Compartmentalized dynamics of cytomegalovirus replication in treated congenital infection.

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All authors have approved the final article.

KEY WORDS

Congenital cytomegalovirus; Virus half-life; virus dynamics; antiviral treatment

Abbreviations:

Basic reproductive number (Ro)

Central nervous system (CNS)

Congenital Cytomegalovirus (CCMV)

Cytomegalovirus (CMV)

Ganciclovir (GCV)

High performance liquid chromatography (HPLC)

Randomised controlled trial (RCT)

Sensorineural hearing loss (SNHL)

Valganciclovir (VGCV)

Viral load and immunology in congenital CMV study (VICC)

Virus half-life (T1/2)

Virus transport medium (VTM)

ABSTRACT

Background: Cytomegalovirus (CMV) is the most prevalent congenital infection in developed countries. A significant number of infected infants develop long-term neurodevelopmental and hearing impairment irrespective of whether disease is detectable at birth. Studies of viral load and replication dynamics have informed the treatment of CMV in adult populations but no similar data exist in neonates.

Objectives: To study CMV virus kinetics in different body fluids of babies treated for congenital infection.

Study design: CMV virus load was sequentially analyzed in blood, urine and saliva in 17 babies treated for symptomatic congenital CMV infection.

Results: Virus was detectable in the urine and saliva of all babies at baseline but in only 15/17 in blood. At the end of 6 weeks of antiviral treatment CMV remained detectable in 9/14 blood samples, 9/12 urine samples and 4/7 salivary swabs. Median half-life ($T_{1/2}$) of virus decline in blood was 2.4 days (IQR 1.9-3.3) and basic reproductive number (Ro) was 2.3. Although $T_{1/2}$ values were similar in urine and saliva to those observed in blood, virus dynamics differed both during and after treatment.

Conclusions: $T_{1/2}$ and Ro in blood in this group of neonates were similar to values derived from studies of immunocompromised adults. The persistent viremia observed in treated neonates cannot therefore be adequately explained by the virus dynamics early in treatment. The different dynamics exhibited in blood and urine suggests that studying changes in distinct body compartments may assist in further understanding long-term manifestations of disease.

23 **Word count 243 (limit 250)**

BACKGROUND

Cytomegalovirus (CMV) is a common congenital infection and an important cause of sensorineural hearing loss (SNHL) [1, 2]. A minority of those infected will have clinically detectable disease at birth, but 13% of those without disease will subsequently develop significant impairments, particularly SNHL [3].

Antiviral treatment improves hearing and neurodevelopmental outcomes when started in the first month of life in symptomatic newborns [4, 5]. There are no randomized studies to support treatment of babies without detectable disease at birth and the search for prognostic markers for adverse long term outcome in these newborns is ongoing .

Natural history studies in adult transplant recipients show that high viral load and viral kinetics in whole blood correlate with the development of CMV end-organ disease [6] with viruria independently associated with disease in renal transplant patients.

High viral load has also been associated with poor long-term outcomes in congenitally infected babies in some studies [7-11] but not others [12]. A major limitation is the lack of adequate numbers of babies without disease at birth that subsequently develop CMV-related morbidity. As SNHL is progressive, the duration of follow-up required to produce meaningful results further impacts on the conduct of such studies [13].

Data in infants largely reports single measurements of viral load rather than sequential monitoring coupled with viral kinetic modelling. A recent study in neonates treated for congenital CMV (CCMV) observed a correlation between higher burden of CMV DNA in the blood in the first 6 weeks of treatment and subsequent SNHL [5]. Given the known

prolonged urinary excretion of CMV in those infected in early life it is possible that virus kinetics differ between body fluids in this group, but no data exist currently.

Further defining the natural history of CMV virus kinetics in different body fluids in those with CCMV could aid our understanding of the pathogenesis of this virus and assist in developing biomarkers.

OBJECTIVES

This study aimed to define the kinetics of CMV replication in blood, urine and saliva in a group of babies receiving treatment.

