ABSTRACT. Human cytomegalovirus (CMV) is the most common cause of viral intrauterine infection and fetal damage largely attributable to maternal primary infection. Most cases of congenital CMV infection in twins reported in the literature involved only 1 twin. We assessed the validity of polymerase chain reaction (PCR) and quantitative PCR on amniotic fluid (AF), at 21 to 22 weeks' gestation and at least 6 to 8 weeks after seroconversion, to predict the outcome of newborns in twin pregnancies. Two pregnant women with twin pregnancies and 1 woman with a triple pregnancy with primary CMV infection defined by the presence of immunoglobulin (Ig) M and low IgG avidity and/or by the presence of clinical symptoms and abnormal liver enzyme values were evaluated. CMV infection was found in 6 fetuses/newborns, 3 of whom were asymptomatic. In the first twin pregnancy with diamniotic-dichorionic separate placentas, CMV symptomatic infection of the female twin was demonstrated by positive virus isolation and high viral load in AF. The male fetus was not infected as demonstrated by negative CMV culture and DNA detection in AF. In the triple pregnancy, the woman had a placenta with 2 monozygotic twins (females) and a separate placenta with a heterozygotic twin (male). The quantitative PCR results were $10^4$ genome equivalents (GE)/mL of females AF and $1.9 \times 10^3$ GE/mL of male AF. Both female twins were asymptomatic at birth, whereas the male presented petechiae, thrombocytopenia, and cerebral ventriculomegaly. In the last twin pregnancy with fused dichorionic placentas, congenital CMV infection of both twins was diagnosed at birth in contrast with prenatal diagnosis. At time of amniocentesis, the left side twin was not infected as shown by negative results of CMV culture and DNA detection in the AF. CMV infection of the right side twin was demonstrated by positive CMV DNA detection with a CMV DNA load of $4.9 \times 10^4$ GE/mL and positive virus isolation in the AF. The morphologic and histologic examinations of the placentas strongly supported a prenatal horizontal acquisition of CMV infection. These twin pregnancies showed a marked difference in the quantity of virus load documented by the prenatal diagnosis suggesting that twin fetuses may react differently to primary maternal infection despite being exposed to the same maternal influences. A high viral load is correlated with congenital CMV infections symptomatic at birth. In such cases, with fetal infection of only 1 twin (at amniocentesis) and fusion of placentas, fetal outcome of both twins needs to be evaluated for the possibility of viral transfer from one fetus to the other. Pediatrics 2003;112:e153-e157. URL: http://www.pediatrics.org/cgi/content/full/112/2/e153; cytomegalovirus, congenital infection, twin pregnancies, placenta, prenatal diagnosis, viral load.

ABBRVIATIONS. CMV, cytomegalovirus; PCR, polymerase chain reaction; AF, amniotic fluid; Ig, immunoglobulin; GE, genome equivalents; qPCR, quantitative polymerase chain reaction; ABR, auditory brainstem-evoked response; PMNL, polymorphonuclear leukocyte; EIA, enzyme immunoassay.

Cytomegalovirus (CMV) is the most common cause of intrauterine infection, affecting from 0.3% to 2% of liveborn infants. Between 10% and 15% of infants infected with congenital CMV exhibit the clinically apparent or symptomatic form of the disease, characterized by petechiae, hepatomegaly, splenomegaly, jaundice, periventricular calcifications, microcephaly, hearing impairment, and chorioretinitis. The remaining 85% to 90% of infected infants are asymptomatic at birth, but $\sim 15\%$ of them will develop delayed sequelae, especially progressive hearing loss.

Primary infection in the mother and as intrauterine transmission during the first 16 weeks of pregnancy have a much greater clinical impact on the fetus than nonprimary infections and infections occurring during the last trimester of pregnancy.

Intrauterine transmission occurs transplacentally during maternal viremia; the placenta acts as a portal of entry for the virus, but it also acts as a barrier because even during maternal primary infection, transmission occurs in only 40% of cases.

Intrauterine transmission can be determined by both polymerase chain reaction (PCR) and virus isolation from amniotic fluid (AF). In single pregnancies, a high viral load in the AF seems to identify fetuses at higher risk of developing a severe infection, although the results should still be considered at an investigational stage because of the relatively low number of cases tested.

A few cases of CMV-infected twin pregnancies are described in the literature, and often only 1 newborn was found infected. In only 1 case was prenatal diagnosis performed. Twin pregnancies represent an interesting model.
because different fetuses are simultaneously exposed to the same maternal influences. This paper describes 3 cases of CMV-infected twin pregnancies in which the maternal viral load in AF correlated with pregnancy outcome.

