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Slow Increase in IgG Avidity Correlates With Prevention of Human Cytomegalovirus Transmission to the Fetus

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Following primary human cytomegalovirus (HCMV) infection, virus-specific IgG antibody shift from low to high avidity with individual variations in the rate of avidity maturation. The kinetics of the avidity maturation of IgG specific for HCMV nuclear antigen in pregnant women with primary infection was investigated. Absorbance values used for avidity index calculation of 286 sequential sera collected from 69 pregnant women with primary HCMV infection were retrieved. Percent difference in absorbance values of IgG antibody bound to the solid phase after urea treatment (IgG avidity) between early (T1, 0–90, median 31 days) and late (T2, 91–180, median 136 days) serum samples was calculated for each woman. Three groups of women were identified: 24/69 (34.8%) women showed high (>100%) avidity increase between T1 and T2 (pattern H), 29/69 (42%) low (<50%) increase (pattern L), and 16/69 (23.2%) intermediate increase (pattern I). Avidity values in T1 samples were significantly higher in women with pattern L compared to women with pattern H ($P = 0.01$). Altogether, 28/69 (40.6%) women transmitted HCMV infection to their fetuses. Fetal infection preferentially occurred ($P < 0.01$) in women with pattern H (15/24, 62.5%) compared with women with pattern L (7/29, 24.1%). In conclusion, different patterns of IgG avidity maturation can be detected following primary HCMV infection. Pregnant women with pattern H (rapid IgG avidity increase) appear to be at higher risk for fetal infection, whereas, pregnant women developing early antibody with high avidity appear to be at a lower risk of vertical transmission. ***J. Med. Virol. 85:1960–1967, 2013.*** © 2013 Wiley Periodicals, Inc.

KEY WORDS: pregnancy; IgG avidity; congenital HCMV infection

INTRODUCTION

After more than 50 years since the first report, congenital human cytomegalovirus (HCMV) infection is still a major, albeit greatly unrecognized, health problem in developed countries [reviewed in Cannon and Davis, 2005; Kenneson and Cannon, 2007; de Vries et al., 2011]. The unavailability of a vaccine as well as of therapeutic intervention or preventative measures of proven efficacy are considered major obstacles to the implementation of serologic screening of women in childbearing age. As a consequence, knowledge and experience of health professionals is hampered and whenever a primary HCMV infection is diagnosed or suspected, seldomly the woman receives correct information.

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On the other hand, in European countries such as Italy, Germany, France, Belgium, where HCMV testing is performed, albeit sporadically, in pregnant women, a few groups/centers have developed a strong specific background either in the diagnosis and/or management of pregnancy complicated by primary HCMV infection. Indeed, major advances have been achieved in the development of new techniques/assays, while the interpretation of serologic/virologic results has greatly improved overtime. One of the major issues addressed in the last few years concerns the interpretation of HCMV-positive IgM antibody results provided by highly sensitive assays. The determination of HCMV-specific IgG avidity index (AI) is considered an important tool for interpretation of IgM positive results. In particular, IgG antibody with low AI are considered indicative of a recent (less than 3–4 months) primary infection, whereas IgG antibody with high AI indicate past infection (dating at least $\geq$6 months).

In addition to the diagnostic setting, HCMV-specific IgG avidity maturation has been considered important also from the pathogenetic standpoint. In fact, IgG of high avidity have been reported to protect the fetus from HCMV infection [Nigro et al., 2005; Maidji et al., 2006].

The main objectives of this retrospective study were: (i) to report distinct patterns of HCMV-specific IgG avidity maturation in pregnant women with primary HCMV infection and (ii) to correlate the IgG avidity maturation rate and the virologic outcome of pregnancy.