STUDY DESIGN

The Viral load and Immunology in Congenital CMV (VICC) study recruited babies into an ethically approved protocol in the UK. 19 babies with CCMV were recruited from 7 study sites between 2008 and 2011. After CCMV diagnosis, participants in the study provided blood, urine and salivary samples at set time-points during and after treatment and up to two years of age. CMV quantitative analysis was performed in the Department of Virology at the Royal Free Hospital. Only the 11 babies that received treatment, with sufficient viral load results for meaningful analysis, are presented here (see supplemental data).

An ethically approved treatment registry for CCMV was also active in the UK during the same time period. Babies in this registry with multiple entries for CMV viral load were included for analysis (N=2)(see supplemental data). The parent(s) or legal guardian(s) of participants in both the above studies provided written informed consent.

Multiple samples were also received at our laboratory from 3 treated babies as part of routine clinical care.

Definitions*:*

CCMV was confirmed if a sample tested positive for CMV within 21 days of life. Symptomatic infection was defined according to criteria used in a previously published randomised controlled trial (RCT) of treatment [4].

Salivary swab acquisition:

Salivary samples were taken using neonatal flocked swabs (Sterilin™ Cambridge, UK) at least one hour after the baby's last feed. Swabs were resuspended in 1ml virus transport medium (VTM) prior to extraction.

Detection and quantitation of CMV DNA:

Total nucleic acid was extracted using the commercial Nuclisense Easymag system (Biomerieux, Basingstoke UK) according to manufacturer's instructions. CMV viral load was then determined using an in-house real-time quantitative PCR as described previously (lower limit of detection being 200 copies/ml, (168 IU/ml)). [14].

An estimate of the volume of saliva held on swabs was obtained by weighing swabs pre- and post- saturation in saliva. The mean of 3 samples gave an estimated volume of 27ul of saliva which allowed for calculations of CMV viral load/ml of saliva.

Measurement of ganciclovir levels:

Ganciclovir (GCV) levels were determined by the Bristol Antimicrobial reference laboratory as described in detail elsewhere [15].

Statistical analysis:

'Baseline' samples were included if they had been obtained before, or within 7 days of, treatment commencing. If multiple samples had been obtained prior to treatment the sample taken closest to treatment onset was used. End of treatment samples were accepted if taken +/- 3 days from the last day of treatment. For analyses involving comparison of virus load between different body fluids samples were only considered if taken within one day of each other.

Viral load measurements of <200 copies/ml were entered as half the limit of detection to enable log conversion and construction of virus decline curves. Mann-Whitney U test was used to compare median values, with Wilcoxon signed rank test used for comparison of paired samples.

Virus decline was calculated using methodology described previously [16]. The slope of decline of $\log_e$ (ln) viral load was computed using segmental regression in GraphPad Prism (GraphPad Software, La Jolla, CA) with X0 constraint for decline set at the point where the phase of most rapid viral decline appeared to end. Virus half-life was then defined using the formula (-ln2)/slope.

For the calculation of the basic reproductive number (Ro) after cessation of therapy the following formula was used:

105 $Ro = 1+ r/\delta e^{rt}$ where r is the growth rate of virus after stopping therapy, δ Is the death rate
106 of a CMV infected cell (taken from Emery et al, 1999) and t is a time delay between infection
107 and production of new virions (set at 2 days)[16].

108 **RESULTS**

109 **Participants:**

110 The study included viral load data from 17 babies treated for congenital CMV. All babies
111 had clinical signs or symptoms of congenital infection with central nervous system (CNS)
112 involvement. SNHL was the only evidence of suspected CNS disease in one neonate.

113 Treatment was with intravenous ganciclovir (iv GCV) at a dose of 5-6mg/kg twice daily (bid)
114 (n=10), oral valganciclovir (VGCV) at a dose of 10-17mg/kg bid alone (n=2) or a combination
115 of iv GCV followed by VGCV (n=5). All babies receiving mixed treatment commenced with iv
116 GCV for a minimum of 6 days.

