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Human Cytomegalovirus UL144 Gene Polymorphisms in Congenital Infections

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The human cytomegalovirus (HCMV) UL144 gene is a tumor necrosis factor-like receptor with the potential to affect HCMV virulence. HCMV strains display genetic variability in the UL144 region, and the analysis of a potential link between UL144 gene polymorphisms and disease severity has scarcely been studied. However, a correlation between the UL144 genotype and congenital-disease outcome has been reported in one previous study, with the observation that all asymptomatic infants had a single UL144 genotype. In order to confirm or refute this finding, we determined the UL144 polymorphisms of HCMV strains recovered from the amniotic fluids of 38 infected fetuses and compared them to HCMV strains obtained from 30 viremic adult controls. The UL144 sequences were distributed among five genotypes (A, B, C, AC, and AB), as previously described. We observed similar percentages of the three major genotypes A (37%), B (33%), and C (27%) in our population. The UL144 genotype distributions were similar among the group of infected adults and the group of infected fetuses and among symptomatic and asymptomatic fetuses ($P < 0.05$). In our series, all five UL144 genotypes could be vertically transmitted from mothers to fetuses, and all could cause symptomatic congenital infection. We concluded that determination of UL144 polymorphisms in cases of congenital infection is not relevant, since it is unlikely to help predict the outcome of the infection.

Human cytomegalovirus (HCMV) is the main cause of congenital viral infection. Around 1% of all newborns are infected by HCMV, but only 10% of these infected children are symptomatic at birth. Mortality is ~30% in symptomatic infants, and brain lesions and subsequent neurological handicaps occur in up to 60% of these cases (8, 19). Children who are asymptomatic at birth can suffer milder morbidity in 5 to 10% of cases. In utero assessment of congenital HCMV infection is difficult. Although brain abnormalities, hepatomegaly, and ascites are objective ultrasound features, difficulty remains in the assessment of infected fetuses when there are no ultrasonographic anomalies. Identification of other prognostic markers is therefore needed. The prognostic value of the HCMV load in amniotic fluid and in fetal blood is under evaluation (9, 10, 17, 18). Characterization of HCMV strains, and particularly the analysis of a potential link between HCMV strain genotypes and disease severity, is also being investigated.

HCMV strains display genetic variability in several regions, especially in the UL144 region, which encodes a structural homologue of the herpesvirus entry mediator, a member of the tumor necrosis factor (TNF) receptor superfamily (6, 7). Herpesviruses possess putative immune evasion genes, which act by targeting TNF superfamily proteins (6, 20). The UL144 protein may therefore contribute to the ability of HCMV to escape immune clearance and may potentially affect its virulence. Phylogenetic analysis shows that nucleotide sequences of the UL144 gene cluster in three major groups (1, 4, 14). The link between the polymorphism of putative virulence genes, such as the UL144 gene, and the outcome of congenital infection has scarcely been studied. However, Arav-Boger et al. (1) reported an interesting association between UL144 genotypes and the outcome of congenital disease, suggesting that UL144 gene polymorphism may have an impact on the severity of the disease. In order to test this hypothesis, we characterized the UL144 polymorphisms of HCMV strains recovered from the amniotic fluid samples of 38 congenitally infected fetuses and in the blood of 30 infected adults enrolled as a control group.

MATERIALS AND METHODS

Patients and samples. Between February 2001 and May 2002, 505 amniotic fluid samples were sent to our laboratory for real-time HCMV PCR assays. Forty-four samples were found to be positive, and 38 of these samples were available for UL144 polymorphism testing. The geographical origins of the 38 pregnant women with primary HCMV infection were Paris and the surrounding area ($n = 32$; 84%) and Reunion Island ($n = 6$; 16%).

The control group consisted of 30 blood samples obtained from 30 infected adults. These 30 adults were either organ or bone marrow transplant recipients diagnosed with active HCMV disease between May 2002 and January 2003 (12). All of the patients were followed in Necker Hospital and lived in Paris and the surrounding area.

Informed consent was obtained from all patients or their parents.

Clinical-data collection. Ultrasonographic reports, outcome, and/or postmortem examination reports were collected. Some of these cases have also been described elsewhere (15). We classified the fetuses into two groups. Group 1 was the group of severely symptomatic fetuses showing the presence of cerebral ultrasounds feature(s), the presence of at least two extracerebral features, or subsequent abnormal neurological development. Group 2 was the group of non-severely symptomatic fetuses with the presence of, at most, only one extra cerebral ultrasound feature or of subsequent normal neurological development.

