Virological and Immunological Correlates of Mother-to-Child Transmission of Cytomegalovirus in The Gambia

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Background. Cytomegalovirus (CMV) is the most common congenital infection and can follow primary and recurrent maternal infection. We studied correlates of vertical transmission of CMV in The Gambia, where most children acquire CMV during the first year of life.

Methods. A cohort of 281 mothers and infants was recruited at birth. Infants were prospectively followed up for CMV infection during the first year of life. Excretion of CMV and antiviral immune response were studied at birth in mothers of children infected in utero, early during infancy, or late during infancy or not infected at 1 year of age.

Results. Congenital infection was diagnosed in 3.9% of newborns, and 85% of children were infected by 1 year. Excretion of CMV in colostrum or in the genital tract was more common in mothers of congenitally (100%) or early infected children (48%) than in mothers of late-infected (20%) or uninfected children (27%). Higher rates of viral excretion were associated with significantly higher levels of serum anti-CMV immunoglobulin G and higher frequencies of CMV-specific CD4+ T cells.

Conclusion. In the context of recurrent maternal infection, transmission of CMV in utero and during early postnatal life is associated with excretion of the virus in colostrum and the genital tract.

Cytomegalovirus (CMV) is the most common congenital infection, with recent estimates of the proportion of pregnancies affected ranging from 0.1% to 5% [1, 2]. Preconceptional immunity provides only partial protection against vertical transmission of CMV, because the virus can be transmitted after both primary and recurrent maternal infections [3–5]. Transmission in utero occurs in ~40% of the cases of primary infections and in 0.2%–5% of the mothers who were immune before pregnancy [2, 6]. Recurrent infections include both reactivation of latent virus and reinfection with a new virus strain, but the relative importance of the two phenomena remains unclear [7]. Mother-to-child transmission of CMV is also common in early postnatal life. Depending on the population, between 10% and 90% of children acquire CMV during their first year of life [6, 8]. Vertical transmission therefore represents an important route of dissemination of the virus in the human population. Approximately 10% of newborns with congenital infection have severe symptoms, and another 10% develop sequelae, including mental retardation and hearing loss. In contrast, peri- or postnatal CMV infection is usually asymptomatic, except in premature infants [8].

Breast-feeding represents the most important route of postnatal infections, with CMV detected in the breast milk of up to 95% of seropositive lactating women [1, 8–10]. This high excretion rate is associated with acquisition of CMV by infants during the first months of life. Genital excretion of CMV is also common and is associated with perinatal infection of newborns [4, 6, 9, 11, 12]. The mechanisms involved in the transmission of CMV in utero remain poorly understood. It is currently believed that transplacental transfer of CMV is a local phenomenon that follows viremia [13]. The development of a vaccine to prevent vertical transmission of CMV during pregnancy will require a better understanding of this phenomenon [14]. This study was un-
DNA was extracted from 200 mothers who were recruited from the larger birth cohort and gave birth to congenitally infected infants. DNA was detected using the method reported by Preiser et al. 

Blood and colostrum were collected from infants (12 weeks). Urinary and colostrum samples were screened by polymerase chain reaction (PCR) for the presence of CMV DNA. Correlates of vertical transmission of CMV were studied in 4 subgroups of mothers defined by the age at which CMV infection was diagnosed in infants: group 1, congenital infection (n = 9); group 2, early infection (5–8 weeks; n = 25); group 3, late infection (17–47 weeks; n = 25); group 4, uninfected at 52 weeks (n = 22). Age ranges for groups 2 and 3 were selected on the basis of the median age at which CMV was detected in infants (12 weeks). Blood and colostrum were collected from an additional 4 mothers who were recruited from the larger birth cohort and gave birth to congenitally infected infants.

**METHODS**

**Study population.** In January 2002, a birth cohort was initiated in the village of Sukuta to study the epidemiology, clinical presentation, virology, and immunology of early CMV infection in an environment of endemcity. The cohort has been described in detail elsewhere [2]. The population included in this study consisted of the first 281 mothers and infants enrolled in the cohort. Urine samples were collected from infants within the first 2 weeks of life, at 2-week intervals to 12 weeks of age, and then at 4-week intervals to 52 weeks of age. Vaginal swabs, colostrum, blood, urine, and saliva were collected from the mothers at or within 2 weeks of birth. All infant urine samples were screened by polymerase chain reaction (PCR) for the presence of CMV DNA. Correlates of vertical transmission of CMV were studied in 4 subgroups of mothers defined by the age at which CMV infection was diagnosed in infants: group 1, congenital infection (n = 9); group 2, early infection (5–8 weeks; n = 25); group 3, late infection (17–47 weeks; n = 25); group 4, uninfected at 52 weeks (n = 22). Age ranges for groups 2 and 3 were selected on the basis of the median age at which CMV was detected in infants (12 weeks). Blood and colostrum were collected from an additional 4 mothers who were recruited from the larger birth cohort and gave birth to congenitally infected infants.