117 **Baseline viral loads:**

118 In blood and urine samples 13/17 and 14/15 were taken prior to, or on the day of,
119 treatment initiation. In saliva 6/8 baseline specimens were acquired after day 0 of
120 treatment (median 3 days).

121 DNAemia was detected in 15/17 (88%) neonates at baseline. All urine and saliva samples
122 were CMV DNA positive. Both the neonates with undetectable DNAemia had samples taken
123 prior to treatment commencing. Median and interquartile ranges (IQR) of CMV loads at
124 baseline in blood, urine and saliva were 3.8 (3.3-4.2), 7.7 (7.0-8.4) and 7.2 (6.8-8.3) $\log_{10}$

genomes/ml with corresponding means of 3.8 (SD ± 0.8), 7.7 (± 0.9) and 7.3 (± 1.5) (*Figures 1 and 2*).

More than one blood sample and more than one urine sample were taken in five neonates before treatment. In 2/5 of these babies viral load in blood and urine decreased by more than 1.0 $\log_{10}$ genomes/ml (blood: range 0.2-1.5 $\log_{10}$ genomes/ml over 6-21 days; urine: range 0.1-1.6 $\log_{10}$ genomes/ml over a period of 1-23 days).

End of treatment viral load:

At the end of a 42 day treatment course CMV remained detectable in 9/14 blood samples (65%), 9/12 urine samples (75%) and 4/7 salivary swabs (57%). Median CMV load in blood, urine and saliva in babies with virus still detectable was 2.8 $\log_{10}$ genomes/ml (IQR 2.5-3.5), 2.9 $\log_{10}$ genomes/ml (IQR 2.7-3.9) and 4.0 $\log_{10}$ genomes/ml (IQR 3.2-5.5) respectively. CMV loads were significantly lower at the end of treatment in blood and urine, but not saliva, compared to baseline values (P = <0.01, 0.02 and 0.13 respectively).

CMV kinetics during therapy:

Baseline CMV loads were approximately 4.0 $\log_{10}$ genomes/ml higher at the start of treatment in urine and saliva as compared with blood but this difference narrowed during the 42 days of treatment (*Figure 1*). In keeping with this observation, viral decline between the start and end of 42 days treatment was higher in urine and saliva compared to blood with an absolute decline of -1.2 $\log_{10}$ genomes/ml (IQR -1.8 to -0.9) observed in 14 paired blood samples compared to -4.4 $\log_{10}$ genomes/ml (IQR -5.5 to -3.8) in urine (N=10) and -4.8 $\log_{10}$ genomes/ml (IQR -5.2 to -3.9) in saliva (N=7)(*Table 1*). In 2/14 paired blood samples no

decline was observed during treatment whereas CMV DNA decreased in all urine and salivary samples.

CMV DNA decline in blood and urine was more rapid during the first 7 days of treatment when compared to the full 42 days of treatment (*Table 1*). Salivary samples from early sampling points were too few to allow for analysis.

Using these data, the half-life of decline ($T_{1/2}$) was calculated using segmental regression of the most rapid phase of virus decline (examples shown in *Figure 3*). The median $T_{1/2}$ in blood of 14 neonates was 2.4 days (IQR 1.9-3.3 days), in urine it was 2.0 days (IQR 1.3-2.6) (N=14) and in saliva 1.5 days (IQR 1.4-2.4) (N=4).

Post therapy kinetics:

Once treatment had stopped, a rebound of CMV DNA levels was observed within 1 week in 4/8 blood, 6/9 urine and 1/5 saliva samples. The median increase in CMV load over the first 7 days post-treatment was 0.52 (blood), 1.04 (urine) and 2.05 (saliva) $\log_{10}$ genomes/ml (*Figure 2*). Where no rebound was observed virus had been undetectable at the end of treatment in 2/4 (blood), 1/3 (urine) and 2/4 (saliva) babies; in the remaining babies virus was still detectable but continued to decrease after treatment discontinuation.