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TABLE 1. Ultrasound findings and UL144 genotypes in 38 congenitally HCMV-infected fetuses

Patient no.	Second-trimester ultrasound[a]	Third-trimester ultrasound[a]	UL144 genotype	Outcome (group)[b]
1	Hyperechogenic bowel	No US findings	A	2
2	Liver calcifications, oligopolyhydramnios	NA	A	NF
3	"Cerebral signs"	NA	A	1
4	Hyperechogenic bowel	Perventricular echogenicity	A	1
5	Hydrops, placentamegaly	NA	A	1
6	Ventriculomegaly	NA	A	1
7	NA	NA	A	NF
8	Hydrops, microcephaly, ventriculomegaly	NA	A	1
9	No US findings	Asymetric ventriculomegaly, hyperechogenic bowel	AB	2
10	IUGR, oligohydramnios	Cerebral calcifications	AB	1
11	Ascitis, ventriculomegaly, porencephaly	NA	AC	1
12	No US findings	No US findings	B	2
13	No US findings	IUGR, ventriculomegaly, microcephaly	B	1
14	No US findings	No US findings	B	2
15	Ventriculomegaly	Ventriculomegaly, cerebral calcifications	B	1
16	No US findings	No US findings	B	2
17	Hyperechogenic bowel	NA	B	2
18	IUGR, oligohydramnios, ventriculomegaly, hyperechogenic bowel, hepatomegaly	NA	B	1
19	No US findings	Ventriculomegaly	B	1
20	Polyhydramnios	NA	B	1[c]
21	Hyperechogenic bowel	NA	B	2
22	No US findings	IUGR, microcephaly, lissencephaly	B	1
23	Hyperechogenic bowel, polyhydramnios	NA	B	1
24	No US findings	Hyperechogenic bowel, ventriculomegaly	C	1
25	Ventriculomegaly, hyperechogenic bowel	NA	C	1
26	No US findings	Cerebral calcifications, microcephaly	C	1
27	No US findings	Cerebral calcifications	C	1
28	IUGR, ventriculomegaly	IUGR, ventriculomegaly	C	1
29	"Cerebral signs"	NA	C	1
30	Hyperechogenic bowel	IUGR	C	1
31	No US findings	No US findings	C	2
32	Microcephaly	NA	C	1
33	IUGR, oligohydramnios	IUGR, oligohydramnios	C	2
34	Ascitis, hepatomegaly	NA	C	1
35	No US findings	No US findings	A	2
36	IUGR, hyperechogenic bowel	NA	A	1
37	No US findings	No US findings	C	2
38	Hyperechogenic bowel, periventricular hyperechogenicity, microcephaly	NA	B	1

[a] US, ultrasound; NA, not available.
[b] Group 1, severely symptomatic fetuses; group 2, non-severely symptomatic fetuses; NF, no follow-up.
[c] Neonatal death secondary to CMV infection.

UL144 gene amplification. Total DNA was extracted from 200 μl of amniotic fluid or blood plasma with the QIamp DNA minikit (QIAGEN S.A., Courtaboeuf, France). The extracted DNA was amplified in 10 mM Tris HCl, 50 mM KCl, 1.5 mM $MgCl_2$ (Applied Biosystems), 1 mM deoxynucleoside triphosphate (Applied Biosystems), 10 μM forward primer (5′-TCG TAT TAC AAA CCG CGG AGA GGA T-3′) and reverse primer (5′-ACT CAG ACA CGG TTC CGT AA-3′) (3), and 1 U of *Taq* polymerase (Applied Biosystems). A nested PCR was performed with forward primer (5′CTT CCG GTA GGC ATG AA-3′) and reverse primer (5′-GAC TTC ATC GTA CCG TGA-5′). Amplification was carried out with a Perkin-Elmer Gene Amp PCR system 2400. The conditions for amplification with all primers sets were 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 55°C for 45 s, and 72°C for 1 min. The 30 cycles were followed by a single extension cycle at 72°C for 5 min. The PCR products were purified using a QIAquick PCR Purification kit (QIAGEN).

