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Immunity Induced by Primary Human Cytomegalovirus Infection Protects against Secondary Infection among Women of Childbearing Age

Stuart P. Adler, Stuart E. Starr, Stanley A. Plotkin, Sue H. Hempfling, Judy Buis, Mary Lou Manning, and Al M. Best

To determine if immunity to human cytomegalovirus (HCMV) protects women from acquiring HCMV from their children, a blinded, randomized protocol was used to monitor seronegative women who received placebo or Towne vaccine (~500 pfu) and seropositive women. Each group was similar for mean maternal (33 years) and child age (18 months) and duration of viral shedding by the child (15 months). Among 19 placebo recipients, 9 developed primary infection; 8 of 19 vaccinees but only 3 of 42 naturally seropositive subjects had evidence of acquiring HCMV from their child. Wild type infection and Towne vaccine induced similar mean lymphoproliferative responses to HCMV antigens, but one dose of Towne vaccine produced mean neutralizing titers 10- to 20-fold lower than those after wild type infection. Thus, a vaccine that induces immune responses equal to those induced by wild type virus may protect healthy women from acquiring HCMV from their children.

Newborns need protection against cytomegalovirus disease. Maternal immunization may provide protection, because newborns who acquire human cytomegalovirus (HCMV) either via postnatal transfusion or transplacentally are protected against HCMV disease if their mothers had antibodies to HCMV before pregnancy. Neonatal transfusion studies first showed that antibodies to HCMV were likely to be protective [1, 2]: Premature newborns with HCMV-seronegative mothers developed symptomatic HCMV infections acquired from transfused blood products but those with HCMV-seropositive mothers remained asymptomatic even if they became infected after receiving the same blood products.

A large study of the natural history of congenital HCMV infections and pregnancy found that maternal immunity protects against symptomatic congenital infection [3]. In infants with congenital infection whose mothers had primary HCMV infection during pregnancy, sequelae occurred in 25% of the 125 [3]. This contrasted with an 8% rate in 64 infants with congenital infection whose mothers were HCMV-seropositive before pregnancy. More important, none of the infected infants with seropositive mothers developed severe sequelae, such as bilateral hearing loss or an intelligence quotient of <70. In contrast, these sequelae occurred in 16% of infants whose mothers had primary infection during pregnancy. These observations suggest that immunity to HCMV before pregnancy will prevent the majority of severe sequelae associated with congenital infection.

Annually, of 4 million live births in the United States, ~8000 infants will develop sequelae or die due to congenital HCMV infection [3]. This low incidence of symptomatic congenital HCMV infection in the general population means that efficacy trials of HCMV vaccines with congenital infection as an end point will require immunization of large numbers of women. As an approach to assessing the potential for vaccine efficacy with a smaller number of subjects, we sought to determine if either wild type virus or vaccine-induced immunity protects against secondary infections among women with a very high risk of acquiring HCMV. Young infants with day care center–acquired HCMV infections excrete virus for 6–41 months and transmit HCMV to their seronegative mothers at an annual rate of 30%–50% [4–6]. We therefore asked if immunization with the Towne strain of HCMV—a live attenuated vaccine that is effective in preventing severe HCMV-associated disease in renal transplant recipients—or immunity from a naturally acquired infection would reduce the rate at which mothers acquire HCMV infection from their children [7, 8].

Materials and Methods

Subjects. Mothers of children <3 years old (each mother having at least 1 child shedding HCMV) were recruited between 1988 and 1993 from day care centers after screening the children for HCMV shedding. Seronegative mothers were enrolled at 2 sites, Richmond and Philadelphia, and were randomized and blinded to receive either a placebo or vaccine. Randomiza-
tion was done in groups of 4 by a random-number generator. Seropositive mothers were all enrolled in Richmond; 1 group wanted to know their serologic status and the other group (blinded) did not. Seropositive mothers in the blinded group all received placebo without knowing if they were seronegative or seropositive or if they had received vaccine or placebo. To avoid enrolling women who were seropositive because of a recent primary infection acquired from their child, seropositive women who had IgM antibodies to HCMV or who were shedding HCMV at entry were excluded.

