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Quantification of Human Cytomegalovirus DNA in Amniotic Fluid of Mothers of Congenitally Infected Fetuses

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A quantitative PCR assay was used to quantitate human cytomegalovirus DNA in amniotic fluid of mothers of 21 fetuses with congenital infection. Seven fetuses presented ultrasound abnormalities or were born with symptoms, whereas 14 fetuses were subclinically infected. Although the median DNA level was higher in symptomatic fetuses, the difference was not statistically significant ($P = 0.09$).

Following primary infection of pregnant women, human cytomegalovirus (HCMV) is transmitted to the fetus in as many as about 50% of cases (1). Infected fetuses can be identified prenatally by HCMV isolation from and/or detection of viral DNA by PCR in amniotic fluid (AF) (9). In addition, it has been shown recently that the overall sensitivity of prenatal diagnosis can be substantially improved when the amount of AF tested by a nested PCR (nPCR) assay is increased (11). However, viral load in AF has never been quantified. We have previously developed a quantitative PCR (Q-PCR) assay which has been widely used to assess the clinical value of HCMV DNA detection in immunocompromised patients with disseminated HCMV infection (3, 4, 7). Therefore, the present study was undertaken to investigate whether the amount of viral DNA in AF could be related to the clinical conditions of infected fetuses (according to ultrasound findings) or to the outcome at birth.

Twenty-four AF samples collected from 21 fetuses (3 fetuses were sampled twice) with congenital HCMV infection were tested by Q-PCR. Fetuses were from 20 mothers (one twin pregnancy) who suffered from primary HCMV infection during pregnancy which was diagnosed by the following criteria: (i) seroconversion, (ii) presence of clinical symptoms and/or abnormal liver enzyme values and presence of virus-specific immunoglobulin M (IgM), or (iii) presence of virus-specific IgM and pp65 antigenemia or leucoDNAemia (14). AF and fetal blood samples were obtained during prenatal diagnosis procedures performed at 18 to 29 (median, 21) weeks of gestation. Diagnosis of fetal HCMV infection was performed by virus isolation from and/or viral DNA detection in AF (11). In addition, HCMV pp65 antigenemia (5), viremia (6), leucoDNAemia (2, 14), and virus-specific IgM (10) in fetal blood were determined. Fetuses were divided retrospectively into two groups: group A included 7 fetuses with morphological alterations at the time of prenatal diagnosis (4 fetuses) or with symptomatic HCMV infection at birth (3 fetuses); group B included 14 fetuses with normal ultrasound findings at the time of prenatal diagnosis and subclinical HCMV infection at birth.

Q-PCR was performed essentially as reported previously (2) with some modifications (9). Briefly, an HCMV genome sequence relevant to exon 4 of the major immediate-early (IE) gene (IE1) was amplified. An internal control of amplification consisting of a fixed amount (100 genome equivalents [GE]) of a recombinant DNA molecule (pAC2) flanked by the target sequence of outer primers used for viral DNA amplification in clinical samples was routinely coamplified to detect PCR inhibitors and to normalize the number of GE of test samples calculated from a standard curve. The standard curve was constructed by densitometric analysis of gel signals of external standards consisting of increasing known amounts of a cloned HCMV IE1 DNA fragment (pCM2) which were amplified in the presence of 100 pAC2 GE. This method gave reproducible HCMV DNA quantification values ranging from 10^1 to 10^4 GE. Samples containing $>10^4$ GE were serially diluted. Positive samples containing 1 to 10 GE were detected by an nPCR assay. An arbitrary value of 5 GE was assigned to AF samples positive only by nPCR. In Q-PCR and nPCR, DNA was extracted from 300 to 800-μl samples of whole AF and multiple 100-μl AF replicates were then independently amplified. Results were expressed as the number of GE per milliliter of AF. For nPCR, 1/100th of the first-round product volume was further amplified for 40 cycles. Specificity of the assay was assessed as reported previously (11).

At the time of prenatal diagnosis, four fetuses of group A showed abnormal ultrasound findings (Table 1): ascites and hydrocephaly (fetus 1), intrauterine growth retardation (fetus 2), ascites (fetus 3), and dilated ventricles (fetus 4). In these fetuses, thrombocytopenia and/or elevated γ-glutamyltransferase (γGT) levels (>2 standard deviations of the mean value for gestational age) were concomitantly detected. Three pregnancies were terminated, while the remaining pregnancy ended in stillbirth at 32 weeks of gestation (Table 1, fetus 2). In this case, postmortem examination did not show any overt malformation or abnormality, whereas viral inclusion bodies were histologically detected in the kidneys, lungs, heart, and pancreas. Fetuses 5 to 7 did not show ultrasound abnormalities at the time of amniocentesis. However, elevated γGT levels were found in all three fetuses, and thrombocytopenia was found in fetus 6. At birth, the three newborns presented a symptomatic HCMV infection. In particular, monolateral chorioretinal atrophy and intracranial calcifications were detected in newborn 5 and 7, respectively, whereas newborn 6 was born with petechiae, hepatomegaly, microcephaly, thrombocytopenia, increased alanine transaminase levels, and hearing impairment. As for the 14 fetuses of group B, ultrasound findings were normal for all of them, whereas γGT values were normal for 9 fetuses and elevated in the remaining 5 (Table 1, fetuses 8, 9, 10, 15, and 16). Three pregnancies were terminated, and 11 went to term. All the newborns were asymptomatic at birth.

