Cytomegalovirus (CMV) shedding is highly correlated with markers of immunosuppression in CMV-seropositive women

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Cytomegalovirus (CMV) enters latency following primary infection and can subsequently reactivate. Reinfection with a different viral strain can also occur. During these events, CMV is shed in bodily fluids. This study examined correlates of CMV shedding in specimens obtained from the HIV Epidemiology Research Study, a multicenter cohort study of US women with or at high risk for human immunodeficiency virus (HIV) infection. Among the women studied, 91.4 % (911/997) were CMV IgG seropositive. Of these women, 2.7 % (25/911) were CMV IgM seropositive. CMV DNA was detected via real-time PCR more frequently in cervicovaginal lavage (CVL) specimens (55/764, 7.2 %) than in peripheral blood mononuclear cells (PBMCs) (26/897, 2.9 %). CMV viral loads in 1 ml CVL (median 534; mean 2598; range=40–74 844) were higher than in 10⁶ PBMCs (median 264; mean 1287; range=35–13 250). CMV DNA in PBMCs was associated with HIV seropositivity [odds ratio (OR) 13.5; 95 % confidence interval (CI) 1.8–100], increasing HIV viral load ($P<0.001$ for trend), decreasing CD4 cell counts ($P<0.001$ for trend) and CMV DNA in CVL (OR 26; 95 % CI 10.7–64). CMV DNA in CVL specimens was associated with CMV IgM seropositivity (OR 4.3; 95 % CI 1.5–12.3), HIV seropositivity (OR 7.3; 95 % CI 2.6–20), increasing HIV viral load ($P<0.001$ for trend) and decreasing CD4 cell counts ($P<0.001$ for trend). The positive predictive value of CMV IgM seropositivity for CMV DNA shedding in either PBMCs or CVL was 20 %. In summary, CMV shedding in CVL and PBMCs was highly correlated with each other and with markers of immune suppression.

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INTRODUCTION

Cytomegalovirus (CMV) is a herpesvirus that generally does not cause disease in immunocompetent individuals

Abbreviations: CI, confidence interval; CMV, cytomegalovirus; CVL, cervicovaginal lavage; HCV, hepatitis C virus; HERS, HIV Epidemiology Research Study; HIV, human immunodeficiency virus; HSV, herpes simplex virus; OR, odds ratio; PBMC, peripheral blood mononuclear cell.

but is responsible for substantial morbidity and mortality among human immunodeficiency virus (HIV)-infected persons (Emery *et al.*, 1999), transplant recipients (Cope *et al.*, 1997) and congenitally infected children (Cannon & Davis, 2005). Compared with CMV infection in HIV-infected individuals and transplant recipients, less progress has been made in the prevention of congenital CMV disease (de Jong *et al.*, 1998; Read *et al.*, 2008), despite a

huge physical, emotional and financial burden (Stratton *et al.*, 2000). Specifically, 30 000–40 000 US children are born with congenital CMV infection annually, causing at least 150 deaths and leaving more than 5500 children with disabilities such as hearing or vision loss, or cognitive disability (i.e. mental retardation) (Dollard *et al.*, 2007; Grosse *et al.*, 2008; Kenneson & Cannon, 2007).

Like other herpesviruses, CMV enters latency following primary infection but can subsequently reactivate (Mocarski *et al.*, 2007). Active virus replication is characteristic of primary infection, reactivation and reinfection with a different CMV strain, but such replication is suppressed during latency. Among pregnant women, virus replication in uterine and placental compartments is probably necessary for fetal infection to occur (Pereira *et al.*, 2005). Because virus replication in these internal compartments is not easily measured, it is important to identify a useful surrogate marker. One potential marker is virus replication in peripheral bodily fluids, such as blood or cervicovaginal secretions, commonly referred to as 'shedding'.

However, the occurrence of CMV shedding in bodily fluids is poorly understood. In particular, it is important to gain a better understanding of the frequency of CMV shedding in adults, the likelihood of shedding in different bodily fluids, the viral loads in these fluids and the factors associated with shedding. The purpose of this study was to address these questions in a large cohort of women of reproductive age.