All subjects were prospectively monitored until either their child ceased shedding HCMV in ≥2 consecutive samples of urine or saliva or until a subject showed evidence of an acquired HCMV infection. Evidence of an acquired HCMV infection was defined either as primary infection (seroconversion) with or without viral shedding or, when the woman was seropositive, as secondary infection: a 4-fold rise in HCMV antibody titers as determined by EIA without viral shedding or maternal shedding of a virus strain with the same restriction endonuclease DNA digestion pattern as the strain shed by her child. For 80 subjects, the average duration of monitoring was 18 months (range, 4–44). After entry, monitoring consisted of testing samples of urine and saliva from each subject and her child for the presence of virus at entry, every 2 weeks for 8 weeks, and monthly thereafter. Samples of serum and plasma were obtained from each mother at entry and 2, 4, and 6 months after entry and then every 3 months up to 1 year.

**Vaccine.** The Towne vaccine was prepared as described and administered subcutaneously in the deltoid region at ~500 pfu/subject [9, 10].

**Laboratory methods.** Urine and saliva samples were cultured before and after concentration in duplicate on MRC-5 fibroblasts as described [11]. White blood cells were prepared from each plasma sample and cultured for HCMV. Isolates were passed two to four times in MRC-5 fibroblasts and incubated for 48 h with [3H]orthophosphate. HCMV DNAs were extracted by a modified Hirt procedure [11]. All isolates were endonuclease-digested with EcoRI and BamHI. Restriction fragments of wild type isolates and of Towne strain of HCMV were separated by electrophoresis through 0.8% agarose as described [11]. Isolates were considered different if they differed by two or more restriction fragments after EcoRI or BamHI digestion.

**SeroLogic assays.** IgG to HCMV was measured by EIA as described [12, 13]. A titer was defined as the highest dilution of serum that produced an OD of >0.1. Seroconversions and 4-fold changes in titer were confirmed by testing paired sera simultaneously in duplicate. IgM to HCMV was measured using a commercial EIA (Abbott, Abbott Park, IL). Neutralizing antibodies were determined by standard plaque reduction assay without complement [14]. A titer was defined as the highest dilution of serum that produced 50% inhibition.

**Lymphocyte proliferation responses (LPR).** HCMV-specific LPR were determined as described [15] and expressed as stimulation indices, defined as the ratio of counts per minute in HCMV antigen–stimulated cultures to counts in control antigen–stimulated cultures. Stimulation indices ≥3 were considered positive.

**Statistical analysis.** Groups were compared by analysis of variance if the dependent variable was continuous or χ² tests if the dependent variable was categoric. Kaplan-Meier survival estimates were also calculated to analyze and plot the time to infection in the different groups. Differences between survival estimates were tested using the log-rank test. All statistical analyses were done using JMP (SAS Institute, Cary, NC) [16].

**Results**

**Serologic and virologic responses.** The study group comprised 80 women, of whom 6 were black and 74 white. Thirty-eight were seronegative; of this group, 19 each received vaccine and placebo. Of the 42 women who were seropositive on entry into the study, 13 were unaware of their serologic status or whether they received vaccine or placebo. The groups were similar for average maternal age at entry into the study, age of the child at entry, and duration of viral shedding by the children after entry (table 1).

Each of the 19 vaccinees seroconverted after vaccination and developed positive LPRs. Vaccination was well tolerated. Mild soreness, erythema, and induration developed at the site of inoculation in 80% of vaccinees in the second week after immunization.

Table 2 lists the overall infection rates by group. Nine vaccinees developed evidence of HCMV infection: 5 had a 4-fold rise in EIA titer to HCMV and shed an isolate with the same DNA pattern as the isolate shed by their respective children, and 3 had a 4-fold titer rise but did not shed virus (figure 1). On the basis of the DNA pattern of the isolates, no vaccinee was viremic with or shed the Towne strain in urine

| Table 1. Characteristics of subjects in a study of maternal HCMV infection. |
|---------------------------------|--------|--------|
| Placebo (n = 19) | Vaccine (n = 19) | Naturally seropositive (n = 42) |
| Maternal age at entry, years | 33 ± 4 | 33 ± 5 | 32 ± 4 |
| Age of child at entry, months | 20 ± 7 | 16 ± 6 | 17 ± 8 |
| Duration of viral shedding by children after entry, months | 13 ± 10 | 17 ± 14 | 18 ± 7 |

