

## INTRAUTERINE TRANSMISSION OF CYTOMEGALOVIRUS TO INFANTS OF WOMEN WITH PRECONCEPTIONAL IMMUNITY

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### ABSTRACT

**Background** Preconceptional immunity against cytomegalovirus (CMV) provides only partial protection against intrauterine transmission of the virus. Whether congenital CMV infection in the offspring of women who are seropositive for CMV can occur after maternal reinfection with a different strain of CMV is unknown.

**Methods** Serum specimens from 46 women with preconceptional immunity against CMV that were obtained during the previous pregnancy and the current pregnancy were analyzed for antibodies against the strain-specific epitopes of CMV glycoprotein H. Virus-neutralizing activity in maternal serum samples was measured against the AD169 laboratory strain of CMV and the CMV isolates available from seven infected infants. In addition, the nucleotide sequences of the glycoprotein H gene from the seven CMV isolates were determined.

**Results** Eleven of the 16 mothers with infected infants (69 percent) had antibodies against the glycoprotein H epitopes present on two laboratory strains of CMV, AD169 and Towne. Ten of the 16 mothers with infected children (62 percent) acquired new antibody specificities against glycoprotein H, as compared with only 4 of the 30 mothers of uninfected infants (13 percent,  $P < 0.001$ ). The samples obtained at the time of the current delivery from four of the seven mothers contained at least twice as many neutralizing antibodies against the CMV isolated from their infants as were present in the samples obtained at the previous delivery. The specificity of the newly acquired maternal antibodies reflected the amino acid sequence of the glycoprotein H epitope of CMV from these four infants.

**Conclusions** In women who are seropositive for CMV, reinfection with a different strain of CMV can lead to intrauterine transmission and symptomatic congenital infection. (N Engl J Med 2001;344:1366-71.)

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**C**ONGENITAL cytomegalovirus (CMV) infection is a leading cause of brain damage and sensorineural hearing loss in children in the United States.<sup>1-3</sup> Unlike preconceptional immunity against rubella or toxoplasmosis, preconceptional immunity against CMV provides only partial protection from intrauterine transmission of the virus,<sup>2,4,5</sup> and a substantial proportion of congenital CMV infections occur in the offspring of women with preconceptional immunity.<sup>6-8</sup> It has been suggested that symptomatic congenital infection and permanent neurologic deficits are rare in the infants of women

with preconceptional immunity,<sup>2,4,5</sup> but these outcomes may in fact be more common than previously thought.<sup>7,9-12</sup>

The factors that are associated with intrauterine transmission of CMV in women with preconceptional immunity have not been defined. This knowledge is crucial for an understanding of the components of protective immunity and for the development of effective vaccines against congenital CMV infections. In this study, we determined whether acquisition of one strain of CMV in women with preexisting immunity against another strain results in intrauterine transmission of CMV. We collected serial serum samples from women with preexisting immunity against CMV and tested them for antibodies against strain-specific neutralizing epitopes within the amino-terminal region of the envelope glycoprotein H of the virus.<sup>13-15</sup> The virus-neutralizing activity of the antibodies from the mothers against the CMV isolated from their infants and the nucleotide sequences of the glycoprotein H genes from the isolated viruses were also analyzed.

### METHODS

#### Study Subjects

Of the 101 women who delivered infants with congenital CMV infection at the University of Alabama Hospital between 1993 and 1995, 40 had previously delivered one or more infants at the hospital. Of these 40 women, 18 (45 percent) were seropositive for CMV at the time of the previous delivery, 12 (30 percent) were seronegative for CMV, and 10 (25 percent) had not previously been tested for CMV. The infants born at the hospital during the study period were screened for congenital CMV infection with the use of a locally prepared monoclonal antibody against CMV immediate-early antigen for the detection of fluorescent foci, as described previously.<sup>16</sup> The results of screening of newborns at the hospital were stored in a data set in our laboratory and retrospectively linked with the obstetrical automated-record-system data base to obtain information on maternal parity, age, race, and marital status.<sup>17</sup> From this information, a comparison group of 30 parous women who delivered uninfected infants during the study period was generated by frequency matching for maternal age, race, and seropositivity for CMV before the current pregnancy. Serial serum samples collected during the previous and current pregnancies were available for 16 of the 18 women who were seropositive at the time of the previous delivery who delivered infants with congenital CMV infections and for all 30 of the women who delivered uninfected infants.