Maximum virus levels following treatment were at age 3 months in blood and age 6 months in urine and saliva samples (*Figure 2*). Median maximum virus load was not significantly different from baseline in blood (3.78 vs 2.96 $\log_{10}$ genomes/ml respectively; P=0.3) or saliva (7.39 vs 7.16 $\log_{10}$ genomes/ml respectively; P = 0.72). Urine CMV load was, however, significantly lower at 6 months of age compared to baseline (median 5.94 vs 7.74 $\log_{10}$ genomes/ml respectively; P= <0.01).

168 The basic reproductive number (Ro) was calculated using the growth rate derived from the
169 post therapy virus rebound and previous estimates of the death rate of a CMV infected cell
170 in vivo (~0.98 day). This calculation revealed a median Ro value of CMV in blood of 2.3 (n=2)
171 in urine of 2.8 (n=2) and in saliva of 4.6 (n=1).

172 **Long-term viral control:**

173 CMV DNA remained detectable in no blood samples (n=6) at month 12 but in most urine
174 (7/7) and saliva (6/8) samples. By 24 months CMV DNA remained undetectable in all blood
175 samples (n=3) but was detectable in 2/3 urine and 1/3 saliva samples. In urine the median
176 CMV load at 12 months was 4.7 $\log_{10}$ genomes/ml (IQR 4.5-5.8)(N=7) which was significantly
177 lower than the baseline load (7.7 $\log_{10}$ genomes/ml ($p<0.01$)). Similarly, salivary viral load
178 was significantly lower at month 12 than at baseline [4.5 $\log_{10}$ (IQR 2.5- 5.1) vs 7.56 $\log_{10}$
179 (IQR 6.76-8.44) genomes/ml respectively ($p <0.01$)].

180 **Ganciclovir levels:**

181 Ganciclovir levels were mostly below quoted reference values of 0.5 mg/L (trough) and
182 7.0mg/L (peak) (*Figure 4).* Plotting $\log_{10}$ virus decline during the first 7 days of treatment
183 against peak and trough GCV levels at day 7 did not reveal any significant association
184 between these two parameters in the 5 babies studied (supplemental data).

185 **DISCUSSION**

186 The results of this study provide insight into the kinetics of CMV in different biological
187 compartments in neonates during and after antiviral therapy. Despite the differences in
188 baseline CMV load, half-lives during the initial phase of treatment were comparable across

compartments (P = 0.1-0.4 for inter-group comparisons) and similar to the 2 days observed in infrequently sampled adult immunocompromised hosts [16].

In contrast to data from studies in adult transplant patients with similar starting virus loads over half of the neonates still had DNAemia detectable at the end of the 6 week treatment course [14]. This observation and that of an initially rapid virus decline followed by a nadir is in keeping with similar observations in treated neonates [17].

The reasons for this incomplete suppression in neonates are unclear. In the setting of CMV replication in HIV infection the efficacy of iv GCV (5mg/kg/bid) has been estimated at 91.5% [18] but where plasma levels are lower the efficacy will be reduced. Therapeutic drug monitoring of GCV levels in the neonates enrolled in this study indicate that plasma GCV levels were low but consistent with other data in children [15]. Higher levels of GCV may be needed in this population to fully inhibit replication. Analysis of the CMV UL97 locus showed no evidence of mutations known to confer GCV resistance.

Alternatively, persistent viremia may represent continued virus excretion from 'sanctuary sites' inaccessible to antiviral agents. Given the increased audiological and neurological morbidity observed in CCMV when compared to immunocompromised adults, the inner ear or CNS would be possible sources of such virus reservoirs and drug penetration at these sites correspondingly suboptimal [19]. Testing such a hypothesis is challenging since no data evaluating virus persistence in CSF exist, nor is this likely to be ethically acceptable.