DNA sequencing. The purified PCR products were sequenced using a fluorescent-dideoxyterminator method (Big Dye Terminator Sequencing kit; Applied Biosystems). The sequencing products were analyzed on a model 377 automated DNA sequencer (Applied Biosystems). The sequences obtained were aligned with Sequence Navigator software and compared to five reference sequences from Arav-Boger et al. (1) (GenBank accession numbers AF498086 to AF498090) and 12 sequences from Lurain et al. (14) (GenBank accession numbers AF084980, AF179206, AF084999, AF084992, AF084990, AF179208, AF084994, AF085003, AF085004, AF084996, AF084999, and AF085002).

The sequences obtained were aligned with Clustal W version 1.6 software. Pairwise evolutionary distances were estimated using Kimura's two-parameter method, and the trees were then constructed by a neighbor-joining method (implemented in PHYLIP (Phylogeny Inference Package version 3.6α). The reliability of each tree topology was estimated from 100 bootstrap replicates. Trees were also inferred by using the maximum-likelihood model.

Statistical analysis. Statistical analyses were performed using XLStat Pro software. Differences in the distributions of UL144 genotypes among the population of symptomatic fetuses, the population of asymptomatic fetuses, and the control population were examined by Fisher's exact test. Only P values of <0.05 were considered significant.

RESULTS

Congenital CMV infection-related symptoms and outcome. Congenital-infection outcome data were obtained in 36 out of 38 cases: 2 cases (numbers 2 and 7) were lost for follow-up, 23