During the study period, 60 parous women became seroposi-

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tive for CMV between the end of their previous pregnancy and the end of their current pregnancy. Serum samples from 12 of these women whose previous pregnancies had occurred before 1987 were not available, and thus serum samples from the other 48 women were obtained at the time of the current delivery and were included in the study. The study protocol was approved by the institutional review board of the University of Alabama at Birmingham, and written informed consent was obtained from the mothers of infants with congenital CMV infection who attended our follow-up clinics.

**Studies of Antiviral Antibodies**

IgG antibodies against CMV and the envelope glycoprotein B were identified with the use of a solid-phase binding assay.<sup>18</sup> Gradient-purified AD169 virions and glycoprotein B recombinant protein derived from baculovirus were used as antigens to measure IgG antibodies against CMV and antibodies against glycoprotein B, respectively.<sup>18</sup>

**Strain-Specific Antibody Response**

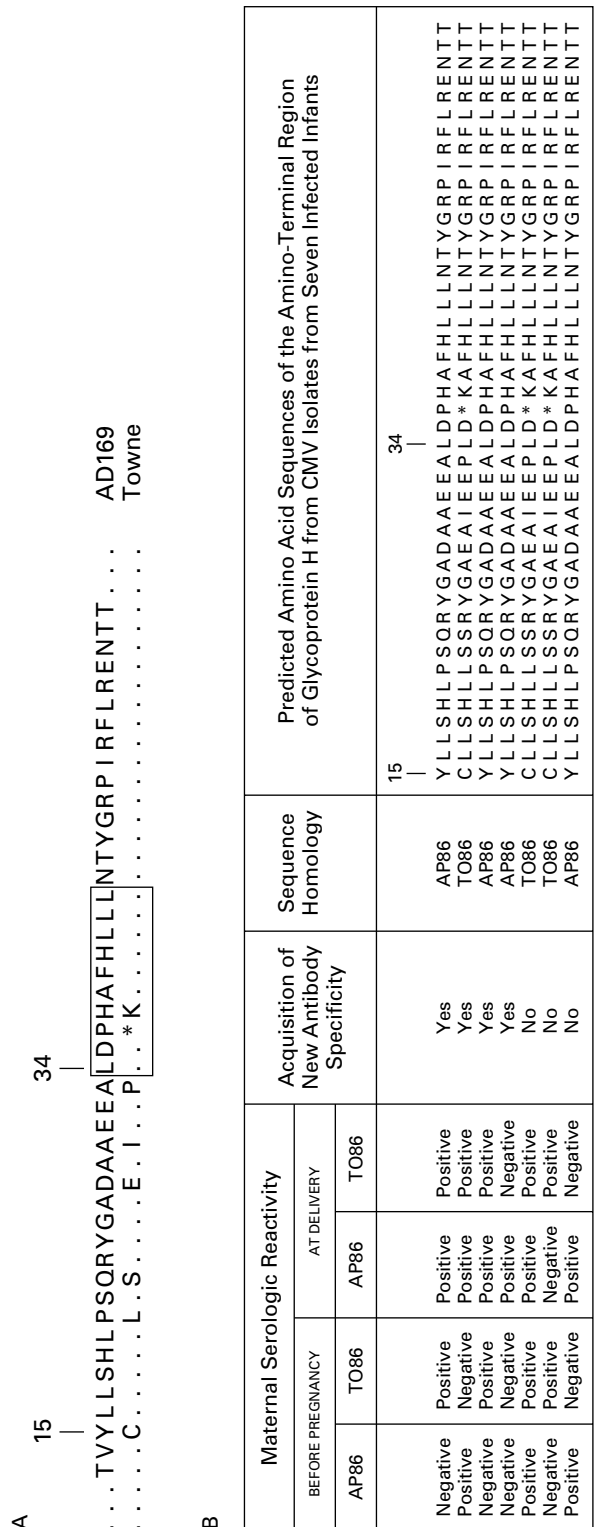
There is sequence heterogeneity within the amino-terminal region of glycoprotein H between two laboratory strains of CMV, AD169 and Towne, which is characterized by the deletion of a proline residue at position 36 and the substitution of lysine for histidine at position 37 in the Towne sequence (Fig. 1A).<sup>19</sup> The amino-terminal regions of glycoprotein H (amino acids 15 to 142) from the AD169 strain and the Towne strain of CMV were expressed in *Escherichia coli* as  $\beta$ -galactosidase fusion proteins (AP86 and TO86, respectively), as described previously.<sup>13,20</sup> Antibodies against the amino-terminal region of glycoprotein H in serum samples were measured with the use of 0.5  $\mu$ g of purified AP86 or TO86 antigens per milliliter in a solid-phase binding assay.<sup>20,21</sup> The results were expressed in terms of the difference in the number of counts per minute in the serum samples from the women and the background levels of the  $\beta$ -galactosidase fusion partner (amino acids 1 to 375), which was purified in the same way. A count per minute more than three times the background value was considered a positive result for antibodies against the antigen tested. Paired serum samples (obtained during the previous pregnancy and the current pregnancy) from all the women in the study were analyzed simultaneously. The serum specimens taken during the previous delivery were retrieved from our serum archive. When the serum specimens obtained at the time of the current delivery contained antibodies against either AP86 or TO86, and these antibodies were not present in the samples taken at the time of the previous delivery, the mothers were considered to have been infected with a different strain of CMV.