METHODS

Study design and population. Participants were enrolled in the HIV Epidemiology Research Study (HERS), a multicenter cohort study of women with or at high risk of HIV infection (Smith *et al.*, 1997; Stover *et al.*, 2003). Recruitment, screening and enrolment for the HERS took place from April 1993 to January 1995. Follow-up visits took place at 6 month intervals. Visits included an extensive interview, a general physical and pelvic examination, and biological specimen collection. For this study, participant visits were stratified on HIV status and CD4 cell count and were selected randomly, one visit per participant, with the sample enriched by a small group of participant visits from women who were pregnant. A total of 997 participant visits were included. Stored serum, peripheral blood mononuclear cells (PBMCs) and cervicovaginal lavage (CVL) specimens were retrieved subject to availability. Informed consent was obtained from all participants and the study was approved by the institutional review boards from each of the authors' institutions.

CMV laboratory methods. All specimens were stored by the HERS repository at -80°C until transport to the testing laboratory in 2005. Specimens were thawed once, tested for other purposes, refrozen and stored at -20°C for 4–6 months, and then tested for CMV DNA for the current study. CMV IgG and IgM antibodies were detected in serum using a VIDAS automated immunoassay test (BioMérieux). Total CMV DNA was extracted from PBMCs and unfractionated CVL specimens using a MagNAPure automated extractor (Roche). CMV PCR used Taqman reagents (Applied Biosystems) and targeted the viral glycoprotein B gene (Boppana *et al.*, 2005). Results were interpreted as described by Kharrazi *et al.*

(2010). Specimens were tested in duplicate, and cycle thresholds of ≤ 40 for both reactions were required to report a specimen as positive.

Statistical analysis. Among CMV IgG-seropositive women, univariate risk factors were examined for CMV DNA shedding in PBMCs and CVL. The variables selected included CMV IgM, demographic variables, variables associated with immunosuppression, and markers of active infection (chlamydia, bacterial vaginosis, syphilis and gonorrhoea) or seropositivity [herpes simplex virus type 2 (HSV-2) and hepatitis C virus (HCV)]. In multivariate analyses, base models included all variables that were associated with CMV shedding in univariate analyses with $P < 0.10$. CMV IgM was included in all models because of its a priori importance. IgM antibody is often used as a marker for active infection because it is present briefly during primary infection and is believed to be generated again by reinfection with a new CMV strain. Due to strong correlation between CD4 count and HIV viral load, these two variables were not included simultaneously in the same model. Each of the other variables was added individually and retained only if it had a $P < 0.10$ or if $\ln(\text{OR}_{\text{without variable of interest}}/\text{OR}_{\text{with variable of interest}})$ was greater than 10% for all levels of any base variable ($\text{OR} = \text{odds ratio}$). Given the small cell sizes, effect measure modification was not assessed. All analyses were performed using SAS v9.2 (SAS Institute).

RESULTS

The 997 women in this study were evenly distributed among the study cities and ranged in age from 17 to 60 years (median 36 years). The majority of the women were black (61.0%), followed by white (19.9%) and Hispanic (17.9%). After oversampling for pregnancy visits, 8.8% of the women were pregnant at the time of their visit. Nearly one in five women had used injection drugs in the 6 months prior to their visit or since their previous visit. The most common infections among the women were HIV (66.2%), HCV (55.9%), HSV-1 (76.0%) and HSV-2 (66.1%). Less common infections included bacterial vaginosis (40.0%), syphilis (10.7%), chlamydia (4.2%) and gonorrhoea (<1%).

CMV IgG antibody was detected in 91.4% (911/997) of the study subjects. Of these 911 women, 2.7% (25/911) were CMV IgM seropositive (Tables 1 and 2). The positive predictive value of CMV IgM seropositivity for CMV shedding in PBMCs or CVL was 20% (5/25). CMV DNA was detected more frequently in CVL specimens (55/764, 7.2%) than in PBMCs (26/897, 2.9%). CMV viral loads in blood were 35–13 250 copies of viral DNA (median 264; mean 1287) per 10^6 PBMCs. CMV viral loads in CVL specimens were 40–74 844 copies (median 534; mean 2598) ml^{-1} .

