trial arm randomization data did not differ between groups with and without CMV viremia, nor did baseline Glasgow Coma Scale scores, hemoglobin levels, or CSF white cell counts (Table 1).

In assessing the dynamics of detectable CMV DNA in plasma, 9 participants had two different, blinded samples tested for longitudinal concordance ($n = 18$ samples). Baseline samples and the subsequent samples were taken a median of 7 days (IQR 5–17) apart. There were 7 subjects who had qualitative concordance of detectable CMV DNA. There were 2 subjects who had no CMV detected in their first sample, but had measurable CMV DNA in their second sample (7 and 8 days later); both subjects were considered CMV positive for our study.

To determine whether CMV viremia was associated with an impaired Th1 response or was a surrogate marker of more severe global immune dysfunction, we assessed the cytokines present in the serum and CSF of 104 participants. We found that 2-fold higher median serum interleukin (IL)-12 (a Th1-related cytokine, protective in cryptococcosis; $P = .035$) and 35% higher median IL-13 (a Th2-related cytokine, nonprotective in cryptococcosis; $P = .028$) measurements were both positively associated with CMV viremia. In the CSF, CMV viremia was marginally associated with a 25% higher median granulocyte macrophage colony stimulating factor (GM-CSF) level, a 35% increase in macrophage inflammatory protein-1 beta (MIP-1β) (CCL4), and a 2-fold increase in monocyte chemoattractant protein-1 (MCP-1) (CCL2; all P values $< .05$) (Supplementary Table 2). Antigen stimulation responses to cryptococcal glucuronoxylomannan or mannoprotein did not differ by CMV status (Supplementary Table 3). These post hoc exploratory analyses revealed some marginal statistical associations with components of the host immune response, but did not reveal markedly impaired Th1 responses or significant immune signature differences among participants with CMV viremia.

DISCUSSION

Our results demonstrate that active CMV replication with viremia is associated with an over 2-fold mortality increase by 10 weeks in severely immunocompromised persons living with HIV who have cryptococcal meningitis. This is a novel finding that supports CMV viremia as a detrimental biomarker in persons with cryptococcal meningitis, irrespective of the presence or absence of end-organ CMV disease. and when combined with similarly observed trends by Ward et al in persons with HIV-associated TB [16], our results posit that CMV may be a risk factor for death in persons with advanced HIV disease. The increased risk for mortality remained significant when adjusting for variables previously shown to be associated with death in cryptococcal meningitis, such as baseline altered mental status and anemia [23–25]. Compared to the cohort living with HIV and with tuberculosis in Ward et al [16], our cryptococcal meningitis cohort had a lower median $CD4^+$ T cell count (64 vs 19, respectively cells/μL) and higher prevalence of CMV viremia (31% vs 52%, respectively). We hypothesize that CMV replication could influence AIDS-related mortality among the severely immunocompromised, and that $CD4^+$ T cell counts >100 cells/μL likely attenuate CMV effects. Interestingly, mortality rates did not correlate with the magnitude of CMV viremia, suggesting the absence of a dose-response relationship. This raises the fundamental question of whether CMV is solely a marker of immune dysfunction or whether CMV contributes (directly or indirectly) to mortality in persons with advanced HIV and concomitant opportunistic infections.

It has been hypothesized that CMV exerts detrimental effects without causing end-organ disease by modulating the host immune system. For example, an increased risk of kidney graft rejection in the setting of active CMV replication is thought to be instigated by immune dysregulation [26]. Interestingly, CMV has been linked to the development of invasive fungal infections in solid organ transplant recipients [27–29]. Invasive fungal infections, including *Cryptococcus*, often occur in the setting of T-cell depletion or dysfunction. $CD4^+$ helper T cells play a critical role in mediating the adaptive immune response to *Cryptococcus*, which is highly predicated on helper T cell polarization to either a Th1 or Th2 phenotype. Studies in mice examining *Cryptococcus*-specific $CD4^+$ T cell polarization demonstrated that Th1-mediated responses are protective and are associated with improved *Cryptococcus* clearance from the lung, whereas Th2-mediated responses are associated with decreased yeast clearance from the CSF and with poorer overall outcomes [30–33].