**PCR detection and quantification of CMV DNA.** CMV DNA was detected using the method reported by Preiser et al. [15]. DNA was extracted from 200 μL of urine (Qiagen columns). The first-round PCR contained 1.25 U of Taq polymerase (Bioline), 1.5 mmol/L magnesium chloride, 5 pmol of oligonucleotide primer, and 200 mmol/L for each dNTP. Second-round PCRs were done using the same reaction conditions in a volume of 25 μL, with the input being 1.5 μL of the first-round PCR product. Primer sequences were 5′-GTACTCGCCCTCGTTTTCGGGTC-3′ (outer sense), 5′-GTCTATTGTTGTCGAGTATCCTACAG-3′ (outer antisense), 5′-TCCGAAGGGCATGAGCTCGATGT-3′ (inner sense), and 5′-CGAGTATCCTACGCTACTGGGA-3′ (inner antisense). These primers amplified 323- and 291-bp fragments, respectively, of the UL50 gene of human CMV. Products were analyzed on an ethidium bromide–stained agarose gel. A negative control sample (PBS) was included for every 7 test samples, and positive controls containing 100 and 10 CMV DNA copies were included in each run. The absolute limit of detection was 25 copies/mL.

The CMV DNA loads were quantified by a real-time PCR method. Samples were quantified by comparison with a standard curve generated from a supernatant of AD169-infected fibroblasts. The top standard was quantified by replicate endpoint dilution PCR. Real-time PCR was done in a working volume of 25 μL containing 5 μL of extracted sample and amplified using the QuantiTect Probe PCR (Qiagen) according to the manufacturer’s instructions, the first-round primers described above, and a dual-labeled TaqMan probe (5′-FAM-AACTCACCTACGTTGCGCGCGGCGGCA-BHQ-3′). The absolute limit of detection was 50 copies/mL (100 copies for vaginal swabs and plasma). To control for nonspecific inhibition of the reaction, 2 μL of each sample was added to a control reaction in which 200 DNA copies of the bacteriophage lambda sequence were amplified in a 10-μL reaction (QuantiTect SYBR Green; Qiagen). Inhibitory samples (cycle threshold >3 SDs above the mean for noninhibitory controls) were reextracted and reassayed.

**Serology.** Plasma concentrations of CMV-specific IgG were quantified using the ETI-CYTOK-G PLUS immunoassays (Dia-Sorin), in accordance with the manufacturer’s instructions. Levels of IgA and IgG in colostrum were determined by ELISA and expressed as arbitrary units.

**Cellular immunology.** Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep (Axis-Shield) and were cryopreserved before testing. The phenotype of CD4+ and CD8+ T lymphocytes was studied by flow cytometry using the following conjugated antibodies: CD4 and CD8 peridinin chlorophyll protein (PerCP), CD27 allophycocyanin (APC), CD28 fluorescein isothiocyanate (FITC), CD38 APC, CD45RA FITC, CD45RO APC, and HLA-DR FITC (Becton Dickinson). Intra-cellular Ki-67 was stained using FITC-conjugated antibodies after permeabilization with FACS-Perm 2 (Becton Dickinson). Cytokine production by CD4+ and CD8+ T lymphocytes was assessed by intracytoplasmic staining and flow cytometry. PBMCs were cultured at 105/500 μL in RPMI 1640 with 10% human AB serum (Sigma-Aldrich) and were stimulated for 18 h with CMV antigens (Viruys); a pool of 138 peptides that were 15 aa long, that overlapped by 11 aa, and that covered the entire CMV pp65 protein (Becton Dickinson); staphylococcal enterotoxin B (SEB, 2.5 μg/mL; Sigma-Aldrich); or medium alone. Brefeldin A (10 μg/mL; Sigma-Aldrich) was added for the last 6 h of culture. After stimulation, cells were stained with anti-CD4– or CD8 PerCP–conjugated antibodies, permeabilized, and stained with the following conjugated antibodies: interferon (IFN)–γ FITC, interleukin-2 PE, and tumor necrosis factor–α APC (Becton Dickinson). Samples were analyzed using a FACSCalibur flow cytometer and CellQuest software (version 5; Becton Dickinson). A minimum of 50,000 CD4+ and 20,000 CD8+ T cells were acquired for cytokine production assays, and...
a minimum of 10,000 CD4+ and CD8+ T lymphocytes were acquired for phenotyping experiments.

