Strong Cell-Mediated Immune Response to Human Cytomegalovirus Is Associated With Increased Risk of Fetal Infection in Primarily Infected Pregnant Women

Alda Saldan,1,a Gabriella Forner,1,a Carlo Mengoli,1,a Nadia Gussetti,2 Giorgio Palù,1 and Davide Abate1

1Department of Molecular Medicine, and 2Padua Reference Center for Infections in Pregnancy, Padua General Hospital, University of Padua, Italy

Background. Human cytomegalovirus (CMV) represents one of the leading causes of congenital infections worldwide. Early diagnosis of fetal infection and consequent rapid therapeutic intervention with immunoglobulin treatment may prevent fetal transmission and virus-related sequelae. In this study, the cell-mediated immunity and immunoglobulin avidity were evaluated as potential predictors of congenital transmission of the infection.

Methods. CMV immunoglobulin G (IgG) avidity and CMV enzyme-linked immunospot (ELISpot) assays were employed in 80 pregnant women including 57 primary and 23 nonprimary CMV infections. Congenital infection was assessed using CMV DNA quantitative polymerase chain reaction on amniotic fluid or offspring urine. Logistic regression and receiver operating characteristic statistical methods were employed to determine the association with congenital infection.

Results. Low CMV IgG avidity (25%) alone correlated with a probability of congenital transmission of 18.2% (95% confidence interval, 7.7%–28.8%). In contrast to the expectations, an increase in CMV ELISpot levels was statistically associated with congenital transmission ($P = .006$). The combined use of CMV ELISpot and low CMV IgG avidity resulted in a higher level of association than either method alone with the incidence of fetal transmission (area under the curve, 0.8685).

Conclusions. CMV-specific cell-mediated immunity represents a relevant marker in assessing the likelihood of congenital CMV transmission, particularly in combination with CMV IgG avidity.

Keywords. human cytomegalovirus; pregnancy; cell-mediated immunity; ELISPOT; fetal infection.
development [12–20], whereas few studies focused on cell-mediated immunity [21, 22]. It is well known from transplantation settings that the cellular arm of immunity, principally T-cell–mediated immunity, is crucial for control of virus infection [23–27]. In this study, the ability of both humoral and cellular immunity to predict congenital transmission was evaluated. It was found that low avidity and high levels of cell-mediated immunity correlate with congenital CMV transmission during primary maternal infection.

Thus, the determination of CMV cell-mediated immunity in pregnant women represents a novel and promising noninvasive biomarker to assess on an individual basis the risk of transmitting the infection to the offspring.

**MATERIALS AND METHODS**

**Patients, Definitions, and Enrollment Criteria**

Eighty pregnant white women were referred to the Padua Reference Center for Infections in Pregnancy for clinical and diagnostic investigations concerning a possible active CMV infection with a risk of transplacental transmission to the fetus. The median age of pregnant women was 31 years (range, 17–42 years). The pregnant women were classified as primary CMV infection (57) and nonprimary CMV infection (relapse or reinfection, 23).

Primary maternal CMV infection was defined by (1) seroconversion in previously seronegative mothers or (2) detection of maternal CMV immunoglobulin M (IgM) and concomitant low maternal CMV immunoglobulin G (IgG) avidity (<25%). Nonprimary CMV infection was defined by the presence of CMV viruria in already CMV IgG-positive pregnant women and detection of CMV IgG avidity >45% within the 14th week of gestation. All the serologic and molecular tests were performed at the Padua General Hospital Microbiology and Virology diagnostic laboratory. Exclusion criteria were (1) the presence of any existing or acquired immunodeficiency and (2) primary CMV infection after the 20th week of gestation. In primarily infected pregnant women, the estimated timing of CMV infection occurred within a median of 6 weeks of gestation (range, 0–20 weeks), and CMV enzyme-linked immunosorbent assay (ELISpot) was performed within a median of 8 weeks (range, 2–17 weeks) after the CMV infection. For nonprimary infections, it was not possible to determine the timing of reactivation/reinfection, and CMV ELISpot was performed within a median of 1 week (range, 1–4 weeks) after the detection of CMV DNA in maternal urine. Of the women experiencing primary CMV infection, 16 of 57 (28%) transmitted the infection to the fetus, 19 of 57 (33%) had episodes of viremia, and 43 of 57 (75%) had viruria. Of the 23 women with nonprimary infections, no case of CMV viremia was reported, all experienced CMV viruria, and no cases of congenital transmission occurred. No statistically significant differences were found in age or presence of comorbidities between the transmitting and nontransmitting women. Moreover, the 2 groups had comparable levels of T cells/peripheral blood mononuclear cells (PBMCs) (approximately 72%). No case of symptomatic CMV infection at birth was found. Active fetal or newborn CMV infection was assessed by CMV DNA detection by quantitative polymerase chain reaction (qPCR) in amniotic fluid at 20–21 gestational weeks or urine at birth [15, 28, 29]. The study was approved by the Padua General Hospital Ethical Committee.