**Virus-Neutralizing Activity**

Serum samples obtained at the time of the previous delivery and the time of the current delivery from all 46 women with preconceptual immunity against CMV were analyzed for neutralizing antibodies against an unrelated laboratory strain of CMV, AD169, with a microneutralization assay.<sup>21-23</sup> CMV isolates from seven infants with congenital CMV infection were available for analysis, and the virus-neutralizing activity against these isolates in the respective maternal serum specimens was determined. The serum samples were serially diluted until a concentration that resulted in a 50 percent reduction in the infectivity of the virus was identified. This assay

**Figure 1.** The Amino Acid Sequence of the Amino-Terminal Region of Glycoprotein H from the AD169 Strain (Fusion Protein AP86) and the Towne Strain (Fusion Protein TO86) of CMV and the Responses of Serum Strain-Specific Antibody against Glycoprotein H in the Mothers of Seven Infants from Whom CMV Isolates Were Available.

In Panel A, the known neutralizing B-cell epitope is enclosed within the box. In Panel B, the predicted amino acid sequences of the viral strains from the seven infants are shown.



has been used in several studies,<sup>21-23</sup> including one in which its reproductibility was confirmed by identical titration curves obtained with repeated testing of a single serum specimen.<sup>23</sup>

#### Analysis of the Nucleotide Sequence Encoding the Amino Terminal of Glycoprotein H from CMV Isolates

CMV isolates were available from 7 of the 16 infants with congenital CMV infection. DNA preparations from the virus with a low number of passages in cell culture were subjected to polymerase chain reaction for amplification of the first 624 base pairs of the glycoprotein H gene. The amplified products were analyzed by standard methods of automated nucleotide sequencing to determine the relation between the predicted amino acid sequence of the viruses from the infants and the epitope-specific antibody response in the mothers.

#### Statistical Analysis

The demographic characteristics of the group of CMV-seropositive women whose infants had congenital CMV infection and the group of CMV-seropositive women whose infants did not have congenital infection were compared with the use of Fisher's two-tailed exact test. Serum levels of antiviral antibodies were compared between the two groups of women with the use of the Wilcoxon rank-sum test.

### RESULTS

Among the women who were seropositive for CMV before the current pregnancy, the characteristics of those whose infants had congenital CMV infection were similar to the characteristics of those whose infants were uninfected (Table 1). Most women in both groups were black and single. The mean interval between pregnancies was not significantly different between the two groups (Table 1). Seven of the 16 infants with congenital CMV infection (44 percent) had clinical abnormalities (jaundice, petechiae, hepatosplenomegaly, microcephaly, seizures, or purpura) during the newborn period, as described in a previous study, and 5 had permanent neurodevelopmental abnormalities.<sup>12</sup> Various demographic findings were similar in the 48 women who became seropositive for CMV between pregnancies (the seroconversion group) and the 46 women who were seropositive for CMV during the previous pregnancy (data not shown).