We assessed cytokine profiles in blood and CSF to explore whether CMV exerted a notable pattern of altered immune response. We did not find any marked differences in baseline immune responses, as assessed by measurements of soluble cytokine or antigen-specific responses. Our results identified

some marginal statistical associations with higher levels of both Th1 (IL-12) and Th2 (IL-13) cytokines in the serum of participants with CMV viremia. Measuring the cytokine milieu in persons with AIDS can be problematic, in that multiple infections or immune processes could be contributing to dysregulation. In particular, tuberculosis coinfection is present in about 15% of first-episode cryptococcal meningitis cases in Uganda [19] and likely influences the cytokine profile. We also assessed the immune responses in the CSF, where CMV viremia was only marginally associated with increases in the chemokines MCP-1 (CCL2) and MIP-1β (CCL4) and the glycoprotein GM-CSF. To better assess *Cryptococcus*-specific immune responses, we developed a whole-blood antigen-stimulation assay. When we stimulated with *Cryptococcus*-specific antigens, we noted no differences in antigen-specific cytokine responses in CMV viremic versus nonviremic participants. Based on our study results, it is unlikely that the increased mortality conferred by active CMV replication is mediated by any attenuating effect of CMV on protective anti-cryptococcal Th1 responses.

CMV has been associated with increased mortality rates in numerous other clinical settings, including systemic lupus erythematosis [34], head and neck cancers [35], and solid organ transplant recipients [36, 37]. Treating symptomatic CMV disease decreases the mortality rate in solid organ transplants patients [38, 39]. CMV prevention in transplant patients demonstrates a survival benefit—using either prophylaxis or preemptive therapy—where treatment is initiated based on the blood CMV DNA concentration [40]. These observations raise the question of whether CMV treatment or prophylaxis has a role in advanced AIDS. We found that half of our severely immunosuppressed participants had active CMV replication and viremia. The high prevalence of CMV viremia, combined with the observed large effect size in mortality, provides a rationale supporting the consideration of a randomized, clinical trial testing CMV antivirals in persons with advanced HIV disease and opportunistic coinfections. The availability of new drugs, such as letermovir, with oral, less toxic formulations further bolsters the feasibility of a clinical trial [41].

Our study had several limitations. First, this study was a retrospective analysis of a randomized, clinical trial in which mortality rates differed by trial arm; however, the mortality association with CMV was present with and without an adjustment for trial arm. Second, we only determined CMV viremia at one given point in time (baseline sample). Future studies should measure CMV plasma DNA across multiple longitudinal time points to better evaluate the kinetics, persistence, and magnitude of viremia over time. Third, our study was not designed to identify and characterize CMV end-organ disease, so we were unable to stratify the differential risks between CMV viremia and confirmed CMV end-organ disease. Lastly, the generalizability of our findings to all persons with AIDS remains unclear, though persons with cryptococcosis and tuberculosis are important subsets in themselves.

In summary, our study demonstrated that the presence of CMV viremia is associated with an over 2-fold greater risk of death in persons with cryptococcal meningitis and, when these data were adjusted for known risk factors, the mortality hazard increased 3.3-fold. The etiology behind the increased risk of death is unclear, but these effects are unlikely to be mediated by attenuation of a beneficial helper T cell response. The lack of a dose-response relationship between the magnitude of CMV viremia and mortality is notable, and perhaps argues for CMV replication as a sensitive marker of severe immune dysfunction, rather than playing a causative role in the increased rate of death. Ultimately, a randomized, clinical trial is required to determine whether treatment of CMV is a modifiable risk factor contributing to mortality in persons with advanced AIDS.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. The authors thank the study team and hospital staff working continuously to provide compassionate care to the participants and patients at Mulago National Referral Hospital.

Financial support. This work was supported by the National Institute of Allergy and Infectious Diseases (grant numbers T32AI055433, U01AI089244, and K23AI138851); the Eunice Kennedy Shriver National Institute of Child Health and Human Development [grant number R01HD079918]); the National Institute of Neurologic Disorders and Stroke (grant number R01NS086312); the Fogarty International Center (grant numbers K01TW010268 and K43TW010718); and a combined National Institute of Neurologic Disorders and Stroke and Fogarty International Center award (grant number D43TW009345) via the Northern Pacific Global Health Fellows Program.

Potential conflicts of interest. G. M. has received grants from the Wellcome Trust and South African Government. M. R. S. has received consulting fees from Sanofi Vaccines and GSK Vaccines, outside the submitted work. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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