MATERIALS AND METHODS

Patients

Serologic and virologic data of 461 pregnant women with primary HCMV infection monitored at the Polyclinic during the period 2003–2009 were retrieved and analyzed. In order to be included in the present study, pregnant women had to satisfy the following criteria: (i) defined onset of primary infection; (ii) primary infection acquired in the first or second trimester of gestation; (iii) serologic follow-up for at least 3 months; and (iv) known virologic outcome of pregnancy. In addition, five non-pregnant immunocompetent subjects (two males) with symptomatic primary HCMV infection and comparable serologic follow-up were included as study controls.

Diagnosis of Primary Infection and Timing of Infection Onset

All sera were tested for HCMV-specific IgG and IgM antibody by ETI-CYTOK-G and ETI-CYTOK-M, respectively (DiaSorin, Saluggia, Italy) according to manufacturer's instruction. IgM results obtained by the ETI-CYTOK-M assay were confirmed by an in house-developed capture ELISA assay [Revello et al., 1991]. In addition, neutralizing (Nt) antibodies preventing infection of human epithelial cells ARPE-19 were determined, as reported [Gerna et al., 2008]. Primary infection was diagnosed based on the presence of at least two of the following four criteria: HCMV-specific IgG seroconversion, presence of IgM antibody, low IgG AI, and DNAemia [Revello et al., 2011]. Timing of infection onset was based on HCMV-specific seroconversion and other serologic (IgM and IgG AI) and virologic (DNAemia) findings, in association with presence of clinical signs/symptoms. In asymptomatic women, IgG and IgM antibody kinetics were considered in association with low IgG AI for determination of infection onset timing.

Definition of Time Points

In order to overcome the problem that multiple sera were collected from each subject at different sampling times, serologic follow-up was divided into two time intervals, namely 0–90, and >90 days after onset of infection. The median blood sampling day for each time interval was calculated for all HCMV-specific IgG-reactive sera. Then, one sample for the <90 (referred to as T1 sample) and one from the >90 (referred to as T2 sample) day interval were chosen for each woman. The T1 sample was the earliest IgG-reactive sample closest to the median day of collection in the 0–90 time interval. The T2 sample was closest to the median day of collection in the >90 days interval. Serologic results at T1 and T2 were used for subsequent analyses.

IgG Avidity Assay and Analysis of Avidity Results

HCMV-specific IgG avidity was determined by using an in-house developed ELISA, as previously reported [Revello et al., 2002, 2010]. Briefly, polystyrene microtiter plate wells were coated overnight with an optimal dilution of glycine-extracted HCMV nuclear antigen [Stagno et al., 1978] or control antigen. Wells were incubated with test sera diluted 1:500 for 1 hr and then washed with either washing buffer or washing buffer plus 6 M urea. After incubation with peroxidase-labeled goat anti-human IgG serum for 2 hr, chromogen/substrate solution was added and absorbance was read at 492 nm. HCMV-specific IgG content in the presence or absence of urea was expressed as net absorbance (difference between absorbance of wells coated with HCMV antigen and control antigen). An absorbance of 0.2 was considered the cut-off value for discriminating IgG-positive from IgG-negative sera. IgG avidity was expressed as absorbance of residually bound IgG after urea treatment (IgG + U), while the AI (used for diagnostic purposes) was calculated by dividing net absorbance in the presence by net absorbance in the absence of 6 M urea. Absorbances obtained in the absence of urea treatment were referred to as (IgG−U). In order to objectively quantify variations in (IgG + U) absorbance between T1 and T2 serum

samples, the following approaches were explored: (i) percent variations were calculated according to the formula [(IgG + U) absorbance at T2−(IgG + U) absorbance at T1/(IgG + U) absorbance at T1] × 100; (ii) IgG + U kinetics slopes including all available samples between T1 and T2 were constructed; and (iii) absolute differences in IgG + U absorbance between T1 and T2 were calculated.

Diagnosis of Congenital Infection

Diagnosis of congenital infection was made either antenatally by rapid virus isolation and DNA detection by PCR in amniotic fluid [Gerna et al., 1990; Revello et al., 1999] or postnatally by rapid virus isolation from urine collected within the first 2 weeks of life.

