Mucosal Antibodies to Human Cytomegalovirus Glycoprotein B Occur following both Natural Infection and Immunization with Human Cytomegalovirus Vaccines

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Because antibodies against human cytomegalovirus (HCMV) glycoprotein B (gB) neutralize, levels of IgG, secretory IgA (sIgA), and mucosal IgA1 antibodies to HCMV were measured in saliva and nasal washes. Ten seronegative adults lacked these antibodies, but of 10 naturally seropositive adults, 10 had IgG to gB, 5 had sIgA, and 0 had mucosal IgA. Among 12 recipients of a live HCMV vaccine, 8 had IgG to gB, 4 had sIgA, and 2 had mucosal IgA in samples collected 10–20 months after immunization; of 10 recipients of a gB vaccine, 8 had IgG to gB, 7 had sIgA, and 7 had mucosal IgA in samples collected just before or 1 month after a booster. IgG to gB and neutralizing titers in serum correlated with IgG to gB in mucosal samples. IgG to gB was in the saliva of 25 of 26 subjects with serum neutralizing titers >1:64. Serum neutralizing titers >1:64, whether induced by vaccine or wild type virus, are associated with mucosal IgG to HCMV.

About 40% of infants born of mothers who acquire a primary human cytomegalovirus (HCMV) infection during pregnancy will be infected in utero and born with a congenital HCMV infection [1]. An important risk factor for maternal acquisition of HCMV infection is the presence of young children in the home <3 years of age with HCMV infections acquired in group day care [2, 3]. Seronegative mothers with such infected children acquire HCMV infections at a rate 20–25 times higher than other women, and at least half of seronegative mothers will become infected within 1 year after their child becomes infected [3]. These observations indicate that prevention of maternal infection acquired from young children is important for reducing the frequency of congenital infection.

We previously observed that the majority (>90%) of naturally seropositive women are protected against acquiring a secondary HCMV infection from a child shedding HCMV in urine and saliva [4]. These women were presumably exposed to a low-dose inoculum on the mucosal surfaces of the upper respiratory tract. Mucosal antibodies derived from serum are presumed to be protective against a broad spectrum of infectious diseases [5]. Therefore, it is reasonable to hypothesize that seropositive women who have children shedding HCMV are protected from secondary infection by mucosal antibodies. For this reason, and because for HCMV the majority of the neutralizing antigenic epitopes occur on the major envelope glycoprotein of the viral particle (gB), we determined the levels of mucosal antibodies to HCMV gB among naturally seropositive persons and recipients of either the live attenuated Towne strain or a purified recombinant gB vaccine.

Methods

Subjects. Serum, nasal washes, and parotid saliva were collected from 42 healthy adults (11 women, 31 men) between 18 and 50 years of age. These subjects comprised 4 groups: seronegative (n = 10); naturally seropositive to HCMV, but not shedding virus (n = 10); seronegative, who received either one, two, or three doses (~500 FFU per dose) of a live attenuated HCMV vaccine (Towne strain) administered subcutaneously with boosters given 4–12 weeks after the first dose (n = 12); seronegative, who received either 5, 30, or 100 µg per dose of a recombinant gB vaccine (gB/MF59) administered intramuscularly (Chiron Biocine, Emeryville, CA) with boosters at 1 and 6 months after the first dose (n = 10). For recipients of the gB vaccine, samples were obtained immediately before (6 months after the first dose) and immediately after (7 months after the first dose) the second booster. For recipients of the Towne vaccine, samples were obtained between 10 and 20 months after their initial immunization. Vaccinees...
were monitored for acquisition of a wild type infection by obtaining serum, urine, and saliva samples at 3-month intervals beginning with their first immunization. During this interval, no subject shed virus in urine or saliva or had a rise in antibody titers to HCMV not associated with vaccine administration.