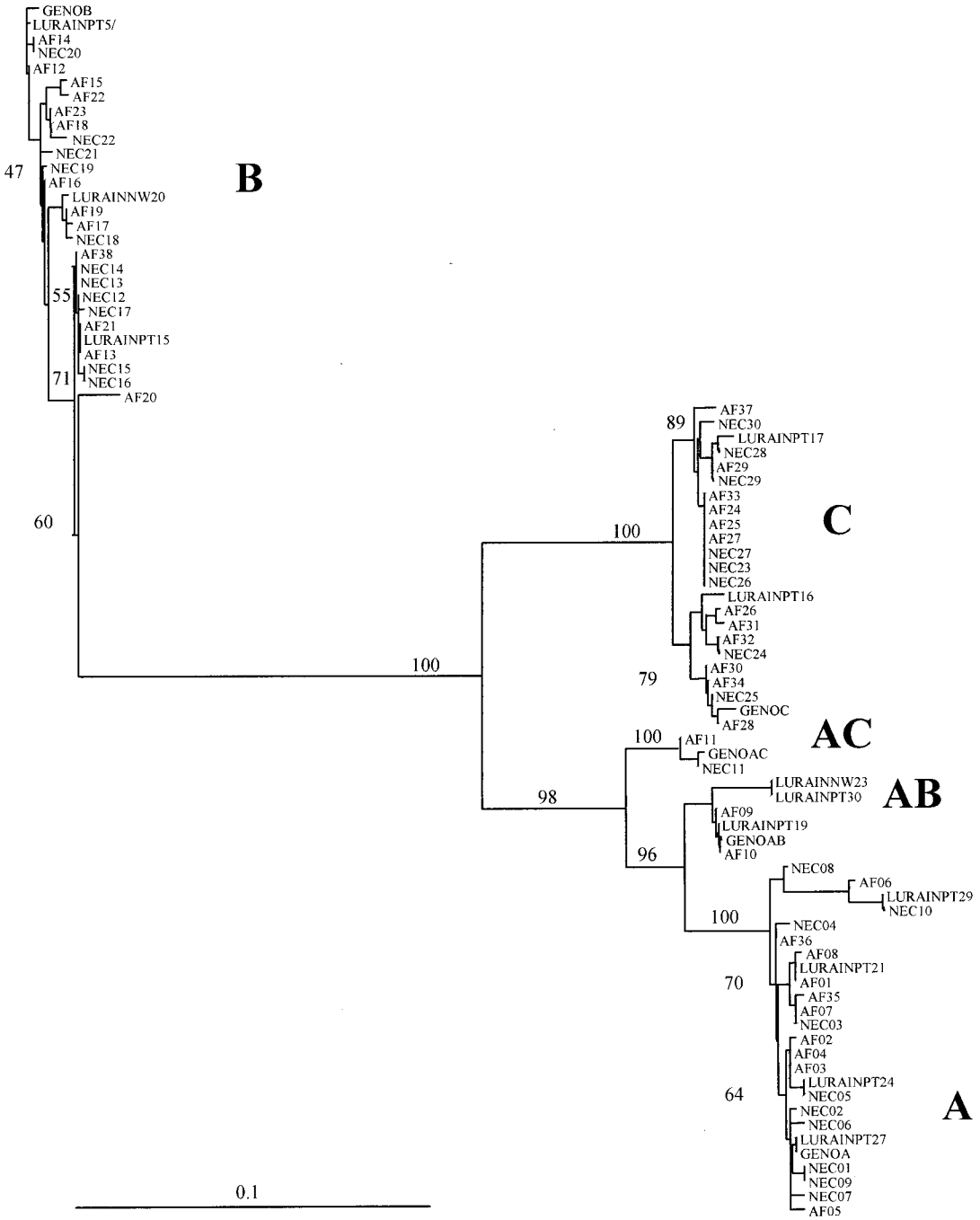

FIG. 1. Phylogenetic analysis of UL144 gene. HCMV strains obtained from congenitally infected fetuses are reported as AF01 to AF38. HCMV strains obtained from the control group are reported as NEC01 to NEC30. Five reference sequences from Arav-Boger et al. (1) are reported as GENOA, -B, -C, -AB, and -AC, and 12 reference sequences from Lurain et al. (14) are reported as LURAINPT5, -PT15, -PT16, -PT17, -PT19, -PT21, -PT24, -PT27, -PT29, -PT30, -NW20, and -NW23. (For GenBank accession numbers, see the text.)

pregnancies were terminated because of ultrasonographic CMV-related severe abnormalities, and 11 pregnancies went to term. Among the 11 neonates, 9 were asymptomatic at birth and had not developed neurological problems at 6 months to 3 years of life. Two neonates were symptomatic at birth and developed severe neurological handicaps.

Detailed ultrasonographic data are reported in Table 1. The main ultrasonographic findings were cerebral ventriculomegaly ($n = 12$), echogenic bowel ($n = 12$), intrauterine growth retardation (IUGR) ($n = 8$), microcephaly ($n = 6$), cerebral calcifications ($n = 4$), and oligohydramnios ($n = 4$). Hydrops, hepatomegaly, cerebral cysts, and hyperechogenic ventricles were less frequent findings. According to our selection criteria, 25 fetuses were considered severely symptomatic and were

TABLE 2. UL144 genotype distribution among French HCMV strains

Case	No. (%) genotype:				
	A	B	C	AB	AC
Congenital infection (n = 38)	10 (26)	13 (34)	12 (32)	2 (5)	1 (3)
Asymptomatic (n = 11)	2 (18)	5 (45)	3 (27)	1 (10)	0
Symptomatic (n = 25)	6 (24)	8 (32)	9 (36)	1 (4)	1 (4)
Unclassified (n = 2)	2	0	0	0	0
Control group (n = 30)	10 (33)	11 (37)	8 (27)	0	1 (3)

classified in group 1, 11 fetuses were asymptomatic and were classified in group 2, and 2 fetuses were not classified because of the absence of follow-up.

UL144 genotyping results. The UL144 gene sequences of the 68 samples studied clustered in five genotypes, as previously described by Arav-Boger et al. and Lurain et al. (1, 14) (Fig. 1). The classification in genotypes A, B, C, AB, and AC used by Arav-Boger et al. (1) was also used in this series. These results were confirmed by a bootstrap value of ≥60%. Variabilities between genotypes A and C, C and B, and A and B were 15, 19, and 22%, respectively. Intravariability within each genotype varied between 0 and 5% and was mainly confined to the 5′ extremity of the gene.

The distribution of UL144 genotypes in the group of infected adults was as follows: 11 (37%), 10 (33%), 8 (27%), 0, and 1 (3%) for genotype B, A, C, AB, and AC, respectively. The UL144 genotype distribution was not significantly different among the three populations studied (symptomatic fetuses, asymptomatic fetuses, and control population) ($P > 0.05$) (Table 2).

DISCUSSION

The UL144 gene encodes a homologue of the herpesvirus entry mediator, which is a member of the TNF receptor superfamily (6), and the UL144 protein might act as a lure to divert the host immunity response. Variability in the UL144 gene nucleotide sequence, therefore, may affect HCMV virulence. Based on this hypothesis, Arav-Boger et al. (1) determined the UL144 genotypes in a population of infected fetuses and reported that infection with the least common UL144 genotypes (A, C, AC, and AB) was associated with an unfavorable outcome of the disease. They therefore suggested that polymorphisms in the UL144 gene may be associated with congenital HCMV disease.