Statistical Analysis

Comparison of raw data between women with early high and low avidity patterns was performed by the Mann–Whitney *U*-test. The Kruskall–Wallis test was adopted for comparison of IgG + U kinetics slope and absolute difference in IgG + U absorbance between T1 and T2 in the three groups. The association between high and low avidity patterns and transmission of the infection to the fetus was analyzed by Fisher's exact test. A logistic regression (both uni- and multivariate) was also performed in order to take into account the possible association of other factors (gestational age, diagnostic criteria, IgG + U at T1 and difference in IgG−U absorbance between T1 and T2) with HCMV transmission to the fetus. Linear and nonlinear regression used to represent kinetics of serological parameters were compared by the extra sum-of-squares of F test.

RESULTS

Patients and Samples

Sixty-nine out of 461 (14.9%) pregnant women with primary infection met the inclusion criteria and were considered for retrospective analysis. Timing of infection onset was achieved in the great majority of women (61/69, 88%) based on seroconversion and other serologic and virologic findings, which were associated with presence of clinical signs/symptoms. In asymptomatic women (8/69, 12%) IgG and IgM antibody kinetics were considered in association with low IgG AI. Overall, 309 sequential sera (median 4, range 3–7 sera per woman) were available. Of these, 286 (92.5%) were HCMV-specific IgG-reactive (OD $\geq$ 0.2). Median T1 and T2 times were 31 (range 11–88) and 136 (range 91–202) days, respectively. As for the five non-pregnant individuals, 24 sequential sera were available, and T1 and T2 were set at 28 (range 13–37) and 139 (range 119–142) days after infection onset, respectively.

Patterns of IgG Avidity Maturation in Pregnant Women

When IgG absorbance values of (IgG + U) antibody (i.e., residual virus-specific IgG antibody binding to the solid-phase after urea dissociating treatment) at T1 and T2 were tested, a marked increase was observed in some women, whereas the increase was much less evident or even absent in some others. In order to quantify this phenomenon, the percent increase in (IgG + U) absorbance between T1 and T2 samples was calculated for each woman. In this way, three groups of women were identified, that is, women with a high (>100%), low ($\leq$ 50%) or intermediate (51–100%) increase (Fig. 1A). The median increase was 180.4% (103.7–610.0%), 12.8% (−31.8% to 50%), and 71.5% (52.4–95.8%) for women with high (n = 24), low (n = 29), and intermediate (n = 16) increase, respectively. These three groups of women were considered representative of different patterns of IgG avidity maturation, and were referred to as pattern H (high increase), pattern I (intermediate increase) and pattern L (low increase). The three groups were also different from each other when considering: (i) the slopes (Fig. 1B) of the regression line reporting the individual IgG + U absorbance kinetics (median 5 [range 2–16] × 10^{-3} OD/day for group H; 3 [0–11] × 10^{-3} for group I and 0 [−2–4] × 10^{-3} for group L; $P < 0.001$) and (ii) the absolute difference (Fig. 1C) between IgG + U absorbance at T1 and T2 (median 0.71 [range 0.28–1.67] OD for group H; 0.27 [0.11–1.00] for group I and 0.06 [−0.16 to 0.35] for group L; $P < 0.01$). Based on the better discriminatory capacity, percent variations in terms of IgG absorbance increase or decrease were used for subsequent analyses.

When HCMV-specific IgG absorbance values in the absence or presence of U treatment and the resulting AI were compared among the three groups at T1 and T2, significant differences were found between groups H and L (Fig. 2). Specifically, it was observed that while (IgG−U) absorbance values were comparable in the two groups at T1, they were significantly higher in group H at T2 (Fig. 2A). In addition, (IgG + U) values were significantly lower in group H at T1 and significantly higher at T2 (Fig. 2B). Finally, AIs were significantly lower in group H at T1 and significantly higher at T2 compared to group L (Fig. 2C). Finally, DNAemia levels were significantly higher at T1 in women of group H compared to women of group L (Fig. 2D).