Vaccines. The Towne vaccine (lot C-107) was produced by Microbiological Associates (Rockville, MD) from the same stock as used for a previous lot of Towne vaccine [6]. Lot C-107 produced serum neutralizing titers 10-fold higher than those previously reported with Towne vaccine [4] (unpublished data). Subjects had mild inflammation at the injection site but no other toxicity.

The gB/MF59 vaccine is a recombinant derivative of HCMV strain Towne gB produced as a secreted protein in Chinese hamster ovary cells. The mature protein was purified from the cell medium. The recombinant gB includes amino acids 1–676 of the extracellular domain. The proteolytic cleavage site at amino acid 437 was blocked by the site-specific mutation of amino acid residues 433,
Before administration, the protein is combined with MF59, an oil-in-water emulsion containing 5% squalene and 0.5% each of sorbitan trioleate and polysorbate 80. After the third dose, the gB/MF59 vaccine was associated with a mild local reaction in 4 subjects.

**Screening assays.** Initial testing of serum for IgG antibodies to HCMV was done by a previously described EIA [8].

**Specimen collection and processing.** PBS (10 mL) was instilled into a nostril followed by aspiration with a catheter and sterile trap using an electric suction pump. Parotid saliva was collected with a parotid saliva collector by stimulation with sour candy [9].

All samples were immediately frozen at -20°C. For use, the samples were thawed and centrifuged at 500 g to remove debris and then concentrated 2- to 4-fold for saliva and 5- to 10-fold for nasal washes by spin dialysis at 5000 g for 30 min (Ultrafree C-MC 30,000; Millipore, Bedford, MA).

**EIAs.** For each assay, the optimal concentration of recombinant gB antigen (Chiron) for adsorption was first determined by serial dilutions of gB antigen. Wells of polyvinyl chloride microtiter plates (Immulon I; Dynatech Laboratories, Chantilly, VA) received 100 μL containing either 20 μg/mL (for mucosal samples) or 10 μg/mL (for serum samples) of purified gB in carbonate buffer (pH 9.6) or 100 μL of control antigen containing either 10 or 20 μg/mL of bovine serum albumin (BSA). After overnight incubation at 4°C, the microwells were washed three times with PBST (PBS with 0.05% Tween 20 and 0.01% NaN₃).

**Sera.** To measure IgG antibodies to gB in sera, sera were serially diluted 2-fold from 1:100 in PBST with 3% BSA, and 100 μL of each dilution was added per microwell precoated with gB. After 90 min at 37°C, the microwells were washed four times with PBST; then 100 μL of a 1:500 dilution of alkaline phosphatase (AP)–conjugated goat anti-human immunoglobulin G (Tago, Burlingame, CA) in PBST with 3% BSA was added to each microwell. After 90 min at 37°C, the microwells were washed four times with PBST, and then 100 μL of p-nitrophenyl phosphate (Sigma, St. Louis), 1 mg/mL, in 10% diethanolamine (pH 9.6) was added to each well. The plates were incubated at room temperature until an OD of 0.200 at 405 nm had developed with a 1:6400 dilution of an HCMV-positive serum (30–60 min). The difference in absorption at 405 nm/490 nm between the control wells (coated with BSA) and the wells coated with gB was measured on a dual wavelength Vmax Microelisa Auto Reader (Molecular Devices, Palo Alto, CA). All assays were run in duplicate. Antibody levels are expressed as optical density units (ODU) per sample volume, with ODUs being the dilution factor of each sample required to produce an OD of 1.0. For all assays, the negative-positive cutoff was the upper limit of the 99% confidence interval for the mean values for the seronegative subjects.

**Mucosal samples.** For measuring IgG antibodies to gB in mucosal samples, the concentrated sample was diluted 1:4 in PBST with 3% BSA and centrifuged at 11,000 g for 20 s at room temperature; next, supernatants were added to microwells precoated with gB. AP-conjugated goat anti-human IgG (Tago) was used at a 1:250 dilution in the EIA, and the assay was done as noted above.