Statistical analysis. All data were stored in a dedicated Access (Microsoft) database, which was used to generate visit and sampling lists throughout the study. Proportions of mothers with detectable viral loads were compared using a χ² test. Viral loads, IgG titers, and proportions of T lymphocytes producing cytokines or expressing markers of activation or differentiation were compared using the Kruskal-Wallis test. Analysis were performed using Stata/SE for Windows (version 8.0; Stata).

RESULTS

Age of acquisition of CMV infection. The cumulative acquisition of CMV in infants, defined as the detection of CMV DNA in 1 or more urine samples, is shown in figure 1. Congenital infection, defined as the detection of CMV DNA in a urine sample obtained within the first 2 weeks of life, was detected in 11 (3.9%) of 281 infants. The median age at which CMV was first detected in the total population was 12 weeks. The proportion of children remaining uninfected at 12 months, defined as no CMV detection in any urine sample, was 14.6% (37/253 subjects).

Maternal excretion of virus. Table 1 shows the rates of detection of CMV DNA in maternal samples collected at or near birth for the 4 subject groups: group 1, congenital infection; group 2, early postnatal infection; group 3, late postnatal infection; and group 4, uninfected at 12 months. Maternal excretion of CMV in colostrum and the genital tract was significantly associated with age at acquisition of CMV in infants. The highest rates of CMV excretion were observed in mothers of newborns with congenital infection. Intermediate rates of excretion were

![Figure 1](https://example.com/figure1.png)

Figure 1. Cumulative prevalence of cytomegalovirus (CMV) infection with age in a cohort of 258 infants, as determined by first detection of CMV DNA in urine.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Congenital infection (%)</th>
<th>Early infection (%)</th>
<th>Late infection (%)</th>
<th>Uninfected (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaginal swab</td>
<td>9/8 (100)</td>
<td>9/22 (41)</td>
<td>3/24 (12)</td>
<td>4/21 (19)</td>
</tr>
<tr>
<td>Saliva</td>
<td>3/9 (33)</td>
<td>4/21 (19)</td>
<td>4/23 (17)</td>
<td>2/21 (9)</td>
</tr>
<tr>
<td>Urine</td>
<td>1/9 (11)</td>
<td>0/22 (0)</td>
<td>0/21 (0)</td>
<td>0/21 (0)</td>
</tr>
<tr>
<td>Plasma</td>
<td>1/13 (8)</td>
<td>0/23 (0)</td>
<td>0/23 (0)</td>
<td>1/20 (5)</td>
</tr>
</tbody>
</table>

NOTE. The 4 subgroups were defined according to the age at detection of CMV DNA in infants: congenital infection, age ≤2 weeks; early infection, age 5–8 weeks; late infection, age 17–47 weeks; uninfected, no infection detected by age 52 weeks.

*P values determined by χ² test.
observed in mothers of children who acquired CMV early during postnatal life. No association was observed between age at acquisition of CMV in infants and viral excretion in maternal saliva or urine. Only 2 mothers had detectable plasma viral loads. The geometric mean virus loads for those samples in which CMV DNA could be detected are shown in table 2. The highest colostrum viral loads were detected in mothers who transmitted CMV in utero. Similar trends were observed in vaginal swabs and urine but these did not reach statistical significance. The relationship between postnatal transmission of CMV and maternal excretion of the virus was examined by collecting samples from the mothers in groups 2 and 3 at the time infection was detected in the infant. At the time of infection, CMV excretion was detected in the breast milk of 24 of 30 mothers (80%), in the saliva of 12 of 33 mothers (36%), and in the urine of 3 of 32 mothers (9%).