In keeping with the above findings (obtained when only time points T1 and T2 were considered), different kinetics were observed when absorbance and AI values of all IgG-reactive sera available from women with patterns H and L were analyzed over the entire follow-up period (Fig. 3). In fact, in pattern H, absorbance of (IgG + U) was shown to increase over time, whereas in pattern L it remained substantially unchanged (Fig. 3, panels I H and I L). Moreover,

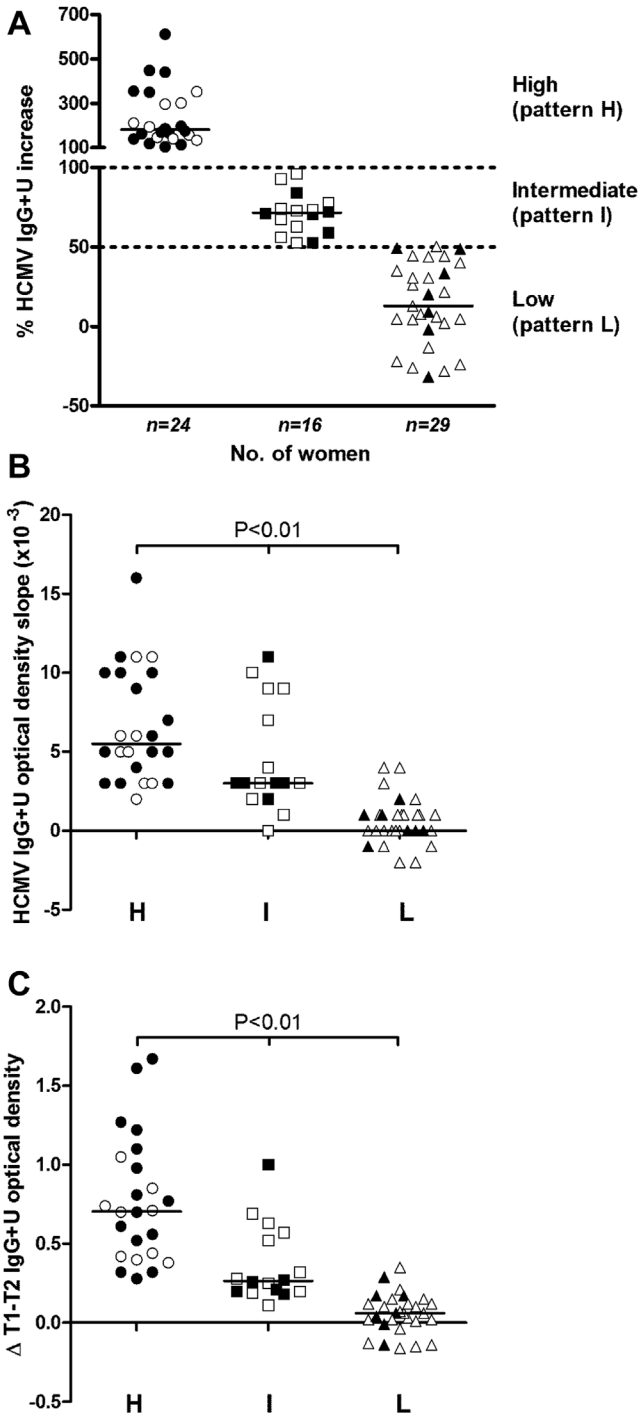

Fig. 1. **A**: ELISA IgG antibody absorbance increase (maturation) following treatment with 6 M urea. Pregnant women with high, intermediate, and low IgG antibody absorbance increase correspond to patterns H, I, and L of IgG avidity maturation as reported in the text. **B**: Slopes of the regression line reporting the IgG + U absorbance kinetics for the three groups. **C**: Absolute difference between IgG + U absorbance at T1 and T2 for the three groups. Women transmitting infection to the fetus are indicated with closed symbols, whereas non-transmitting women are indicated with open symbols.