**Detection of CMV DNA, CMV IgM and IgG, CMV IgG Avidity, and CMV ELISpot Tests**

Maternal CMV IgM and CMV IgG (Siemens Immulite) and Maternal CMV IgG avidity (Technogenetics) were determined according to manufacturer instructions. CMV IgG avidity was calculated as percentage of undissociated CMV IgG/total CMV-specific IgG. CMV DNA detection in maternal whole blood, amniotic fluid, and maternal and newborn urine was performed using real-time qPCR [30]. The lowest limit of CMV DNA detection is <1000 copies/mL. PBMCs were Ficoll purified and stimulated with overlapping peptides spanning the CMV pp65 (ppUL83) protein (Autoimmun Diagnostika) in an interferon (IFN)-γ ELISpot assay as previously described [31]. The maximum CMV pp65-ELISpot value was limited to 1000 spots per 2 × 10^5 PBMCs, as 1000 spots represent the saturating concentration of the ELISpot well. The reported CMV ELISpot data are expressed as number of spots per 2 × 10^5 PBMCs.

**Statistical Analysis**

All data were statistically analyzed using Stata 13 (StataCorp 2013). The adjusted predictions included the mean value and 95% confidence interval (CI).

All analyses using the binary variable “transmission” as outcome were performed on the 80 pregnant women with primary or nonprimary CMV infection. The logistic regression of congenital CMV transmission (dependent variable) under the effect of CMV ELISpot and low avidity (<25%) was applied to determine statistically significant associations. A P value <.05 was considered significant.

The diagnostic accuracy toward the clinical condition of congenital CMV transmission (active neonatal infection within the first 3 weeks of life) was also evaluated. A conventional nonparametric receiver operating characteristic (ROC) curve analysis was performed on CMV ELISpot and CMV IgG avidity. The best cut-point was estimated by looking for the highest Youden index, which is sensitivity – (1 – specificity), at each candidate cutoff [32]. As reference, the score at Q-point was reported as well; the Q-point is the point on the ROC curve, where sensitivity equals specificity and is located where the diagonal line running from the top left corner to the lower right corner intersects the ROC curve [33]. Predictive margins with 95% CI, after
logistic regression, where the dependent variable was the CMV transmission and the explanatory variables were CMV ELISpot value and CMV IgG avidity, were employed to assess the risk of congenital CMV transmission under the circumstances where low CMV IgG avidity scored 1 (avidity <25%) or 0 (≥25%). This CMV IgG avidity cutoffs were chosen after the distribution analysis of this variable. In addition, the combined diagnostic utility (AUC ROC) of the 2 tests was explored after logistic regression. In this case, the combined predictive value of CMV ELISpot and low avidity (<25%) on the active CMV neonatal infection, using the parameter congenital CMV transmission as reference outcome variable, was employed.

RESULTS

The probability of giving birth to an actively infected offspring as predicted by anti-CMV IgG avidity assay alone is shown in Figure 1A. As expected, the probability declined with the avidity increment. At avidity = 25%, the probability was 18.2% (95% CI, 7.7%–28.8%). The probability of giving birth to an actively infected infant by CMV ELISpot is shown in Figure 1B. As expected, the probability declined with the ELISpot increment. At ELISpot = 200, the probability was 18.2% (95% CI, 7.7%–28.8%).