In univariate analyses (Tables 1 and 2), the presence of CMV DNA in PBMCs was most strongly associated with the presence of CMV DNA in CVL, HIV seropositivity, higher HIV viral load and lower CD4 count. The presence of CMV DNA in PBMCs was also associated with having sex with a male in the past 6 months. The presence of CMV DNA in CVL was most strongly associated with the presence of CMV DNA in PBMCs, HIV seropositivity, higher HIV viral load, lower CD4 count, CMV IgM

Table 1. Univariate analyses of CMV DNA in PBMC specimens in CMV IgG-seropositive women

Data were not available for all women for all variables.

Variable	No. of women	CMV DNA in PBMCs [n (%)]	OR (95 % CI)	P value
CMV DNA in CVL				
No	695	9 (1.3)	1	
Yes	55	14 (25.5)	26 (10.7–64)	<0.001
CMV IgM				
Seronegative	872	24 (2.8)	1	
Seropositive	25	2 (8.0)	3.1 (0.7–13.8)	0.12
Age at visit				
≥42 years	222	5 (2.3)	1	
37–41 years	217	7 (3.2)	1.5 (0.5–4.6)	0.53
32–36 years	247	10 (4.0)	1.8 (0.6–5.4)	0.27
<32 years	211	4 (1.9)	0.8 (0.2–3.2)	0.80
Pregnancy status				
Not pregnant	811	25 (3.1)	1	
Pregnant	80	1 (1.3)	0.4 (0.05–3.0)	0.35
HIV status				
Seronegative	306	1 (0.3)	1	
Seropositive	591	25 (4.2)	13.5 (1.8–100)	0.001
HIV viral load				
0 (HIV negative)	306	1 (0.3)	1	
<1000	154	1 (0.6)	2.0 (0.1–32)	
1000–10 000	175	3 (1.7)	5.3 (0.6–52)	
≥10 000	195	18 (9.2)	31 (4.1–234)	<0.001*
CD4 cell count				
≥500	485	2 (0.4)	1	
200–499	231	3 (1.3)	3.2 (0.5–19.1)	
51–199	134	10 (7.5)	19.5 (4.2–90)	
≤50	47	11 (23.4)	74 (15.8–345)	<0.001*
Sex with male in past 6 months				
No	219	12 (5.5)	1	
Yes	645	13 (2.0)	0.4 (0.2–0.8)	0.008
Injection drug use†				
No	720	22 (3.1)	1	
Yes	165	4 (2.4)	0.79 (0.27–2.32)	0.67
Chlamydia‡				
Negative	684	20 (2.9)	1	
Positive	30	0 (0)	– (–)	–
Bacterial vaginosis‡				
No	527	17 (3.2)	1	
Yes	350	9 (2.6)	0.8 (0.3–1.8)	0.58
HSV-2‡				
Seronegative	297	12 (4.0)	1	
Seropositive	578	14 (2.4)	0.6 (0.3–1.3)	0.18
HCV‡				
Seronegative	374	12 (3.2)	1	
Seropositive	471	12 (2.5)	0.8 (0.4–1.8)	0.57

*Test for linear trend.

†Injected drugs included speedball, cocaine, heroin, amphetamines and other opiates injected since the last visit or within the previous 6 months.

‡Result from enrolment visit.

Table 2. Univariate analyses of CMV DNA in CVL specimens in CMV IgG-seropositive women

Data were not available for all women for all variables.