(IgG–U) absorbance values were high and did not vary over the entire period for women with pattern H, whereas they slowly decreased over time in women with pattern L (Fig. 3, panels II H and II L). As a consequence, a steady maturation process from low to high AI values was observed for women with pattern H, whereas in women with pattern L the maturation was much less pronounced, and AI values tended to linger in the intermediate range over the entire follow-up period, showing a significantly ($P < 0.0001$) different lower slope (Fig. 3, panels III H and III L). Intermediate kinetics were observed in women of group I (data not shown).

No significant difference in age, parity, trimester of gestation at HCMV infection, or time interval between T1 and T2 was noted among the three groups (Table I). However, and most notably, a striking difference between group H and L was observed in the virologic outcome of pregnancy. In fact, as many as 15/24 (62.5%) women from group H transmitted the infection to their fetuses compared to 7/29 (24.1%) women from group L ($P < 0.01$, odds ratio 5.2). Six out of 16 (37.5%) women with an intermediate increase (pattern I) transmitted the infection (Table I). The criteria adopted for diagnosis of primary infection (laboratory findings alone versus association with clinical signs/symptoms), gestational trimester at the onset of infection, and IgG + U absorbance at T1 were not associated with transmission of the infection to the fetus, whereas ΔT1–T2 IgG–U absorbance showed a trend towards a possible correlation with HCMV transmission (Table II). However, in multivariate analysis, the IgG + U maturation pattern remained the only parameter (Table II) associated with the outcome ($P = 0.02$; odds ratio 4.7). No significant difference was observed between transmitter (n = 28) and non-transmitter women (n = 41) at T1 and T2 time points for both IgM and neutralizing antibody (data not reported).

Of the five non-pregnant subjects examined, two had increases of 400% and 357%, respectively, in (IgG + U) absorbance (pattern H), two showed <50% increase (pattern L), while in the remaining subject, the absorbance increase (pattern I) was intermediate (62%, data not shown). All five immunocompetent individuals had clinical symptoms and/or signs, that is, high grade fever, elevated liver enzymes, severe headache, and prolonged asthenia. No relationship between pattern of IgG avidity increase and clinical outcome was observed in these patients.

DISCUSSION

Finding maternal prognostic markers of HCMV transmission to the fetus during primary HCMV infection is one of the major concerns of researchers working in this area. In this study, it was found that the calculation of the percent variation in IgG absorbance values following urea treatment at two time points (T1 and T2) allows identification of different