### CASE REPORTS

#### Case A
A 23-year-old primiparous woman had a twin pregnancy (a female and a male fetus) that was without complication until 15 weeks’ gestation, when routine CMV serologic tests revealed suspected CMV infection (Table 1). Further investigations for CMV were performed. CMV-immunoglobulin (Ig) G with low avidity and CMV-IgM were demonstrated. Isolation of CMV from urine and saliva was negative, and viral antigenemia and DNAemia were negative. At gestation week 22, AF samples were collected from both sacs. CMV infection of the female twin was demonstrated by positive CMV DNA detection (3 × 10^6 genome equivalents [GE]/mL of AF) and positive virus isolation. The male fetus was not infected as shown by negative CMV culture and DNA detection in the AF. At 31 weeks’ gestation, a discordant growth between the fetuses was diagnosed by ultrasound examination, revealing an intrauterine growth restriction in the female fetus. Pregnancy was complicated by in utero death of the female twin at gestation week 34. A cesarean section was performed 2 days later. The surviving infant had Apgar scores of 7 at 1 minute and 9 at 5 minutes and weighed 1700 g. At birth, no clinical evidence of congenital infection was detected, and the urine and saliva culture for CMV was negative. At autopsy, the female fetus showed hepatosplenomegaly and cerebral ventriculomegaly. Microscopic examination disclosed enlarged, inclusion-bearing cells in the lungs, liver, and kidneys. The overall features were characteristic of generalized cytomegalic inclusion disease. Placentas were diagnostically-dichorionic and separate. No histopathologic examination of placentas was done.

#### Case B
A 28-year-old woman had a triple pregnancy. She was a second gravida and had a placenta with 2 monozygotic twins (females) and a separate placenta with a heterozygotic twin (male; Table 1). At week 13 she had both anti-CMV-IgM and IgG with a low IgG avidity value, indicating a recent primary infection. Isolation of CMV from urine and saliva was negative, and the viral antigenemia and DNAemia were negative. AF was obtained at 21 weeks gestation from the fluid compartment of the heterozygotic fetus and from only 1 fluid compartment of the monozygotic twins. AF from the third gestational sac was not obtained for technical reasons. CMV DNA detection and virus isolation were positive in both AFs. The quantitative PCR (qPCR) results were: 10^5 GE/mL of female AF and 1.9 × 10^6 GE/mL of male AF. Delivery by cesarean section was planned at the 33rd week of pregnancy. The

### TABLE 1. Detailed Results of Maternal and Neonatal Findings in 3 Twin Preganancies

<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>Symptoms</td>
<td>DNAemia</td>
<td>Sex / Mz Dz</td>
<td>Tests on amniotic fluid</td>
</tr>
<tr>
<td>A</td>
<td>Healthy</td>
<td>–</td>
<td>F / Dz</td>
<td>+ 3 × 10^6</td>
</tr>
<tr>
<td>B</td>
<td>Healthy</td>
<td>–</td>
<td>M / Dz</td>
<td>F / Mz</td>
</tr>
<tr>
<td>C</td>
<td>Nausea, fever, muscle weakness</td>
<td>1.7 × 10^3 GE/10^5 PMNLs</td>
<td>F / Mz (left side)</td>
<td>+</td>
</tr>
</tbody>
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Mz indicates monozygotic; Dz, dizygotic; F, female; M, male; nd, not determined.
months of life, the infant presented a monolateral sensorineural hearing loss.

The other infant had normal ABR and at 12 months of life was healthy, without auditory impairment.

Gross and histologic examination of the placenta showed a dichorionic, diamniotic-fused placenta. At submacroscopic examination, performed with a stereomicroscope and on histology, blood vessels crossing the 2 placentas were detected. Both placentas were CMV-positive, as detected by in situ hybridization.

METHODS

Serologic Tests

Anti-CMV IgG was evaluated with a commercial kit (Enzygnost anti-CMV/IgG enzyme immunoassay (EIA) a method; Behring, Marburg, Germany). Plates were read on a micro-EIA automatic reader (Behring). Anti-CMV IgM was evaluated using an anti-CMV/IgM kit (Enzygnost; Behring). Both kits were used and the results interpreted as suggested by the manufacturer. IgG avidity was determined using a commercial kit (Cytomegalovirus IgG Avidity EIA Well; Radim, Rome, Italy). The results were interpreted as suggested by the manufacturer; in particular, an avidity index (absorbance reading after urea wash/absorbance reading without urea wash × 100) <35% was considered low, >45% was considered high, and between 35% and 45% was considered moderate.