**NOTE.** Data are mean ± SD.

| Table 2. HCMV infections among subjects by group. |
|---------------------------------|--------|--------|
| Group (n) | No. shedding same strain as child | No. with 4-fold titer rise | Total no. infected (%) |
| Vaccine (19) | 5 | 8 | 8 (42.0) |
| Placebo (19) | 4 | 9 | 9 (47.0) |
| Naturally seropositive | | | |
| Open (29) | 0 | 1 | 1 (3.4) |
| Blinded (13) | 2 | 1 | 2 (15.0) |
| Total (42) | 2 | 2 | 3 (7.0) |
or saliva. The 42% infection rate for the vaccine group was similar to the 47% rate for the 19 who received placebo. Among the placebo recipients, 9 seroconverted, and 4 of them shed an HCMV isolate with the same DNA pattern as the isolate shed by their respective children (figure 1).

Among 42 subjects who were seropositive at entry as a result of naturally acquired HCMV infection, 3 showed evidence of having acquired infection from their respective children. One woman, who knew her serologic status and never shed HCMV, had constant EIA titers (1:20,480) over the first 6 months of the study. In the ninth month, her EIA titer increased to 1:81,920. A second woman, whose status was blinded at entry, shed an HCMV isolate with a DNA pattern identical to that of her child 4 months after entry. Her EIA titer remained at 1:819,200 over the first 9 months of the study and gradually declined over the next 7 months. A third seropositive subject, also blinded, had a rise in EIA titer from 1:20,480 to 1:163,840 between the second and fourth month after entry and shed a small amount of HCMV (<10 pfu/mL) in a single urine specimen. The DNA pattern of that isolate was the same as the DNA pattern of the isolate shed by her child (figure 1).

After entry, 2 of 42 seropositive women shed HCMV with a DNA pattern different from that of the isolate shed by their children (figure 1). In the first, the isolate that differed was shed 6 months after entry. Her EIA titers were stable over a 2-year observation period. The second subject had HCMV infection at entry with an isolate unrelated by DNA pattern to that shed by her child. Her EIA titer at entry was 1:20,480 and increased to 1:81,920 by 2 months after entry, at which

Figure 1. Restriction endonuclease fragments of DNAs of HCMV isolates after digestion with EcoRI (A) and BamHI (B). Isolates were obtained from 13 mothers (M) and 19 children (C) and are grouped in pairs according to whether mothers received vaccine (V) or placebo (P) or were naturally seropositive (NS). T, digest pattern of Towne vaccine DNA; arrows, fragments that differ between mother and child.
time her sera also contained IgM antibodies to HCMV. Over the next 27 months, her EIA titers stabilized between 1:20,480 and 1:40,960 and she did not shed HCMV. The remaining 37 women had no evidence of infection.

When either maternal shedding of the same viral isolate as shed by her child or a 4-fold rise in EIA titer without viral shedding was considered an indication of secondary maternal HCMV infection acquired from a child, 3 (7%) of 42 initially seropositive women had evidence of a secondary infection. This was significantly lower \( \left( \chi^2 = 13.1; 1 \text{ df}; P < .001 \right) \) than the 45% rate of infection in seronegative women who received placebo or vaccine.

To determine if immunization with Towne vaccine affected the rate over time at which women acquired HCMV from their children, we used survival estimates (figure 2). For the recipients of either placebo or vaccine, nearly all infections were acquired within the first year after entry and the vaccine had no impact on the rate of infection \( \left( \chi^2 = 0.18; P = .67 \right) \). Among the seropositive women, infection rates were similar between those who knew their serologic status and those who did not \( \left( \chi^2 = 1.99; P = .16 \right) \). However, as shown in figure 2, natural seropositivity did have an impact on the rate of acquisition of HCMV over time from the children. Women who were naturally seropositive differed significantly from those who received vaccine or placebo \( \left( \chi^2 = 13.22; P < .001 \right) \).

**Immune responses.** To determine why vaccine failed to protect against secondary infection, we first compared the geometric mean neutralizing antibody titers, EIA titers, and LPR stimulation indexes between 2 groups: vaccinees who remained uninfected and vaccinees who became secondarily infected (table 3). The LPR stimulation indexes were similar in both groups, as were the peak geometric mean EIA titers. The peak geometric mean neutralizing antibody titers induced by Towne vaccine, although low in both groups, were two times higher among those who did not become secondarily infected after vaccination than among those who were secondarily infected (table 3). However, this difference lacked statistical significance \( \left( t = 1.77; 17 \text{ df}; P < .1 \right) \).