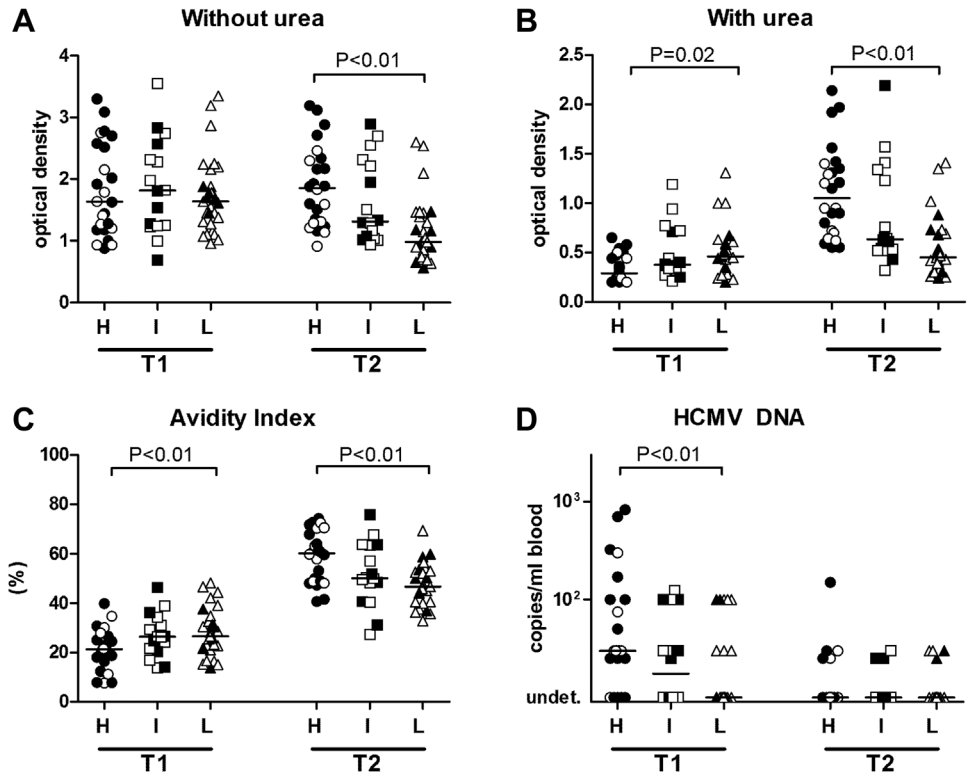

Fig. 2. HCMV-specific ELISA IgG antibody reactivity in the absence or presence of urea dissociating treatment in three groups of pregnant women with H, L, and I avidity patterns at T1 and T2 time points. T1 and T2 represent the median blood sampling time at 31 (range 1–88) days for T1, and at 136 (91–202) days for T2. The Mann–Whitney *U*-test was used for statistical analysis of patterns H and L. *P*-values were indicated when significant. Closed symbols indicate women transmitting virus to the fetus, while open symbols refer to women not transmitting infection.

patterns of IgG avidity maturation. These patterns seem to be associated with a different HCMV transmission rate. In pattern H, an increase greater than 100% in the amount of IgG bound to the solid phase after urea treatment was observed between the two time points, which was associated with a rather stable amount of IgG in control (urea-untreated) wells. As a result, AI values increased in about 3–4 months. On the other hand, in pattern L, (IgG + U) absorbance showed little variation (<50% increase), while IgG absorbance values in the absence of urea treatment declined slowly overtime. Hence, the resulting AI eventually increased, although at a significantly slower rate compared to pattern H. Pattern I was intermediate, in that kinetics of IgG avidity maturation was less defined. The majority of women with a suitable follow-up were shown to fall within either pattern H or L with a comparable frequency (about 40% each).

The most remarkable finding, however, was observed when the vertical transmission rate in group H and group L was analyzed. In fact, vertical transmission occurred at a significantly higher rate in women with pattern H (62.5%) compared to women with pattern L (24.1%). Thus, apparently, women with a slower maturation of IgG avidity were less prone to transmit the infection. However, it must be noted that the amount of IgG that remained bound to the solid phase after urea removal at T1 (i.e., early after the onset of infection) was significantly higher in pattern L (i.e., in women less likely to transmit the infection) than in pattern H. Moreover, since IgG absorbance in the absence of urea was comparable in pattern H and L at T1, also the resulting AIs were significantly higher at T1 in women with pattern L as compared to women with pattern H. Opposite results were observed at T2 in that AIs were significantly higher in women with pattern H as compared to women of pattern L.

A possible reason for the higher IgG antibody reactivity observed at T2 in transmitter mothers could be that the immune system of pregnant women carrying infected fetuses is more stimulated resulting in a more intense IgG antibody production [Alford et al., 1988]. However, the fact that in our study pattern H could be observed both in pregnant women who did not transmit the infection, as well as in non-pregnant subjects (although numbers are too small to allow conclusive remarks), potentially argues against such an explanation.