Variable	No. of women	CMV DNA in CVL [<i>n</i> (%)]	OR (95 % CI)	<i>P</i> value
CMV DNA in PBMCs				
No	727	41 (5.6)	1	
Yes	23	14 (60.9)	26 (10.7–64)	<0.001
CMV IgM				
Seronegative	743	50 (6.7)	1	
Seropositive	21	5 (23.8)	4.3 (1.5–12.3)	0.003
Age at visit				
≥42 years	189	7 (3.7)	1	
37–41 years	189	15 (7.9)	2.2 (0.9–5.6)	0.08
32–36 years	209	20 (9.6)	2.8 (1.1–6.7)	0.02
<32 years	177	13 (7.3)	2.1 (0.8–5.3)	0.12
Pregnancy status				
Not pregnant	696	50 (7.2)	1	
Pregnant	67	5 (7.5)	1.0 (0.4–2.7)	0.93
HIV status				
Seronegative	262	4 (1.5)	1	
Seropositive	502	51 (10.2)	7.3 (2.6–20)	<0.001
HIV viral load				
0 (HIV negative)	262	4 (1.5)	1	
<1000	127	7 (5.5)	3.8 (1.1–13.1)	
1000–10 000	144	12 (8.3)	5.9 (1.9–18.5)	
≥10 000	175	31 (17.7)	13.9 (4.8–40)	<0.001*
CD4 cell count				
≥500	409	13 (3.2)	1	
200–499	193	5 (2.6)	0.8 (0.3–2.3)	
51–199	121	20 (16.5)	6.0 (2.9–12.5)	
≤50	41	17 (41.2)	22 (9.4–50)	<0.001*
Sex with male in past 6 months				
No	182	17 (9.3)	1	
Yes	554	36 (6.5)	0.7 (0.4–1.2)	0.20
Injection drug use†				
No	609	49 (8.0)	1	
Yes	146	6 (4.1)	0.49 (0.21–1.17)	0.11
Chlamydia‡				
Negative	578	38 (6.6)	1	
Positive	27	5 (18.5)	3.2 (1.2–9.0)	0.02
Bacterial vaginosis‡				
No	459	34 (7.4)	1	
Yes	299	20 (6.7)	0.9 (0.5–1.6)	0.71
HSV-2‡				
Seronegative	247	16 (6.5)	1	
Seropositive	498	36 (7.2)	1.1 (0.6–2.1)	0.71
HCV‡				
Seronegative	311	28 (9.0)	1	
Seropositive	406	20 (4.9)	0.5 (0.3–1.0)	0.03

*Test for linear trend.

†Injected drugs included speedball, cocaine, heroin, amphetamines and other opiates injected since the last visit or within the previous 6 months.

‡Result from enrolment visit.

seropositivity, a younger age, the presence of chlamydia and the presence of HCV antibody. For both PBMCs and CVL, the association of CMV shedding with increasing

HIV viral load and decreasing CD4 cell count had a strong biological gradient ($P<0.001$ for trend in all cases). Women who were missing PBMCs or CVL were similar

to those who had available PBMCs or CVL with regard to HIV serostatus, HIV viral load and CD4 count (data not shown).

In multivariate analyses (models not shown), the strongest risk factors ($P < 0.001$ for trend) for CMV shedding were the markers of immunosuppression (i.e. low CD4 count and high HIV viral load). For CMV in PBMCs, shedding was significantly less likely if the woman was HSV-2 seropositive [OR=0.4; 95 % confidence interval (CI) 0.2–0.9]. For CMV in CVL, shedding was significantly more likely if CMV IgM (OR=4.9; 95 % CI 1.1–22) or chlamydia (OR=4.8; 95 % CI 1.4–16.5) were present. However, due to the small numbers in some variable categories, CIs tended to be very wide for all variables in the multivariate analyses.

DISCUSSION

In this, one of the largest studies to examine risk factors for CMV DNA shedding, shedding in CVL or PBMCs was found to occur in a low proportion of women. When CMV shedding did occur, it was more frequent in CVL than in PBMCs, although shedding in the two specimen types was highly correlated. Viral loads were also higher in CVL than in PBMCs. However, viral loads were relatively low for both specimens, suggesting some degree of control by the immune system. It is not known why CMV shedding occurs more frequently in some specimen types than in others. Previous studies of adults have found that CMV shedding is usually more frequent in genital secretions or urine than in blood (Kaye *et al.*, 2008; Novak *et al.*, 2008; Spano *et al.*, 2004; Zanghellini *et al.*, 1999).

The prevalence of CMV shedding in CVL in this study was at the lower end of the range compared with other studies, where estimates typically ranged from ~5 to ≥ 30 % (Bello & Whittle, 1991; Biri *et al.*, 2006; Broccolo *et al.*, 2008; Chandler *et al.*, 1985; Clarke *et al.*, 1996; Collier *et al.*, 1995; Demmler *et al.*, 1987; Kaye *et al.*, 2008; Numazaki *et al.*, 1970; Pass *et al.*, 1982; Shen *et al.*, 1993a, c; Spano *et al.*, 2004; Stagno *et al.*, 1975; Tanaka *et al.*, 2006; Zanghellini *et al.*, 1999). The high variability in CMV shedding in CVL across these studies may be due in part to differences in geography or the type of population studied, with many of the highest estimates coming from populations enrolled at sexually transmitted disease clinics. Based on some of these previous studies, one might expect a higher prevalence of CMV shedding among a population with HIV or HIV risk factors. However, the enrolment criteria of the HERS included women with moderate sexual activity (five partners in total over 5 years) such that many of the study participants did not have a history of high sexual risk activity and did not have sexually transmitted diseases (Cannon *et al.*, 2001; Smith *et al.*, 1997). Nevertheless, the pattern of CMV shedding in this population should not be presumed to be representative of that which might be found in the general population. An additional limitation of the study was the retrospective design and use of