Figure 1. Adjusted prediction of fetal transmission by cytomegalovirus (CMV) immunoglobulin G (IgG) avidity (A) or CMV enzyme-linked immunospot assay (ELISpot) (B) alone. CMV IgG avidity is expressed as percentage of undisassociated CMV IgG/total CMV IgG. CMV ELISpot is expressed as number of spots per $2 \times 10^5$ peripheral blood mononuclear cells. The central line displays the mean; gray areas represent 95% confidence interval (CI).
infected offspring was examined using the CMV ELISPOT assay alone (Figure 1B). As evident, the probability of congenital transmission increased as the CMV ELISPOT increased. The line of the mean crossed the probability of 0.5 at an approximate value of 568 per $2 \times 10^5$ PBMCs. Results to pp65 antigen was consistent with the response to the CMV whole virus lysate.

**Figure 2.** Receiver operating characteristic (ROC) curve of immunoglobulin G (IgG) avidity (A) or cytomegalovirus (CMV) enzyme-linked immunospot assay (ELISPOT) (B) in predicting congenital CMV infection. The diagonal line is the reference line of no diagnostic effectiveness. The numbers above the CMV ELISPOT and IgG avidity indicate a series of candidate cutoffs. To avoid the CMV IgG avidity curve below the reference diagonal line, it was mathematically transformed into the complementary value $100 -$ avidity percentage; however, the numbers in the figure indicate the CMV IgG avidity percentage, not its complement.
Flow cytometry analysis revealed that the vast majority of CMV ELISpot responses were CMV pp65-specific CD3+CD8+IFN-γ+ T cells (data not shown). Moreover, the levels of CMV-specific T cells of transmitting women remained consistently high over time (data not shown). To assess the ability of the assays to detect a neonatal active CMV infection, the nonparametric ROC analysis was applied both to the CMV IgG avidity and CMV ELISpot; the results in Figure 2 showed that CMV IgG avidity had an AUC of 0.72 (95% CI, .60–.83; standard error [SE] = 0.06), whereas the CMV ELISpot had an AUC of 0.81 (95% CI, .69–.92; SE = 0.06). The best estimated cutoff using the maximum Youden index was 185 per 2 × 10^5 PBMCs for CMV ELISpot (74% of correct classification) and 21% for CMV IgG avidity (66% of correct classification). The Q-points were 187 per 2 × 10^5 PBMCs and 16% for CMV ELISpot and CMV IgG avidity, respectively. The logistic regression, performed on 80 pregnant patients with primary or nonprimary CMV infection, detected a significant correlation between high CMV ELISpot levels and low avidity in relation to neonatal infection (Table 1). The cutoff of low avidity was set at <25% after the distribution analysis of CMV IgG avidity, displaying a clear bimodal pattern (Supplementary Figure 1). The congenital CMV transmission has been evaluated considering as a dependent variable the congenital transmission, and as explanatory variables CMV ELISpot values and CMV IgG avidity (Figure 3); when avidity was low (<25%), at CMV ELISpot values = 445 spots per 2 × 10^5 PBMCs, the probability of an actively infected neonate crossed the line of 50%. With avidity ≥25%,

Table 1. Logistic Regression of Cytomegalovirus Transmission

<table>
<thead>
<tr>
<th>Congenital CMV Transmission</th>
<th>Coefficient</th>
<th>SE</th>
<th>z</th>
<th>P Value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV-ELISpot</td>
<td>0.004</td>
<td>0.001</td>
<td>2.75</td>
<td>.006*</td>
<td>.001–.007</td>
</tr>
<tr>
<td>Low CMV IgG avidity (&lt;25%)</td>
<td>2.515</td>
<td>1.090</td>
<td>2.31</td>
<td>.021*</td>
<td>.378–4.652</td>
</tr>
<tr>
<td>Intercept</td>
<td>−4.255</td>
<td>1.100</td>
<td>−3.87</td>
<td>.000</td>
<td>−6.412 to −2.099</td>
</tr>
</tbody>
</table>

Logistic regression of CMV transmission (dependent variable) under the effect of CMV ELISpot and low avidity (<25%). Log likelihood = −26.944688, LR $\chi^2(2) = 23.38$, $P = .0000$. Pseudo $R^2 = 0.3026$.