Although rebound of virus was common in all body fluids in the first week post-treatment, maximal rebound occurred earlier in blood when compared to urine and saliva; the rebound in DNA-emia is consistent with other recent reports during neonatal treatment [5]. Only

211 virus in urine rebounded to a level significantly lower than baseline in our study. This is an
212 important observation if the 'threshold' concept of CMV disease proposed in adults applies
213 to CCMV [20].

214 If virus is not in a steady state at the initiation of therapy then the dynamic models adopted
215 may not be fully applicable. However, congenital infection often occurs months before birth
216 and the values obtained here are consistent with those derived in adults. The growth of
217 CMV during the rebound phase allowed us to estimate Ro for CMV during this resurgence in
218 replication. The Ro values are relatively modest at 2.3 and 2.8 for blood and urine
219 respectively, consistent with those observed in D+R- solid organ transplant recipients [21].

220 Overall the data presented here imply that initial viral response to treatment is similar to
221 that observed in adult immunocompromised hosts. However, following this initial response,
222 CMV replication patterns differ in neonates when compared to immunocompromised
223 adults. In keeping with this altered virus kinetics is the ongoing audiological damage and
224 neurological damage unique to this age group. The reasons for this remain to be elucidated
225 but are likely a complex combination of host and virus factors, including immunological
226 immaturity and a possible increased susceptibility of the rapidly dividing cells in early life to
227 viral damage.

228 It is possible that even longer periods of treatment or antiviral drugs with better CNS
229 penetration will be needed if the continued detection of high amounts of virus in urine is of
230 relevance for subsequent neurological outcomes. The challenge must now be to evaluate
231 whether current antiviral agents reach the body compartments relevant for disease at
232 sufficient levels to prevent viral replication and/or damage and whether monitoring virus

load in multiple body compartments can assist in further defining viral parameters of importance for future prognosis.

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Table 1: Median viral decline in different body fluids over time in 17 babies treated for congenital CMV.

Figure 1

Mean viral load over time in different body fluids in 17 babies treated for congenital cytomegalovirus.

CMV viral load was measured in blood, urine and saliva using quantitative real-time PCR. Treatment was with either ganciclovir or valganciclovir in all babies and for a duration of 42 days +/- 1 day in 16/17 babies.

Figure 2

CMV virus load over time in different body compartments in 17 babies treated for congenital CMV

Quantitative CMV viral load measured in (A) blood, (B) urine and (C) saliva at different time points during and after treatment.

Baseline = start of treatment; End treatment = end of treatment course; D3 and D7 Post = 3 and 7 days after treatment discontinued respectively; M3, 6, 12 = age 3, 6 and 12 months of life respectively.

Error bars represent median and interquartile range.

Figure 3: Example of segmental regression of log_e blood viral load in 6 babies treated over 42 days for congenital cytomegalovirus.

Plots were constructed using GraphPad Prism software to define 2 phases of virus decline. Examples are shown for 6 babies. Plots in the remaining 8 babies and in other body fluids were constructed in a similar way.

Figure 4: Pre- (A) and Post- (B) dose ganciclovir levels in babies treated for congenital CMV

Ganciclovir (GCV) levels measured in babies aged <6 months of age (<6mo) and <28 days of age (<28 days) being treated for congenital CMV. Levels are compared between those derived from anonymized data received from the British Antimicrobial reference laboratory and described in detail elsewhere (Luck et al IJAA 2011 [15]) and those obtained during the viral load and immunology in congenital CMV (VICC) study.

Supplemental data: Relationship between virus decline in blood (A. and B.) and urine (C. and D.) and ganciclovir levels over the first 7 days of antiviral treatment for congenital cytomegalovirus infection.

Data are shown for day 7 pre- (trough: B. and D.) and post- (peak: A. and C.) ganciclovir levels taken on day 7 of treatment in 5 babies. Treatment was with ganciclovir in 4 and valganciclovir in 1 baby.

Supplemental data: Viral load at each time point in different body fluids in 17 babies treated for congenital cytomegalovirus (CMV)