In this study, we could identify the five UL144 genotypes previously described (1, 4, 14). Genotype B was also the most prevalent genotype recovered among French HCMV strains, as it had been previously described in the United States (1, 4, 14). However, the B genotype was less frequent in our population than in the U.S. population: 37% in our control group versus 51 (23 of 45) and 69% (34 of 49) in U.S. studies (4, 14). Alternatively, the prevalences of genotypes A and C were higher in the French population than in the United States, with 33 and 27%, respectively, in our control group versus 22 (10 of 45) and 9% (4 of 45) in the first U.S. study (14) and 4 (2 of 49) and 10% (5 of 49) in the second U.S. study (4). The prevalences of genotypes AB and AC were as low among French strains as in the United States (4, 14).

In our study, UL144 genotype distributions were similar among strains recovered from infected fetuses and among those obtained from infected adults. Thus, the information obtained on UL144 genotypes suggests that the strains recovered in congenital infection reflect the strains circulating in the French population and that no UL144 genotype is particularly associated with congenital HCMV infection. All genotypes except AC, which was found in only 4% of all strains, could be recovered both in asymptomatic and in symptomatic cases of congenital infection. Our results are therefore different from those of Arav-Boger et al. (1), who described an association between UL144 genotypes A and C and the severity of congenital infection. In our study, 45 (5 of 11) and 60% (15 of 25) of the strains detected in 11 asymptomatic fetuses and in 25 symptomatic fetuses, respectively, were genotype A or C. In the U.S. study, no strains of genotypes A and C were recovered from 10 asymptomatic infected fetuses, although 46% of the strains found in 13 symptomatic fetuses were genotype A or C. These results are probably explained by the conjunction of a lower prevalence of genotypes A and C in the U.S. population (4 to 22 and 9 to 10%, respectively) and the small size of the study groups. However, collecting samples from congenitally HCMV-infected fetuses in the absence of national screening policies is challenging, explaining the difficulty in gathering a larger series of samples.

In conclusion, the UL144 genotype does not seem to carry any definite prognostic value in infected fetuses. All five UL144 genotypes can be vertically transmitted from mothers to fetuses, and all can cause symptomatic congenital infection. It is therefore not relevant to test for UL144 genotypes in this context. Other HCMV polymorphisms, such as glycoprotein B genotypes, have been evaluated in congenital infection and also proved to be disappointing (2, 3, 5, 13, 15, 21). Moreover, it was recently demonstrated that the inter- and intragenic variability of HCMV clinical isolates leads to an infinite number of genetic combinations, probably explaining the failure to use sequence information to predict disease outcome (16). Alternatively, specific host genetic factors probably explain the severity of congenital HCMV infection in some individuals (11), and more efforts probably should target this approach.

REFERENCES

1. **Arav-Boger, R., R. E. Willoughby, R. F. Pass, J. C. Zong, W. J. Jang, D. Alcendor, and G. S. Haymard.** 2002. Polymorphisms of the cytomegalovirus (CMV)-encoded tumor necrosis factor alpha and beta chemokine receptors in congenital CMV disease. J. Infect. Dis. **186:**1057–1064.
2. **Arista, S., S. De Gracia, G. M. Giammanco, P. Di Carlo, and E. Iannitto.** 2003. Human cytomegalovirus glycoprotein B genotypes in immunocompetent, immunocompromised, and congenitally infected Italian populations. Arch. Virol. **148:**547–554.
3. **Bale, J. F., J. R. Murph, G. J. Demmler, J. Dawson, J. E. Miller, and S. J.**