#### Data on Serum Antiviral Antibodies

IgG antibodies against CMV and glycoprotein B were detected in the serum samples obtained at the time of the current delivery in the mothers with pre-conceptional immunity against CMV, independently of the presence or absence of CMV infection in their infants (Fig. 2).

#### Strain-Specific Antibody Responses

Antibodies reactive with at least one of the antigens (AP86 or TO86) were present in the serum specimens obtained at the time of the current delivery from 14 (88 percent) of the 16 women whose infants had congenital CMV infection and 22 (73 percent) of the 30 women whose infants were not infected (Table 2). However, a greater proportion of mothers of infants

**TABLE 1.** CHARACTERISTICS OF MOTHERS WHO WERE SEROPOSITIVE FOR CMV BEFORE BECOMING PREGNANT, ACCORDING TO WHETHER THEIR INFANTS HAD CONGENITAL CMV INFECTION.\*

CHARACTERISTIC	MOTHERS OF INFECTED INFANTS (N=16)	MOTHERS OF UNINFECTED INFANTS (N=30)
Race — no. (%)		
Black	15 (94)	28 (93)
White	1 (6)	1 (3)
Other	0	1 (3)
Single marital status — no. (%)	15 (94)	24 (80)
Age — yr	20±4	21±3
Age ≤20 yr — no. (%)	7 (44)	12 (40)
Interval between pregnancies — mo	29±15	34±13
Previous pregnancies — no. (%)		
1	13 (81)	21 (70)
≥2	3 (19)	9 (30)

\*Plus-minus values are means ±SD. Maternal race was determined from the information recorded in the obstetrical automated-record-system data base.<sup>17</sup> Because of rounding, not all percentages total 100.

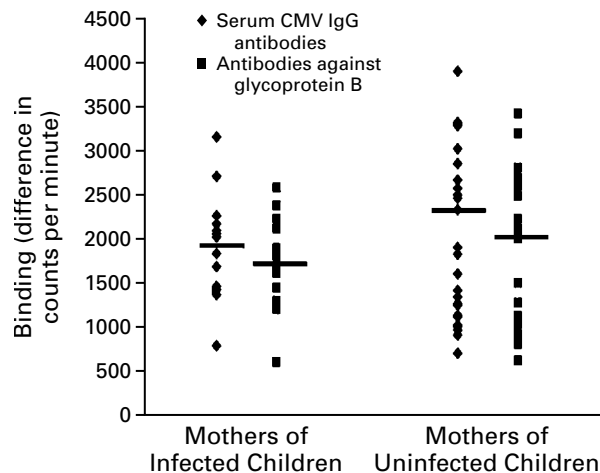
with congenital CMV infection had antibodies against both AP86 and TO86 than did mothers of uninfected infants ( $P<0.001$ ). Only 3 of the 48 mothers in the seroconversion group (6 percent) had serum antibodies against both AP86 and TO86; the majority of women in this group (65 percent) had antibodies against only one of the antigens (Table 2).

#### Appearance of New Antibody Specificities between Pregnancies

Of the 11 mothers whose infants had congenital CMV infection and whose serum at the time of the current delivery contained antibodies against both AP86 and TO86 (Table 2), 7 (64 percent) had antibodies against only one of the antigens in the sample obtained during the previous pregnancy, and 1 did not have antibodies against either AP86 or TO86 in the previous serum sample. In addition, two mothers with antibodies against one of the antigens at the current delivery had no detectable antibodies against either antigen at the previous delivery. In contrast, new antibody specificities were detected in only 4 of the 22 women whose infants were not infected and who had serum antibodies against one or both antigens at the time of the current delivery (Table 3). Thus, 62 percent of the mothers with congenitally infected infants had acquired antibodies with different specificities between pregnancies, as compared with only 13 percent of the mothers with uninfected infants ( $P<0.001$ ) (Table 3).