Maternal antibody response to CMV. Anti-CMV specific IgG was detected in all maternal plasma samples tested ($n = 169$). Plasma antibody concentrations were compared in the 4 study groups (figure 2). A significant association was observed between maternal anti-CMV antibody concentrations and age at acquisition of CMV in the infants. Mothers who gave birth to infants infected in utero (group 1) or early during postnatal life (group 2) had significantly higher antibody concentrations than did mothers of infants uninfected at 12 months (group 4). A similar trend was observed for mothers of infants infected late during postnatal life (group 3), but this did not reach statistical significance. No significant association was detected between anti-CMV IgG concentrations in cord blood or colostrum or anti-CMV IgA concentrations in colostrum and viral excretion in the mothers or age at acquisition of the virus in infants (data not shown).

Maternal T cell response to CMV. The cytokine response of maternal CD4$^+$ and CD8$^+$ T lymphocytes to CMV antigens is shown in figure 3. Maternal CD4$^+$ T cell response to CMV was significantly associated with age at acquisition of CMV in infants. Mothers in groups 1 and 2, who gave birth to children infected in utero or early during postnatal life, had higher frequencies of CD4$^+$ T cells producing IFN-$\gamma$ in response to CMV antigens than did mothers in group 4, who gave birth to uninfected children (figure 3A). The frequencies of CD8$^+$ T cells producing IFN-$\gamma$ in response to CMV pp65 peptides were higher in groups 1 and 2, but this difference did not reach statistical significance (figure 3B). To evaluate the specificity of these differences, cells were stimulated with the superantigen SEB. The frequencies of maternal CD4$^+$ T cells producing IFN-$\gamma$ in response to SEB were significantly associated with age at acquisition of CMV in infants, with the highest frequencies observed in mothers in group 2 (figure 3C). A similar but statistically insignificant trend was observed for CD8$^+$ T cell responses (figure 3D).

Activation and differentiation of maternal T lymphocytes. The phenotype of maternal T lymphocytes is shown in figure 4.
A significant association was observed between the expression of some markers of activation by CD4\(^+\)/H11001 and CD8\(^+\)/H11001 T cells and the age at diagnosis of CMV in infants. Higher frequencies of CD8\(^+\)/H11001 T cells expressing Ki-67, a nuclear protein expressed during the entire cell cycle, were detected in mothers of infants infected before the age of 1 year than in mothers of uninfected infants (figure 4B). Higher frequencies of Ki-67\(^+\)/CD4\(^+\)/H11001 T cells were also observed in mothers in groups 1 and 2 (figure 4A). Similar results were obtained with CD38, a marker of T cell activation (figure 4C and 4D). In contrast, no significant differences were observed between the 4 study groups in the proportions of T lymphocytes expressing HLA-DR or CD45RO, a marker of differentiation. Similarly the proportions of maternal CD4\(^+\)/H11001 and CD8\(^+\)/H11001 T cells expressing CD27 and CD28, markers that are down-regulated on differentiated cells, were similar in the 4 study groups.

**DISCUSSION**

In The Gambia, most infants acquire CMV during the first year of life. By the age of 1 year, >90% of the children excreted CMV in the urine. This high prevalence of infection in the general population was confirmed by the fact that all mothers were seropositive at the time of delivery. Therefore, we can assume that mother-to-child transmission of CMV is primarily related to recurrent maternal infections in the Gambian population. The median age at which CMV excretion was first detected in the urine of infants was 12 weeks. In a large proportion of children, CMV excretion was observed between 5 and 8 weeks of age. Because the incubation period of CMV infection is \(\approx 4\) weeks, these children were presumably infected during the early postnatal period [9]. High rates of CMV excretion were detected at birth in the mothers of these children, confirming findings of previous studies indicating that the genital tract and breast-feeding are important routes for transmission of CMV in the perinatal and postnatal periods [6, 9, 11, 16]. Low rates of CMV excretion were detected at birth in mothers of children in whom CMV excretion was first detected between 17 and 47 weeks of age. In contrast, these mothers frequently excreted CMV in breast milk at the time the virus was detected in their children. This temporal association further supports the concept that breast milk represents an important route of CMV transmission during postnatal life [9]. The frequent detection of CMV in Colostrum contrasts with previous reports indicating that excretion in breast milk is uncommon.
during the first week after delivery compared with later during the postpartum period [9, 10].