To measure IgA and secretory IgA (sIgA), an EIA was used. For mucosal samples, concentrated nasal washes and parotid saliva were diluted 1:4 in PBST with 3% BSA containing 25% goat anti-human IgG antibodies (product I-1136; Sigma). After incubation for 60 min at 37°C, samples were centrifuged at 11,000 g for 20 s at room temperature to remove IgG complexes, and supernatants were then added to duplicate microwells containing 2 μg of...
gB/well. IgA (IgA1) was detected with 1:250 AP-labeled goat antibody to human IgA (Tago). sIgA was detected with a 1:2000 dilution of mouse monoclonal anti-human secretory component C clone GA-1 (product 1-6635; Sigma) and with a 1:250 AP-labeled goat antibody to mouse IgG (Tago).

To confirm specificity, positive samples were retested using a 1:1000 dilution of mouse monoclonal anti-human secretory component C (clone Hp 6130, lot 041294p; Hybridoma Reagent Laboratory, Baltimore), and this monoclonal produced the same results as the Sigma monoclonal antibody. For sIgA, no absorbance was detected if any component was omitted either singularly or in combination. To exclude sIgA reactivity due to cross-reactions of monoclonal antibodies with IgG antibodies bound to gB, the mucosal samples were replaced with purified serial dilutions of a seropositive pooled human IgG (intravenous immunoglobulin, lot 6.371.266.0; Sandoz, East Hanover, NJ), and no reactivity was detected.

Total mucosal immunoglobulin levels. To confirm the adequacy of each sample, total IgG and IgA levels were measured in each sample by an EIA. Microwells were first coated as described above with 10 μg/mL anti-human IgG or anti-human IgA (Tago). To develop a reference curve of concentration versus optical density, after overnight incubation at 4°C, human IgG (intravenous immunoglobulin, lot 6.371.266.0; Sandoz) or human IgA (Accurate Chemical and Scientific, New York) were serially diluted 10-fold from 100 μg/mL, and 100 μL of each dilution was added per microwell. After 60 min at 37°C, the microwells were washed, and 100 μL of a 1:500 dilution of an AP-conjugated goat anti-human IgG or anti-human IgA (Tago) was added for 60 min at 37°C. To measure total IgG and IgA in saliva and nasal washings, concentrated samples were first centrifuged at 11,000 g for 20 s at room temperature; supernatants were serially 4-fold diluted from 1:100 and added to microwells precoated with either anti-human IgG or anti-human IgA as described above for the reference curve. Each assay was done as noted above. The lower limit of detectability was 10 ng of immunoglobulin/mL of sample.

Neutralization assays. Neutralizing activity in sera was determined without complement as described [4]. A titer was defined as the highest dilution of serum that produced 50% inhibition of cytopathic effect.

Statistical analysis. Correlation coefficients and regression equations were determined using least squares and matrix linear regression [10]. For each regression coefficient, P values were calculated using SEs and a t test.

Results

All mucosal samples had detectable levels of IgG and IgA. In parotid saliva, the mean concentration (±SD) for IgA was 27 ± 15 μg/mL and for IgG, 1.2 ± 2.2 μg/mL. For nasal washes, the mean concentration (±SD) for IgA was 30 ± 28 μg/mL and for IgG, 7.5 ± 9.4 μg/mL. For both nasal washes and saliva, there were no significant correlations between the initial sample volumes, total amounts or concentrations of IgG and IgA recovered per subject, and the concentration of specific IgG, sIgA, or mucosal IgA against gB. Antibody responses to gB were not associated with the number of doses of Towne vaccine or the amount of gB/MF59 vaccine administered.