#### Neutralizing-Antibody Responses

When virus-neutralizing activity was assayed with the AD169 laboratory strain of CMV, no significant



**Figure 2.** Results of Binding Assays of Serum CMV IgG Antibodies and Antibodies against Glycoprotein B at the Time of Delivery in Mothers Who Were Seropositive for CMV before Pregnancy, According to Whether Their Infants Had Congenital CMV Infection.

The results are expressed in terms of the difference in the number of counts per minute in the serum samples from the women and the background level of activity of the  $\beta$ -galactoside fusion partner used in the assay. The horizontal bars represent the median difference in counts per minute.

differences were found between the serum of the mothers whose infants had congenital CMV infection and that of the mothers whose infants did not (data not shown). The neutralizing-antibody titers against the AD169 laboratory strain were similar in the serum samples obtained at the end of both pregnancies in all women (Table 4). In contrast, the serum samples obtained at the current delivery from four of the seven mothers contained at least twice as much neutralizing antibody against the CMV isolated from their infants as was present in the serum samples from the previous pregnancy. The four women with this increase in neutralizing-antibody titers had acquired antibodies with new specificities against glycoprotein H between the two pregnancies. In contrast, serum specimens from the three mothers without serologic evidence of new infection did not show a similar increase in neutralizing activity.

#### Nucleotide-Sequence Analysis

The nucleotide sequence of the region of the glycoprotein H gene encoding the amino-terminal region of the protein was determined in the CMV isolates from seven infants with congenital CMV infection. The predicted amino acid sequence of CMV isolates obtained from four of the seven infants correlated with the newly acquired antibody specificities from the serum of their mothers (Fig. 1B). Of these four infants, three had symptomatic congenital infection, and there

**TABLE 2.** STRAIN-SPECIFIC SERUM ANTIBODY RESPONSES TO THE AMINO-TERMINAL REGION OF GLYCOPROTEIN H AT THE TIME OF DELIVERY IN MOTHERS WHO WERE SEROPOSITIVE FOR CMV BEFORE THE CURRENT PREGNANCY AND MOTHERS IN THE SEROCONVERSION GROUP.\*

ANTIBODY RESPONSE	MOTHERS WITH PRECONCEPTIONAL IMMUNITY AGAINST CMV		SEROCONVERSION GROUP (N=48)
	MOTHERS OF INFECTED INFANTS (N=16)	MOTHERS OF UNINFECTED INFANTS (N=30)	
	number (percent)		
Response to a single strain	3 (19)	18 (60)	31 (65)
AD169	2 (12)	8 (27)	13 (27)
Towne	1 (6)	10 (33)	18 (38)
Response to both AD169 and Towne	11 (69)	4 (13)†	3 (6)
Response to neither AD169 nor Towne	2 (12)	8 (27)	14 (29)

\*Because of rounding, not all percentages sum to the totals shown.

† $P < 0.001$  for the comparison with the mothers of infected infants.

were permanent neurodevelopmental deficits in all three of these children.

## DISCUSSION

There has been much debate about the relative contributions of the reactivation of endogenous virus and reinfection with a different strain of CMV to intrauterine transmission of CMV to the infants of women who were previously seropositive for the virus. We found that 62 percent of the mothers of infants with congenital CMV infection had acquired antibodies with new specificities, as compared with 13 percent of the mothers whose infants were not infected. This finding supports our hypothesis that the acquisition of a new strain of CMV can lead to intrauterine transmission of CMV to the infants of women with preconceptional immunity against the virus.

Serologic assays provide only indirect evidence of reinfection; a more direct indication would be the isolation of different strains of CMV over time. However, infectious virus or viral DNA can be recovered in only a minority of seropositive subjects.<sup>24,25</sup> Moreover, sampling bias could result in the recovery of a single viral genotype from subjects infected with multiple strains. The serologic assay we used requires infection with a second, genetically distinct virus that induces an antibody response and thus is a more reliable method for demonstrating exposure to viruses with different antigenic compositions.

On the basis of the finding that viral isolates from six mother–infant pairs had identical restriction-fragment patterns, intrauterine transmission of reactivated CMV was considered the cause of congenital CMV

**TABLE 3.** COMPARISON OF STRAIN-SPECIFIC ANTIBODY RESPONSES AGAINST GLYCOPROTEIN H IN SERIAL SERUM SAMPLES FROM MOTHERS WITH PRECONCEPTIONAL IMMUNITY AGAINST CMV, ACCORDING TO WHETHER THEIR INFANTS HAD CONGENITAL CMV INFECTION.