Second, we compared the peak values for EIA titers, neutralizing titers, and LPR in 4 groups: women who received placebo and then had a primary wild type infection, vaccinee who acquired wild type infection, subjects who were initially seropositive, and vaccinees who did not acquire wild type infection (table 4). The differences among groups in the mean LPR stimulation indexes were insignificant \( \left( F \left[ 2, 66 \right] = 1.45; P < .24 \right) \). Significant differences occurred for EIA and neutralizing titers. The women who were naturally seropositive had a geometric mean EIA titer of 1:20,000 and a

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**Table 3.** Serologic and cellular responses of vaccinees secondarily infected and those who remained uninfected.

<table>
<thead>
<tr>
<th>Response</th>
<th>Infected ( (n = 8) )</th>
<th>Uninfected ( (n = 11) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIA titer</td>
<td>1974 (1280–5120)</td>
<td>1280 (640–2560)</td>
</tr>
<tr>
<td>Neutralizing titer</td>
<td>16.1 (4–64)</td>
<td>44.0 (16–256)</td>
</tr>
<tr>
<td>Lymphocyte response</td>
<td>10.7 (4–17)</td>
<td>10.0 (3–21)</td>
</tr>
</tbody>
</table>

**NOTE.** Titers are given as reciprocal geometric mean. Lymphocyte response as stimulation index. Values are maximum after immunization but before secondary infection and are given as mean (range). No comparisons were significant.
Table 4. Serologic and lymphoproliferative responses of women with wild type infection compared with vaccine recipients.

<table>
<thead>
<tr>
<th>Response</th>
<th>Initially naturally seropositive (n = 40)</th>
<th>Vaccines before secondary infection (n = 19)</th>
<th>After wild type infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIA titer</td>
<td>20.557 (10.240–80.420)</td>
<td>1531 (640–5120)</td>
<td>11,000 (5120–40,960)</td>
</tr>
<tr>
<td>Neutralizing titer</td>
<td>235* (32–1024)</td>
<td>29 (4–128)</td>
<td>322† (64–1024)</td>
</tr>
<tr>
<td>Lymphocyte response</td>
<td>13.16 (3–32)</td>
<td>10.3 (3–21)</td>
<td>ND</td>
</tr>
<tr>
<td>Placebo recipients</td>
<td></td>
<td></td>
<td>34,400 (10,240–163,840)</td>
</tr>
<tr>
<td>Vaccines</td>
<td></td>
<td></td>
<td>300 (64–512)</td>
</tr>
</tbody>
</table>

NOTE. Titors are given as reciprocal geometric mean, lymphocyte response as stimulation index. ND, not done. Values are highest after either immunization or secondary infection (for seropositive women, values are those obtained at entry) and are given as mean (range).

* n = 14.
† n = 3.

geometric mean neutralizing titer of 235, similar to the titers observed for originally seronegative women with primary HCMV infection, regardless of immunization status (table 4). These titers were 10- to 20-fold higher than the vaccine-induced titers (table 4).

After a wild type infection, subjects who had previously received vaccine developed geometric mean EIA antibody titers 3-fold higher (P < .02) than those of subjects who received placebo and then acquired a wild type infection, suggesting that the latter had an anamnestic response induced by the secondary infection (table 4).

Discussion

The major finding of this study is the relative protection afforded by a primary infection due to a wild type virus and the association of this protection with high levels of neutralizing antibodies to HCMV. Only 3 of 42 women with naturally acquired HCMV infection and high levels of antibody to HCMV showed possible evidence of having acquired their infection from their children. In contrast, nearly half of the women with vaccine-induced immunity and low levels of antibody to HCMV developed evidence of infection acquired from their children. Because the infection rate in the placebo recipients was similar to that of vaccinees, these observations suggest that high antibody levels derived from a wild type infection are associated with protection.