Petheram. 2000. Intrauterine cytomegalovirus infection and glycoprotein B genotypes. J. Infect. Dis. **182:**933–936.
4. **Bale, J. F., S. J. Petheram, M. Robertson, J. R. Murph, and G. Demmler.** 2001. Human cytomegalovirus A sequence and UL144 variability in strains from infected children. J. Med. Virol. **65:**90–96.
5. **Barbi, M., S. Binda, S. Caroppo, V. Primache, P. Dido, P. Guiditto, C. Corbetta, and D. Melotti.** 2001. CMV gB genotypes and outcome of vertical transmission: study on dried blood spots of congenitally infected babies. J. Clin. Virol. **21:**75–79.
6. **Benedict, C. A., K. D. Butrovich, N. S. Lurain, J. Corbeil, I. Rooney, P. Schneider, J. Tschopp, and C. F. Ware.** 1999. A novel viral TNF receptor superfamily member in virulent strains of human cytomegalovirus. J. Immunol. **162:**6967–6970.
7. **Cha, T. A., E. Tom, G. W. Kemble, G. M. Duke, E. S. Mocarski, and R. R. Spaete.** 1996. Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains. J. Virol. **70:**78–83.
8. **Gaytant, M. A., E. Steegers, B. A. Semmekrot, H. Merkus, and J. M. Galama.** 2002. Congenital cytomegalovirus infection: review of the epidemiology and outcome. Obstet. Gynecol. Surv. **57:**245–256.
9. **Gouarin, S., E. Gault, A. Vabret, D. Coite, F. Rozenberg, L. Grangeot-Keros, P. Barjot, A. Garbarg-Chenon, P. Lebon, and F. Freymuth.** 2002. Real-time PCR quantification of human cytomegalovirus DNA in amniotic fluid samples from mothers with primary infection. J. Clin. Microbiol. **40:**1767–1772.
10. **Lazzarotto, T., S. Varani, B. Guerra, A. Nicolosi, M. Lanari, and M. P. Landini.** 2000. Prenatal indicators of congenital cytomegalovirus infection. J. Pediatr. **137:**90–95.
11. **Lee, S. H., A. Zafer, Y. de Repentigny, R. Kothary, M. L. Tremblay, P. Gros, P. Duplay, J. R. Webb, and S. M. Vidal.** 2003. Transgenic expression of the activating natural receptor Ly49 confers resistance to cytomegalovirus in genetically susceptible mice. J. Exp. Med. **197:**515–526.
12. **Leruez-Ville, M., M. Ouachée, R. Delarue, A. S. Sauget, S. Blanche, A. Buzyn, and C. Rouzioux.** 2003. Monitoring cytomegalovirus infection in adult and pediatric bone marrow transplant recipients by real time PCR assay performed with blood plasma. J. Clin. Microbiol. **41:**2040–2046.
13. **Lukacsi, A., B. Tarodi, E. Endreffy, A. Babinszki, A. Pal, and R. Pusztai.** 2001. Human cytomegalovirus gB genotype 1 is dominant in congenital infections in south Hungary. J. Med. Virol. **65:**537–542.
14. **Lurain, N. S., K. S. Kapell, D. D. Huang, J. A Short, J. Paintsil, E. Winkfield, C. A. Benedict, C. F. Ware, and J. W. Bremer.** 1999. Human cytomegalovirus UL144 open reading frame: sequence hypervariability in low-passage clinical isolates. J. Virol. **73:**10040–10050.
15. **Picone, O., J. M. Costa, M. Leruez-Ville, and Y. Ville.** Cytomegalovirus glycoprotein B genotypes and CMV DNA viral load in amniotic fluid of congenitally infected fetuses: are these prognosis factors? Prenat. Diagn., in press.
16. **Rasmussen, L., A. Geissler, and M. Winters.** 2003. Inter- and intragenic variations complicate the molecular epidemiology of human cytomegalovirus. J. Infect. Dis. **187:**809–819.
17. **Revello, M. G., M. Zavattoni, M. Furione, F. Baldanti, and G. Gerna.** 1999. Quantification of human cytomegalovirus DNA in amniotic fluid of mothers of congenitally infected fetuses. J. Clin. Microbiol. **37:**3350–3352.
18. **Revello, M. G., M. Zavattoni, A. Sarasini, F. Baldanti, C. De Julio, L. De-Giuli, U. Nicolini, and G. Gerna.** 1999. Prenatal diagnostic and prognostic value of human cytomegalovirus load and IgM antibody response in blood of congenitally infected fetuses. J. Infect. Dis. **180:**1320–1323.
19. **Revello, M. G., and G. Gerna.** 2002. Diagnosis and management of human cytomegalovirus infection in the mother, fetus and newborn Infant. Clin. Microbiol. Rev. **15:**680–715.
20. **Smith, C. A., T. Farrah, and R. G. Goodwin.** 1994. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell **76:**959–962.
21. **Trincado, D. E., G. Scott, P. A. White, C. Hunt, L. Rasmussen, and W. D. Rawlinson.** 2000. Human cytomegalovirus strains associated with congenital and perinatal infections. J. Med. Virol. **61:**481–487.