IgG antibodies to gB. None of 10 seronegative adults had IgG antibodies to gB in either nasal washes or saliva. All 10 naturally seropositive adults had IgG antibodies to gB in saliva and 7 of 10 in nasal washes (figure 1A). Among recipients of the Towne vaccine, 8 of 12 had IgG antibodies to gB in either saliva or nasal washes (5 in saliva and 8 in nasal washes). The highest levels of IgG antibodies against gB in both serum and mucosal samples occurred in the recipients of gB/MF59 vaccine 1 month after the second booster (7 months after the first dose) (figure 1A). For those naturally seropositive, the average concentration of IgG antibodies to gB in serum was 5236 ODU/mL of serum (range, 2074–8090); for recipients of Towne vaccine, 1722 (range, 813–5491); and for recipients of gB/MF59 vaccine, 2000 (range, 218–6323) before the second booster (6 months after the first dose) and 23,000 (range, 6426–67,917) 1 month after the second booster (7 months after the first dose).

Considering samples (n = 42) from vaccinees and seropositive subjects, there was a significant (P < .001) linear correlation between the concentration of IgG antibodies to gB in serum obtained simultaneously with mucosal specimens and the respective concentration in saliva and, considered separately, the amount in nasal washes (figure 2A, B). To confirm an association between neutralizing titers and levels of antibodies against gB, neutralizing titers were measured in 35 of the 42 sera but were not measured in 7 recipients of Towne vaccine. Neutralizing titers in sera correlated linearly with IgG antibodies to gB in saliva (figure 2C) but not in nasal washes (data not shown). IgG antibody to gB was detected in the saliva of 25 of 26 subjects with serum neutralizing titers >1:64 but in only 6 of 9 saliva specimens from subjects with neutralizing titers ≤1:64 (P = .044, Fisher’s exact test, two-tailed).

sIgA antibodies to gB. Figure 1C shows the levels of sIgA detected in mucosal specimens by group. One seronegative subject had a low level of sIgA antibodies to gB (OD = 0.145) in nasal wash, but none had sIgA antibodies to gB in saliva. Among the naturally seropositive subjects, low but significant (OD > 0.1) levels of sIgA were detected in 5 subjects: 5 had sIgA antibodies to gB in saliva, and 4 of these 5 had sIgA antibodies to gB in nasal washes. None of 12 recipients of Towne vaccine had sIgA antibodies to gB in saliva, but 4 had sIgA antibodies to gB in nasal washes. Among recipients of the gB/MF59 vaccine at 1 month after the third dose (7 months after the first dose), 7 of 10 subjects had detectable levels of sIgA antibodies to gB in saliva (5 subjects) or nasal washes (5 subjects). There was no correlation between gB-specific sIgA in mucosal specimens and serum levels of IgG antibodies to gB (r = .38, P > .1).

Mucosal IgA to gB. Figure 1B shows the levels of gB-specific IgA detected in mucosal specimens by group. Among those either seronegative or naturally seropositive, none had detectable levels of mucosal IgA antibodies to gB. Mucosal IgA antibodies to gB were detected in the nasal washes of only 2 subjects who received Towne vaccine but was detected in saliva and nasal washes of 7 of 10 recipients of the gB/MF59 vaccine at 1 month after the third dose (7 months after the first dose). IgA against gB in nasal washes was also significantly correlated with IgA and IgG antibodies to gB in sera (r = .8, P < .001).
Saliva -- 0.0. units/O.025ml

Nasal wash -- 0.0. units/O.025ml

Figure 2. Relationship between serum antibodies and IgG antibodies to HCMV gB in mucosal samples. Solid lines are best-fit linear regression; dotted lines are 95% confidence intervals for each regression: A: \( r = 0.87, n = 42, P < 0.001 \); B: \( r = 0.69, n = 42, P < 0.001 \); C: \( r = 0.73, n = 34, P < 0.001 \).