ACQUISITION OF NEW ANTIBODY SPECIFICITIES BETWEEN PREGNANCIES	MOTHERS OF INFECTED INFANTS (N=16)	MOTHERS OF UNINFECTED INFANTS (N=30)
	no. (%)	
Yes	10 (62)	4 (13)*
No	6 (38)	26 (87)

\*P<0.001 for the comparison with the mothers of infected infants.

infection in women who had previously been seropositive for the virus.<sup>26</sup> Although different restriction-fragment patterns reliably indicate sequence differences between isolates, similarity or even identity between restriction-fragment patterns does not prove sequence homology. Also, the difference between the rate of congenital CMV infection in infants born to low-income women (1 to 2 percent) and the rate in those born to middle-income or high-income women (0.1 to 0.2 percent) cannot be explained solely by reactivation.<sup>4</sup>

A major limitation of this study is that our strain-specific antibody assay distinguishes between only AD169-like and Towne-like strains of CMV and most likely underestimates the true frequency of new infections. This limitation is illustrated by the finding that

27 percent of the women with uninfected infants had no serum antibodies against either AP86 or TO86 at the current delivery (Table 2). The distribution of strain-specific serologic responses in the 48 women in the seroconversion group was similar to the distribution of responses in women who were previously seropositive and had uninfected infants (Table 2). A possible explanation for the similar strain-specific antibody data from the mothers in the seroconversion group and the seropositive mothers of uninfected infants is that these two groups had similar risks of exposure to new strains of CMV.

The appearance of new antibodies against glycoprotein H between pregnancies could have been due to the selection of an antibody-resistant viral mutant over time,<sup>27-30</sup> but such mutations have only been found in viruses obtained from immunocompromised hosts. Furthermore, the genetic difference between the AD169 and Towne strains with respect to the neutralizing epitope on the amino-terminal region of glycoprotein H consists of changes in at least two codons and therefore probably represents two independent mutational events, a rate of mutation that is inconsistent with the genetic stability of CMV.<sup>31</sup> Thus, the selection of antibody-resistant mutants seems an unlikely explanation for the appearance of strain-specific antibodies against glycoprotein H in the mothers of infected infants. In addition, the predicted amino acid sequence of glycoprotein H from the infants of the four mothers who had serologic evidence of new infection reflected the newly acquired antibody specificities, and three of these four infants had symptomatic congenital CMV infection that led to permanent neurodevelopmental deficits.

In conclusion, among women with preexisting im-

**TABLE 4.** MATERNAL VIRUS-NEUTRALIZING ACTIVITY AGAINST AD169 AND THE STRAIN OF CMV ISOLATED FROM THE INFECTED INFANT.

SUBJECT NO.	ANTIBODY TITER AGAINST AD169*		ACQUISITION OF NEW ANTIBODY SPECIFICITIES BETWEEN PREGNANCIES	ANTIBODY TITER AGAINST THE STRAIN OF CMV ISOLATED FROM THE INFANT*	
	SERUM OBTAINED AT PREVIOUS DELIVERY	SERUM OBTAINED AT CURRENT DELIVERY		SERUM OBTAINED AT PREVIOUS DELIVERY	SERUM OBTAINED AT CURRENT DELIVERY
	1	600		720	Yes
2	1200	1800	Yes	120	660†
3	820	1600	Yes	420	2400†
4	1400	920	Yes	440	1100†
5	3600	2800	No	1600	2200
6	900	800	No	480	600
7	820	1600	No	600	800

\*The titer of neutralizing-antibody activity is given as the reciprocal of the serum dilution that resulted in a 50 percent decrease in the infectivity of the virus.

†The neutralizing-antibody titers against the virus strain isolated from the infant at least doubled between pregnancies.

munity against CMV, intrauterine transmission of CMV to their infants occurred most often in those who had acquired a different strain of CMV between pregnancies. However, the frequency of new infection with CMV in previously seropositive women from various populations and the consequences of such reinfection will need to be defined in prospective studies with sufficient follow-up of the infants with congenital CMV infection.

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