However, although we tried to avoid enrolling seropositive women whose primary infection was recently acquired from their child, in no case could we determine precisely when seropositive women first became infected or the source of infection. If reinfection rates are strain related, this may be important. Different strains of HCMV induce variable titers of both EIA and neutralizing antibodies in both humans and animals [13, 17, 18]. Presumably, the highest titers and hence the highest level of protection against a secondary infection will occur against the strain that caused the primary infection. However, it is unlikely either that a substantial portion of the women seropositive at entry had a primary infection acquired from their children or that a substantial portion of the children acquired HCMV congenitally from their mothers. In Richmond, 40% of women of childbearing age are seropositive, a rate similar to that we observed among mothers with children in day care who were shedding HCMV, and <2% of seropositive mothers give birth to infants with asymptomatic congenital HCMV infection [19].

In the current study, a 4-fold elevation in EIA titer was the best indication of a secondary infection. All vaccinees had detectable levels of both neutralizing and EIA antibodies to HCMV before acquiring a wild type infection from their children. Each vaccinee who shed a wild type isolate also had a concomitant 4-fold rise in antibody, and no vaccinee shed HCMV without a serologic response. Among the recipients of placebo, the rate of seroconversion and shedding was similar to that observed in a previous study: about half of the women with infected children seroconverted, and of these, half had HCMV recovered from urine or saliva [4].

Even though for purposes of data analysis in this study we considered 3 seropositive women to have had evidence of HCMV acquisition from their children, only 1 seropositive subject demonstrated entirely convincing evidence of a secondary infection acquired from her child: at 4 months after entry she both had a serologic response and shed an HCMV isolate with the DNA pattern of the isolate shed by her child. One of the other 2 women considered infected shed the same isolate as her child but did not have a serologic response. Thus, she may have had reactivation of an infection acquired before entry. The other woman had a serologic response to HCMV but no virus isolated. Thus, the source of her secondary infection could not be determined.

Two seropositive women were infected with isolates unrelated to their child’s isolate. One of these may have been undergoing a primary infection at entry with an isolate that differed from that of her child, and the other was IgM-negative and had stable EIA titers. Over 2 years, neither developed evidence of having acquired CMV from her child.

The frequency of secondary infection in previously in-
fected persons is related to the route of challenge and inoculum of the challenging virus. Women in this study were presumably challenged with a low-dose inoculum by the mucosal route. Those seropositive from naturally acquired infections may not be protected against secondary infection when challenged by larger doses or by other routes, for example, virus transmitted by semen or cervical secretions. In analysis of HCMV genomes, three types of observations have identified reinfecion in transplant recipients, patients with AIDS, those with multiple sex partners, and children in day care. First, subjects have shed ≥2 HCMV isolates simultaneously [20–26]. In most cases, a single subject shed a single isolate from one site (e.g., urine, saliva, cervical secretions) and a different isolate from a second site. Second, serial HCMV isolates from a single subject have had different DNA patterns [20, 21, 24–27]. Third, seropositive transplant recipients frequently have become reinfected with latent HCMV in donor kidneys or other organs [25].

Our finding that Towne vaccine did not protect against secondary infections as well as did wild type infection was not entirely unexpected. In a previous study, we observed that only 1 of 5 naturally seropositive persons, when challenged with 100 pfu of the nonattenuated Toledo strain of HCMV by the subcutaneous route, developed serologic or virologic evidence of postchallenge HCMV infection [28]. When 7 subjects previously immunized with the Towne strain were similarly challenged, 4 developed HCMV infection after challenge; 100 pfu of the Toledo strain produced infection in all seronegative volunteers.

Our data suggest that the Towne strain of HCMV did not protect against secondary infection because it induced lower titers of vaccine-induced neutralizing and EIA antibodies than did wild type virus. Moreover, within the vaccinated group, those who later became secondarily infected had lower neutralizing titers than did those who remained uninfected. However, neutralizing antibodies may be a marker for some other protective component of the immune response, for example, mucosal secretory IgA and IgG.

Additional unpublished studies in progress suggest that the weak humoral response induced by the Towne vaccine in this study may be related to the use of only a single dose of vaccine.

The practical implications of our data relate to vaccine evaluation. The ability of wild type infection to protect persons who are challenged naturally means that either live or recombinant HCMV vaccines can be evaluated rapidly, in small groups of subjects, for their ability to afford similar protection. If a vaccine induces high antibody titers and affords protection of nonpregnant women against acquisition of HCMV from infected children, then it is logical to suppose that without large and lengthy clinical trials involving pregnant women, a vaccine could be considered likely to protect newborns against cytomegalovirus disease.

Acknowledgments

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References