Virologic Tests

CMV Isolation

The shell vial procedure was used for CMV isolation from urine, saliva, and AF. The cells were fixed 24 to 48 hours after inoculation and were stained by an indirect immunofluorescence assay with a monoclonal antibody reacting with the CMV IE1 and EA gene product (E13 + 2A2; Argene, Varilhes, France).

Antigenemia

The presence of CMV pp65 (ppUL83) in 2 × 10⁶ PMNLs of patients was determined, as recently described, using a CMV pp65-specific pool of 2 monoclonal antibodies (IC3 and AYM-1; Argene) in indirect immunofluorescence test.

PCR in AF

CMV DNA was individually extracted from 3 to 6 aliquots of AF (100 µL each) with an IsoQuick Nucleic Acid Extraction kit (Orca Research, Bothell, WA), and PCR was conducted as described in detail previously. AF was considered positive if at least 1 of the aliquots was positive.

PCR in Blood

CMV-DNA was extracted from aliquots of 1 × 10⁶ PMNLs by CMV-Ibridoquant Extraction kit (Bioline, Turin, Italy). Nested PCR was performed for amplification of a highly conserved region of the viral genome (major immediate-early) using 2 primer sets described elsewhere. PCR products were separated by electrophoresis on a 2% Nusieve-Seakem (2:1) gel (FMC Bioproducts) and visualized with transillumination. The sample was regarded as positive when a band corresponding to a 110-bp DNA fragment was detected and visualized with transillumination. The sample was regarded as positive when a band corresponding to a 110-bp DNA fragment was detected and visualized with transillumination.

qPCR in Blood and in AF

Competitive qPCR was conducted with a commercially available kit (Amplification set CMV-Ibridoquant, Bioline).

DNA was extracted from aliquots of 1 × 10⁶ PMNLs by CMV-Ibridoquant Extraction kit (Bioline) and from AF as previously described.7 PCR was run as suggested by the manufacturers. In this test, an internal control construct, which is amplified by the same primer pairs but recognized by a different molecular probe, is amplified and detected. This method allows reproducible amplification of a minimum of 100 GE/1 × 10⁶ PMNLs and of 940 GE/mL of AF.

The quantitative determination of CMV DNA in the AF of at least 10⁵ GE gave a 100% certainty of detecting an infected fetus/newborn. Higher viral loads (≥10⁶) were associated with fetuses or newborns with symptoms.12,13 To estimate the interassay variability of the qPCR assay, DNA extracted from the AF was quantified in 3 independent PCR runs. The mean GE value ± the standard deviation was 1827 ± 187 GE, and the coefficient of variation was 10.2%.

Morphologic and Histologic Examinations

The placenta was formalin fixed and then sectioned with a large knife perpendicular to fetal membranes.

For 3-dimensional tissue reconstruction, large sections (macrosections) were obtained including the insertions of the 2 umbilical cords and the membrane separating the 2 fetal cavities. Macrosections were fixed flat overnight, washed in tap water, dehydrated through a graded series of alcohol to xylol, and finally cleared in methylsalicylate.11 Macросections were examined under a stereomicroscope (Nikon, Tokyo, Japan) and subsequently were paraffin-embedded and prepared for histologic examination.

For conventional histologic examination, small blocks of placentas were obtained and paraffin was embedded with a routine procedure. Blocks were cut and histologic sections stained with hematoxylin and eosin. From selected blocks, 4-µm sections were cut and pretreated for in situ hybridization as previously described. For in situ hybridization, a biotinylated DNA CMV probe was employed (Enzo Diagnostics Inc, Farmingdale, NY). The probe was denatured at 95°C for 10 minutes. Hybridization was performed overnight at 37°C in a humid chamber. The probe was diluted 1/10 in hybridization buffer supplied by Enzo Diagnostics. To enhance positivity, 2 cycles of catalyzed amplification system (Dako GenPoint; Dako) were performed. Negative controls consisted in omitting the probe; sections of known positivity were added as positive controls.

DISCUSSION

Maternal factors are thought to explain the variable outcome of congenital CMV infection in newborns after primary CMV infection during pregnancy.24 Some have suggested that women who transmit CMV to the fetuses might have defective immunologic responses and, therefore, are unable to limit replication of the virus.24,25 In contrast, the results of our study are in agreement with literature reports15,16 showing that twin fetuses may react differently to primary maternal CMV infection, although exposed to the same maternal influences. Therefore, the defective immunologic response in the mother cannot alone account for the different outcome of transmission.

Congenital CMV infection is almost invariably accompanied by positive histopathologic findings in the placenta, indicating that the placenta is the main entrance for CMV to the fetuses and that placental infection following maternal viremia is the initiating step of fetal infection. The placenta could have a more important role as a protective factor than maternal immunologic reactivity.