Abbreviations: CI, confidence interval; CMV, cytomegalovirus; ELISpot, enzyme-linked immunospot assay; IgG, immunoglobulin G; LR, likelihood ratio; SE, standard error.

*Indicates significant effect.

Figure 3. Predictive margins after logistic regression, where the dependent variable was the cytomegalovirus (CMV) transmission, and the explanatory variables were CMV enzyme-linked immunospot assay (ELISpot) score and CMV immunoglobulin G avidity. The bars show the 95% confidence interval (CI). CMV ELISpot is expressed as number of spots per 2 × 10^5 peripheral blood mononuclear cells.
the curve was never significantly different from probability level = 0. Thus, at high avidity, a low CMV ELISpot value correlated with a low likelihood of neonatal infection; whereas if CMV ELISpot value was high, a prediction was not possible. With linear regression likelihood ratio test applied to logistic regression, the combined use of CMV ELISpot and low CMV IgG avidity as predictors of the congenital CMV infection was more effective than either CMV ELISpot alone \((P = .0031)\) or IgG avidity alone \((P = .0018)\). A logistic ROC curve considering the combined predictive value of CMV ELISpot value and low IgG avidity \(<25\%)\) was employed to evaluate the congenital CMV transmission endpoint (Figure 4). The combined assays had an AUC of 0.8685, which was greater than those of the mentioned variables used singly.

DISCUSSION

The major finding of this study is that congenital transmission of primary CMV infection is associated with strong cell-mediated responses to CMV in the presence of low CMV IgG avidity. Additional studies should be made to assess the role of CMV cell-mediated immunity in nonprimary CMV infections. There are several hypotheses and speculations that could be made to explain this phenomenon: In this study, the cell-mediated immunity assay was performed after the confirmed primary or nonprimary maternal infection. It is possible that the high levels of cell-mediated immunity associated with congenital transmission may be consequentially correlated to the duration and magnitude of the maternal viremia [34], so that higher and prolonged viral load in blood and/or urine may induce a stronger cell-mediated immune response. The second potential hypothesis is an indirect immune-mediated damage: high cell-mediated immune responses may induce a local proinflammatory status at the placental level sequentially leading to the expression of atypical molecules and receptors that may facilitate the transplacental virus passage [18, 35, 36]. A third speculation regards the potential dual role of the cell-mediated immunity during the primary or nonprimary infections: in primary infections, a strong cell-mediated immune response promotes congenital CMV infection, whereas in the nonprimary infections, the preexisting cell-mediated immunity may limit or impede congenital transmission. All these hypotheses and speculations need to be experimentally verified. This study highlights the relevance of the cell-mediated immunity to CMV and the consequences for congenital transmission and could also provide crucial insights in view of the CMV vaccine development. Moreover, this study shows that the detection of CMV specific cell-mediated immunity may be useful for the diagnosis of congenital CMV infection. The CMV ELISpot is a noninvasive procedure performed on blood-isolated PBMCs, and thus is much less invasive and risky compared with the amniocentesis presently required to assess congenital CMV transmission.

Supplementary Data

Supplementary materials are available at Clinical Infectious Diseases online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank Padua General Hospital–Azienda Ospedaliera di Padova for providing us with the IFN-γ ELISpot plates; Dr Edward S. Mocarski of Emory University School of Medicine, for the critical reading of the manuscript and discussion of the data; Drs Daniel Tinto and Federica Franceschi for technical assistance; and nurses Gelinda Chies, Alessandra Degli Agostini, Elisa Palazzin, and Cristina Sansone for their help with sample collection.

Author contributions. D. A., A. S., and C. M. analyzed the laboratory and clinical data and wrote the manuscript. A. S. and G. F. collected and analyzed the cytomegalovirus ELISpot and clinical data. G. P. and N. G. supervised the study. C. M. performed the statistical analysis.

Financial support. This work was supported by a University of Padova 60% grant to D. A. (60A07-8071/14 and 60A07-8934/13).

Potential conflicts of interest. All authors: No potential conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References