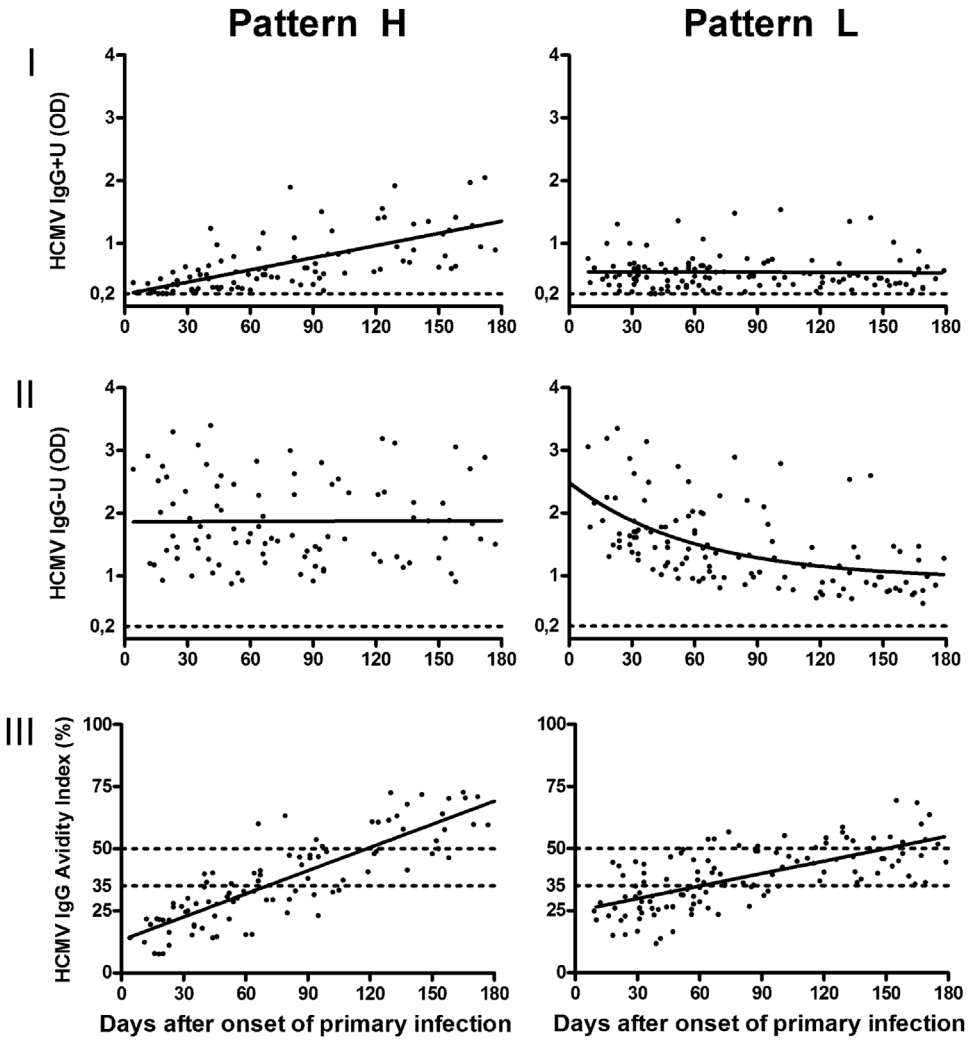

Fig. 3. HCMV ELISA absorbance (OD) of IgG antibody after urea treatment (IgG + U; panels I H, and L), IgG antibody without urea treatment (IgG–U; panels II H, and L), and IgG avidity index (AI, panels III H, and L) during the follow-up period of 24 women with pattern H and 29 women with pattern L. Discontinuous horizontal lines indicate ELISA IgG cut-off in panels I H and L, and II H and L, while they indicate the upper limit of the low (35%) and the intermediate (50%) avidity index in panels III H and L, respectively. Patterns H and L were different in the presence of urea (I) and in the absence of urea (II), while the linear regression curves representing the kinetics of IgG avidity index in patterns III H and L had a significantly different slope ($P < 0.0001$, extra sum-of-squares of F-test).