A high incidence of congenital CMV infection was observed in this study population [2]. The association between congenital CMV infection and high rates of maternal excretion at birth was unexpected. Mothers of children infected in utero excreted CMV more commonly in colostrum and in the genital tract than did mothers of children infected during the early postnatal period. As observed for early postnatal infection, CMV was not commonly detected in the urine, saliva, or plasma of mothers who gave birth to congenitally infected children. These results indicate that in utero transmission of CMV after recurrent maternal infection is associated with increased viral replication in the breast and genital tract. This association has important implications for the evaluation of anti-CMV vaccine candidates. To prove the efficacy of vaccine candidates against congenital CMV infection, clinical trials including thousands of women will have to be organized [14]. The availability of a laboratory end point would considerably reduce the required sample size and would thus allow more vaccine candidates to be evaluated. Blood viral load is among the virological end points that could be considered, but studies have shown a poor correlation between viremia and in utero transmission of CMV [17, 18]. Viral excretion in colostrum and the genital tract may represent more sensitive end points, with genital excretion being a potential end point for efficacy trials in nonpregnant women.

Vaccination against CMV may be considered in countries with very high rates of seropositivity (such as The Gambia) to prevent the morbidity associated with congenital infections [19]. A vaccine that prevents CMV infection would be interesting for early life immunization. Immunization of already-infected subjects would be more challenging, because it would have to induce an immunity that would be of better quality or greater magnitude than that induced by natural infection [1]. Congenital infection after primary maternal CMV infection represents the main target for an anti-CMV vaccine. It will therefore be important to determine whether the observations made in our study population can be extrapolated to this setting. Historical studies suggest that this may be the case. Pass et al. reported higher excretion rates in black American mothers transmitting CMV in utero after primary infection than in control subjects [10, 20]. It is not possible to determine from these studies

Figure 4. Expression of markers of activation by maternal T lymphocytes at birth according to the age at which cytomegalovirus (CMV) infection was diagnosed in children. The expression of the nuclear factor Ki-67 (A and B) and the membrane antigen CD38 (C and D) by CD4+ and CD8+ T lymphocytes was measured by flow cytometry. Frequencies of T lymphocytes expressing the markers are shown as geometric means and 95% confidence intervals. The Kruskal-Wallis test was used for statistical comparison; *P* values are noted for significant differences.
whether the high excretion rates were correlates of transmission or simply the consequence of recent infection. Stern and Tucker provided more direct evidence supporting an association between in utero transmission of CMV after primary infection and maternal excretion of the virus in urine [21].

The mechanisms involved in CMV excretion in chronically infected subjects remain unclear. Reactivation of CMV in immunocompromised patients indicates that CMV replication is controlled by the host cell–mediated immune response [14, 22]. We observed that viral excretion in mothers in groups 1 and 2 was associated with increased frequencies of CMV-specific CD4+ T lymphocytes producing IFN-γ. Although we cannot rule out the possibility that immune responses were defective before CMV excretion, the data suggest that viral replication stimulated specific CD4+ T cells. This hypothesis is further supported by the observation that CMV-specific antibody levels were also significantly increased in mothers in the same groups. The observation that mothers who transmitted the virus in utero or during the first year of life displayed high frequencies of activated T cells is intriguing. This activation phenotype was characterized by high proportions of CD4+ and CD8+ T lymphocytes expressing CD38 and the proliferation marker Ki-67. CMV replication may play an important role in the activation of T lymphocytes.

The differences in the level of T cell activation in the 4 study groups may reflect different degrees of viral replication. However, the phenotype of T cells observed in our study is not typical of active CMV infection, suggesting that other mechanisms may be involved. The proportions of T lymphocytes expressing the activation marker HLA-DR and the proportions of CD4+ and CD8+ T cells expressing the advanced state of differentiation (CD27+CD28+) that is typical of CMV-specific cells were similar in the 4 study groups [23]. Pregnancy may have played a role because it is associated with activation of the innate immune system and of T lymphocytes [24, 25]. This state of T cell activation appears to be regulated by a number of suppressive factors derived from the placenta [26, 27]. Finally, T cell activation may have been at least partly related to coinfections, such as malaria. The observations that the proportions of activated T lymphocytes were low in mothers who did not transmit the virus during the first postpartum year and that lymphocyte activation preceded viral excretion in mothers in group 3 suggest that excretion of CMV may at some related least in part to immune activation. The mechanisms involved in replication of latent CMV remain unclear. In vitro studies have shown that activation of allogeneic T cells induces the replication of CMV in latently infected mononuclear cells [28, 29]. A similar phenomenon may occur during pregnancy and the postpartum period.

This prospective cohort study demonstrates that congenital CMV infection is associated with increased excretion of the virus in colostrum and in the genital tract at birth. The results suggest that common pathogenic mechanisms underlie in utero and perinatal transmission of CMV and have important implications for the development of anti-CMV vaccines.

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