Discussion

Although systemically administered vaccines are generally considered poor inducers of sIgAs, sIgA antibodies were detected in some of the recipients of the HCMV vaccines. Rubella and inactivated poliovirus vaccines also induce specific sIgAs, but in most cases, induction of specific sIgA requires immunization with an antigen that a subject has previously encountered at mucosal surfaces [11, 12]. Previous mucosal exposure will induce a specific sIgA response with systemic administration of inactivated bacterial (Streptococcus pneumoniae, Haemophilus influenzae, Neisseria meningitidis) and viral (influenza virus) vaccines [13]. Before immunization, the recipients of the two HCMV vaccines may have been exposed to either wild type HCMV or another antigen cross-reactive with gB. The 5 vaccinees who developed sIgA antibodies to gB did so immediately after the third dose (second booster). This type of response may be more common than generally appreciated, since a recent study reported that systemic administration of a third booster of H. influenzae type b capsular polysaccharide conjugate vaccine also induced a specific sIgA against capsular polysaccharide in saliva [14].

Although a majority of the recipients of the gB/MF59 vaccine also had nonsecretory IgA antibodies to gB in mucosal samples, our most important observation may be that IgG antibodies to gB were frequently present in mucosal specimens of those who either received an HCMV vaccine or were naturally seropositive. The linear correlation between concentration of
that they were present via transudation of a small percentage of serum antibodies into mucosal specimens. The concentration of IgG antibodies to gB in the mucosal specimens was ~0.1% of the serum concentration.

Robbins et al. [5] recently proposed that the effectiveness of all US Food and Drug Administration-licensed vaccines directed against viruses, bacteria, or toxins can be accounted for by their ability to stimulate a critical level of serum IgG antibodies against one or more antigens. Small quantities of these antibodies on mucosal surfaces may inactivate a low-dose inoculum of the pathogen. These investigators noted that the only vaccine-induced immune response that has been associated with the ability of all vaccines to prevent acquisition of an infection are serum IgG antibodies, although other immune mechanisms, such as IgM, IgA, or T cells, may also contribute to protection. This implies that for a vaccine to confer protection, a critical level of IgG antibodies must be present when the host is exposed to the pathogen.

This hypothesis agrees with our current and previous observations concerning HCMV. In previous studies, we observed a low rate of secondary infection in those seropositive to HCMV from naturally acquired wild type HCMV infections [2, 4]. Among those naturally seropositive, the incidence of secondary infection will depend on the challenge dose and route of infection. For example, virus transmitted by semen or cervical secretions may result in a secondary infection rate different from that following a challenge by the oral mucosal route. We observed reinfection with a second strain of HCMV in some children in day care (who presumably become infected via the oral route), but the incidence was low [2]. Only 2 of 160 infected children became reinfected with a second HCMV strain. Recently, Shen et al. [15] observed a child who shed two consecutive strains of HCMV. In another study, we observed that only 3 of 42 seropositive women whose children were shedding HCMV may have become reinfected from their children [7]. Thus, existing data indicate that for seropositive children and their seropositive mothers, reinfection with HCMV is infrequent.

Clinical trials evaluating both Towne HCMV vaccine and the subunit gB vaccine in MF59 adjuvant for the prevention of congenital HCMV disease are in their nascent stages. Even though we obtained mucosal specimens from recipients of Towne vaccine between 10 and 20 months after immunization, a majority of subjects still had detectable levels of IgG antibodies to gB in mucosal specimens. The 10 subjects who received the gB/MF59 vaccine were the first to receive this new vaccine, and their samples were obtained sooner after immunization than samples from the Towne vaccinees. Even though our data indicate that the gB/MF59 vaccine with appropriate booster doses will induce high levels of serum neutralizing antibodies to HCMV as well as mucosal antibody, it is unknown if these levels will be sustained. The levels of antibodies required to prevent HCMV acquisition among vaccine recipients are also unknown. For these reasons, comparisons between the two vaccines and inferences regarding which vaccine is likely to be more effective are not yet possible.

Mothers and children in day care are presumably exposed to a virus by the oral mucosal route. For naturally seropositive women, either IgA or IgG with neutralizing activity may create an immune barrier to secondary infection on the mucosal surfaces. If serum-derived IgG antibodies alone are sufficient to prevent secondary infection, our data indicate that immunization with either a recombinant subunit vaccine or the attenuated Towne vaccine may be effective.

References