archived specimens such that it was not possible to conduct tissue culture on clinical specimens to determine the proportion of CMV DNA that was infectious virus.

The relatively low prevalence of CMV DNA positivity we found may have been due in part to the long-term storage of specimens and the single freeze–thaw cycle prior to testing, but we would expect only slight DNA degradation under these storage conditions. Another possible contributor to the lower prevalence may have been the use of an automated DNA extraction method (MagNA Pure; Roche), which was the best available DNA extractor at the time but which has since been replaced by methods providing higher yields of DNA. The lower prevalence may also have been related to testing of CVL specimens rather than cervical swabs, which have shown higher sensitivity for HIV RNA and HSV-2 DNA (Delany *et al.*, 2008).

The strongest risk factors for CMV shedding were markers of immunosuppression such as HIV seropositivity, high HIV viral load and low CD4 cell count. These associations showed a strong biological gradient, with shedding prevalence substantially increasing as HIV viral load increased and CD4 cell count decreased. These findings emphasize the fundamental role of immune control in maintaining CMV latency and represent one of the largest-scale *in vivo* demonstrations of such a role. The infrequency of CMV shedding in immunocompetent adults suggests that adult-to-adult transmission may be much more frequent when the source adult is immunocompromised. Alternatively, CMV transmission may be driven primarily by recently infected adults whose immune system has not yet brought the virus under control (Coonrod *et al.*, 1998; Natali *et al.*, 1997; Revello *et al.*, 1998; Zhang *et al.*, 2006).

Although the pregnant women in this study did not have an elevated risk of CMV shedding, the overall frequencies of shedding and pregnancy were too low to assess this risk adequately. Other studies have found that CMV shedding increases with advancing gestational age (Shen *et al.*, 1993b; Stagno *et al.*, 1975), but no studies have shown whether pregnancy itself is a risk factor for shedding. Presumably the altered immune response during pregnancy could lead to lack of immune control and subsequent reactivation of CMV (Szekeres-Bartho, 2002). Such increased risk of shedding during pregnancy could be responsible for transmission to the fetus among women who are CMV seropositive prior to conception.

CMV IgM antibody was found in 2.7 % of the CMV IgG-seropositive women. Most women who were IgM positive did not have detectable CMV DNA in their PBMCs or CVL; the positive predictive value of IgM for CMV shedding was only 20 %. Several factors may have contributed to the low correlation of IgM and viral shedding, including the relatively low viral loads seen in this study, intermittent shedding typical of CMV infection (Griffiths *et al.*, 1978; Nankervis *et al.*, 1984; Rosenthal *et al.*, 2009), persistence of IgM after virus replication has

ceased (Kangro *et al.*, 1982) and limitations in sensitivity and specificity of CMV IgM assays (Lazzarotto *et al.*, 1992). Whatever the reason, CMV IgM positivity may be a poor marker for active CMV replication.

Finally, CMV shedding in CVL but not PBMCs was associated with the presence of chlamydia. CMV shedding in CVL was not associated with bacterial vaginosis, in contrast to findings from a smaller study of CMV genital tract shedding (Ross *et al.*, 2005). Nevertheless, various studies have shown that CMV is associated with the presence of genital tract infections, and that interaction among these agents could be occurring, perhaps mediated through low-grade inflammation or other immunomodulatory effects (Chandler *et al.*, 1987; Coonrod *et al.*, 1998; Fowler & Pass, 1991; Pereira *et al.*, 1990; Shen *et al.*, 1994). Such interactions could increase the potential for sexual transmission of infections such as CMV and HIV.

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