One of the factors in limiting viral spread could be the ability of the placenta to produce interferon when challenged.27 Interferons are constitutively expressed by several tissues, notably in the placenta of several species including humans.26 Fisher et al.30 observed that CMV infection impairs cytотrophoblast expression of HLA-G, an important component of the mechanism that protects fetal cells from removal by maternal immune cells.

Another theory is that the placenta could act as a nonspecific barrier.31 In our dizygotic twins (case A) with separate pla-
centas, only 1 twin was severely infected, and the finding of high viral load in AF had predicted the presence of a symptomatic infection.

Assuming that both placentas were exposed to the virus at the same time and at the same viral load and that neonatal infection is most likely attributable to maternal viremia with transplacental spread of the virus, the placenta of the surviving uninfected twin may have acted as a barrier to viral transmission.

In monozygotic twins with monochorionic placenta (case B), infection occurred with the same severity in both female twins. Supplied by a common placenta and probably equipped with identical genetic codes, the fetuses reacted to maternal CMV infection as a unit, ie, as a single fetus would have done. There is also a separate placenta with a heterozygotic twin. This infant was more severely affected by the intrauterine acquired infection than the female twins. It shows that the clinical outcomes of congenital infection differ in dizygotic infants even if both placentas are infected. These twins experienced a marked difference in the quantity of virus load documented by prenatal diagnosis, suggesting that viral load in the AF taken at 21 weeks’ gestation and at least 8 weeks after seroconversion is correlated to pregnancy outcomes, a high viral load being linked with congenital infections symptomatic at birth.

The placenta could also serve as a reservoir in which virus replicates before reaching the fetus, influencing the risk of clinical symptoms in the fetus/newborn.

In our last case with fused dichorionic placentas (case C), a congenital CMV infection of both twins was diagnosed by virus isolation in the urine collected in the first week after birth. Both placentas were CMV-positive, in contrast with prenatal diagnosis that had documented CMV infection in only 1 fetus.

Our hypothesis is that initially (15 weeks’ gestation) maternal viremia caused infection of the least resistant placenta (the right-side placenta). As the placenta is a dynamic organ whose structure and function change throughout pregnancy, twin placentas may fuse together, creating vascular anastomoses. Placenta fusion, together with a local increase in virus in the infected placenta/fetus, leads to viral transfer from the infected fetus to the more resistant sibling. The possibility that what we observed is attributable to a late intrauterine viral transmission from the mother to the second fetus is very unlikely because the viremia phase in immunocompetent subjects is very short (<30 days), at the time of amniocentesis maternal blood was CMV DNA negative and during the third trimester trophoblasts are much more resistant to CMV infection than early pregnancy trophoblasts. Moreover, under our experimental conditions, a negative result of prenatal diagnosis indicates the absence of an infection of fetus/newborn.

In this study, establishment of maternal primary CMV infection in early pregnancy was responsible for invasive prenatal diagnosis. Amniocentesis was performed at 21 to 22 weeks’ gestation and at least 6 to 8 weeks after seroconversion to discriminate between fetuses who are infected but fail to develop the congenital syndrome and those with CMV disease. The limitations of noninvasive ultrasound examinations are well known: they detect a maximum of only 5% of all CMV-infected fetuses.

According to our findings, a positive culture result in AF indicated congenital infection, but a high CMV DNA load in AF predicted symptomatic congenital infection.

This result is not surprising, as qPCR determination of AF viral load predicted both the infectious and clinical outcome of maternal CMV infection in fetuses and newborns. More recently, Gouarin et al reported that CMV load in AF correlates with fetal clinical outcome, and they also observed how viral replication tends to increase more rapidly in symptomatic fetuses than in asymptomatic ones during the course of intrauterine CMV infection.

CONCLUSIONS

This study shows that maternal factors play a very limited role in influencing CMV transmission, as fetuses simultaneously exposed to the same maternal influence had a completely different outcome. Therefore, even sophisticated studies in the mother will not be sufficient to predict congenital infection in the fetus, and studies in the fetus are a necessary complement. In case of serologic diagnosis of maternal primary infection in early pregnancy, prenatal diagnosis should be offered to detect or exclude fetal infection and a high viral load predicts symptomatic congenital infection. Furthermore, in twin pregnancies with fetal infection of only 1 twin (at amnioncensis) and with fusion of placentas, prenatal diagnosis has limited value and the outcome of both twins needs to be evaluated for the possibility of a prenatal horizontal acquisition of the infection.

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