TABLE I. Characteristics of the Three Groups of Pregnant Women Showing Different HCMV-Specific IgG Avidity Patterns

IgG + U pattern	No. of women	Median age in years (range)	Parity			Trimester of gestation at onset		Median day after the onset (range)		Congenital HCMV infection (%)	
			0	≥1	NA	I	II	T1	T2		
H	24	32 (17–41)	10	13	1	18	6	29 (11–56)	138 (91–202)	15/24 (62.5)	$P < 0.01$[a]
L	29	34 (19–38)	9	18	2	20	9	31 (12–67)	135 (93–169)	7/29 (24.1)	
I	16	31 (20–39)	8	7	1	14	2	36 (13–88)	126 (100–152)	6/16 (37.5)	
Total	69	33 (17–41)	27	38	4	52	17	31 (11–88)	136 (91–202)	28/69 (40.6)	

NA, not available.
[a]Fisher exact test. All the other parameters (age, parity, gestational age at onset, T1 and T2) were not significantly different between women with H and L IgG + U patterns.

TABLE II. Logistic Regression Analysis of Factors Possibly Associated With HCMV Transmission to the Fetus

	Univariate		Multivariate	
Parameter	Odds ratio	*P*-value	Odds ratio	*P*-value
Diagnostic criteria of primary HCMV infection (laboratory findings only vs. laboratory findings + signs/ symptoms)	1.04	0.97	NA	
Gestational age at onset of infection (2nd trimester vs. 1st trimester)	1.03	0.95	NA	
IgG + U absorbance at T1	0.44	0.46	NA	
ΔT1–T2 IgG–U absorbance	2.04	0.10	1.16	0.76
IgG + U increase pattern (H vs. L)	5.23	<0.01	4.74	0.02

NA, not applicable.
Only parameters with *P*-value ≤ 0.10 were included in multivariate analysis.

Another hypothesis takes into consideration the clinical relevance of primary infection. A more intense and prolonged IgG antibody response has been reported in subjects with clinically apparent primary infection compared to patients with subclinical infection [Hayes et al., 1987; Alford et al., 1988]. Again, our data do not fully support such an explanation as clinical findings in our population of pregnant women (as well as in healthy controls) were distributed in the three groups.

Finally, it is possible that the differences in IgG kinetics observed between pattern H and L could reflect individual variation in IE-specific IgG response, which in turn, could be related to active viral replication [Gerna et al., 1978; Alford et al., 1988]. In the present study, higher amounts of viral DNA in blood were detected at T1 in women with pattern H compared to women with pattern L.

The considered hypothesis is that a higher and more sustained viral replication (i.e., higher antigen load) occurring in women with pattern H, leads to a more prolonged stimulation of antibody production (greater IgG–U level at T2) by both short-living plasma cells (that are continuously generated) and long-living plasma cells, and to a higher avidity (i.e., IgG + U absorbance) maturation. Instead, in women with pattern L, more rapid antigen clearance occurs that, in turn, induces an early disappearance of short-living plasma cells. Thus, the antibody production is sustained to a lower level by long-living plasma cells only, and antibody production is maintained at high levels only for the first months after infection onset. In parallel, the higher viral load of group H women could also lead to a higher risk of virus transmission to the fetus.

In addition, it should be taken into account that the assays used in this study measure IgG directed to HCMV nuclear antigen (mainly comprising IE antigens and pp65) and these antibodies are likely not to have a protective role against HCMV dissemination, but just reflect the level of viral replication. At this time, studies on the protective role of IgG specific for the HCMV glycoprotein complexes (gB, gH/gL, gH/gL/pUL128-131) are ongoing [Lilleri et al., 2012, 2013].

In conclusion, different kinetics in IgG antibody response and avidity increase were found in immunocompetent (pregnant and non-pregnant) individuals following primary HCMV infection. Moreover, vertical transmission appeared to be associated with a particular pattern of IgG antibody response. When considering that >90% fetuses become infected within 6–8 weeks after maternal infection [Revello and Gerna, 2004], it is reasonable to hypothesize that differences in the early maternal antibody response might have a critical impact on the virologic outcome of pregnancy. Ad hoc prospective studies are warranted to confirm findings of the present report.

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