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TABLE 1. Quantification of HCMV DNA in AF of congenitally HCMV-infected fetuses by quantitative PCR

Group and fetus	Time (wks of gestation) of:		No. of HCMV DNA GE/ml of AF	Outcome[a] (symptoms)
	Maternal infection	Prenatal diagnosis		
Group A				
1	<10	19	1.25×10^5	TOP
2	10	27	1.25×10^8	Stillbirth
3	<8	22	6.25×10^7	TOP
4	<13	21	1.25×10^9	TOP
5	<10	21	1.25×10^8	Term (yes)
6	8	23	1.25×10^8	Term (yes)
7	11	22	1.25×10^6	Term (yes)
Group B				
8	6	21	6.25×10^6	TOP
9	6	21	3.75×10^7	TOP
10	9	21	3.75×10^6	Term (none)
11	9	21	1.25×10^8	Term (none)
12[b]	11	18/23[c]	$3.7 \times 10^1/6.2 \times 10^1$[c]	Term (none)
13[b]	11	18/23	$2.5 \times 10^1/1.6 \times 10^1$	Term (none)
14	22	31	1.25×10^6	Term (none)
15	<12	24	6.25×10^5	
		29	2.5×10^6	Term (none)
16	<14	23	1.25×10^8	Term (none)
17	18	21	8.3×10^1	Term (none)
18	8	18	3.75×10^6	TOP
19	7	21	6.25×10^6	Term (none)
20	8	21	3.75×10^6	Term (none)
21	12	21	3.75×10^6	Term (none)

[a] TOP, termination of pregnancy; Term, full-term pregnancy.
[b] Fetuses 12 and 13 were twins.
[c] Procedure done twice at 18 (before the slash) and 23 weeks (after the slash) of gestation.

Detailed results of HCMV DNA quantification in AF samples of 21 congenitally infected fetuses are reported in Table 1. Median GE levels were 1.25×10^8 (range, 1.25×10^5 to 1.25×10^9) and 3.75×10^6 (range, 1.6×10^1 to 1.25×10^8) in groups A and B, respectively. The difference was not statistically significant ($P = 0.09$ by the Mann-Whitney test). In particular, in some fetuses, the same DNA level was observed despite the severity of HCMV infection (e.g., 1.25×10^8 GE was detected in fetuses 5 and 6 of group A and in fetuses 11 and 16 of group B). Moreover, a fourfold increase in the DNA amount was found in fetus 15 during two procedures performed 5 weeks apart. Finally, it is noteworthy that fetuses 12, 13, and 17 showed <100 GE/ml of AF. This low viral DNA level was possibly due in fetus 17 to the brief period of time between maternal infection and prenatal diagnosis (3 weeks). However, in the remaining two fetuses (fetuses 13 and 14), two procedures were performed 6 and 11 weeks after maternal infection, respectively, and in both cases, low levels of viral DNA were found to linger in both AF. Thus, from the data reported in Table 1, the amount of viral DNA detected in AF does not appear to correlate with the time interval between maternal infection and the prenatal diagnosis procedure.

As reported in more detail elsewhere (13), virologic and serologic examinations performed on fetal blood samples showed that positive results for antigenemia, viremia, and leucoDNAemia were observed in 6, 5, and 7 fetuses of group A ($n = 7$), respectively, compared to 7 of 14, 5 of 11, and 9 of 11 fetuses of group B, respectively. As for the presence of virus-specific IgM, all seven fetuses of group A and 5 of 14 fetuses of group B were IgM positive.

To our knowledge, the quantitation of viral DNA in AF of congenitally HCMV-infected fetuses has never been reported so far. Our data indicate that the amount of viral DNA in AF does not correlate with the presence of ultrasound abnormalities at the time of prenatal diagnosis or with the outcome at birth. This finding is in keeping with previous results obtained by our group showing that, although higher levels of HCMV pp65 antigenemia, viremia, and DNAemia were detected in blood samples from fetuses with abnormal ultrasound and biochemical or hematologic findings compared to those of fetuses with normal findings, the difference was not statistically significant, except for antigenemia (13). On the other hand, we have shown that infants with symptomatic congenital HCMV infection do have significantly higher levels of HCMV load in blood at birth and that clearance of the virus takes longer than in subclinically infected infants (12). Moreover, it has also been reported that infants with symptomatic congenital HCMV infection excrete larger amounts of virus in the first few months of life than those with asymptomatic infection (15). These data seem to indicate that quantitation of different virological parameters may be of clinical value only after birth. Finally, our numbers are too low to hypothesize that the presence of very small amounts of viral DNA such as those detected in AF of three fetuses of group B correlate with asymptomatic infection at birth. However, it is important to stress that all fetuses with PCR-positive AF, including the three with the smallest amounts of viral DNA, were born with the infection. This finding is in contrast with the results of Lazzarotto et al. (8), who recently reported that congenital HCMV infection was diagnosed at birth in only 12 of 27 fetuses with PCR-positive AF. This latter study hypothesized that the finding "was probably due to the high sensitivity of the procedure (100%), which detects a viral load so low as to be cleared by the defense of the mother or fetus. Quantitative PCR is in progress to verify this hypothesis." Our Q-PCR data do not support this interpretation.

In conclusion, different variables such as gestational age of maternal infection, timing of intrauterine transmission of the infection, timing of prenatal diagnosis, and most importantly, the unfeasibility of a follow-up of the infection during the fetal life remain major obstacles to the identification of a reliable prenatal marker of symptomatic congenital HCMV infection.

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