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Salivary Antibodies to Cytomegalovirus (CMV) Glycoprotein B Accurately Predict CMV Infections among Preschool Children

JIAN BEN WANG AND STUART P. ADLER*

Division of Infectious Diseases, Department of Pediatrics, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

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Among preschool children, we found an association between cytomegalovirus (CMV) infection and salivary immunoglobulin G antibodies to CMV glycoprotein B. All of the 20 infected children had immunoglobulin G to CMV glycoprotein B in their saliva, whereas 38 of 38 uninfected children lacked these antibodies. Testing saliva provides a sensitive and specific alternative to obtaining serum.

Following a primary cytomegalovirus (CMV) infection, children less than 3 years of age excrete CMV in urine and saliva for a mean of 18 months, with a range of 6 to 40 months (3). This prolonged excretion is associated with an increased rate of CMV transmission to CMV-seronegative caretakers and mothers (1, 2, 4, 7–9). Seronegative mothers with infected children acquire human CMV infections at a rate 20 to 25 times higher than other women, and at least half of seronegative mothers will become infected within 1 year after their children become infected (2, 4, 7–9). Approximately 40% of infants born of mothers who acquire a primary human CMV infection during pregnancy will be infected in utero and born with a congenital human CMV infection (10). We have recently observed that CMV-seronegative pregnant women who are aware that they have young children shedding CMV can significantly reduce their risk of acquiring CMV from their infected children if they perform hygienic measures to reduce child-to-mother transmission of CMV (5). For these reasons, rapid identification of preschool children likely to be shedding CMV and who have CMV-seronegative pregnant mothers or caretakers may be important.

We recently observed that adults who are either naturally seropositive to CMV or recipients of CMV vaccines have immunoglobulin G (IgG) antibodies to CMV glycoprotein B (gB), the major envelope protein of the CMV viral particle, in parotid saliva and nasal washes (11). Since serum specimens are often difficult to obtain from young children and viral isolation from urine and saliva may be slow and expensive, we evaluated the association between viral infection and salivary IgG antibodies to CMV gB.

The subjects were 61 children who attended one of six day care centers in Richmond, Va. These children were part of ongoing studies of day care transmission of CMV (5). Urine and saliva samples for viral isolation were obtained every 3 months from each child. Before oral mucosal samples were obtained for antibody assays, the children had had an average of 4 sets of samples obtained for CMV culture, with a range of 1 to 10 culture sets per child. When the oral mucosal samples were obtained for antibody assay, the mean age of the children was 21 months, with a standard deviation of 12 months.

To isolate CMV, urine and saliva samples (mouth swabs) were inoculated onto confluent monolayers of MRC5 fibroblasts. A 0.2-ml aliquot of urine was cultured directly, and the remainder of the specimen was centrifuged at 20,000 × *g* for 60 min. After the supernatant was aspirated, the pellet was suspended in 1 ml of minimal essential media and placed into culture. All cultures were incubated at 37°C for 6 weeks and observed weekly.

To obtain oral mucosal samples for antibody determinations, Calgiswabs (Baxter C8853-14; Spectrum Laboratories Inc., Houston, Tex.) were used to swab the buccal mucosa and then placed into 15-ml tubes containing 1 ml of phosphate-buffered saline (PBS). The tubes were vortexed before aliquots were removed. Undiluted saliva was also collected by aspiration from the mouth with soft plastic large-bore transfer pipettes (SAMCO Scientific Inc., San Fernando, Calif.) and transferred to sterile 15-ml conical tubes. All samples were frozen at −20°C until assayed.

Mucosal samples were assayed by enzyme immunoassay for IgG to CMV gB. Wells of polyvinyl chloride microtiter plates (Immulon I; Dynatech Laboratories, Chantilly, Va.) received 100 μl of carbonate buffer (pH 9.6) containing 20 μg of purified gB (Chiron Corp., Emeryville, Calif.) per ml or 100 μl of control antigen containing either 10 or 20 μg of bovine serum albumin (BSA) per ml. After overnight incubation at 4°C, the microtiter plate wells were washed three times with PBS containing 0.05% Tween 20 and 0.01% NaN_3 (PBST).

For measuring IgG to gB in mucosal samples, the sample of saliva was diluted 1:4 in PBST with 3% BSA and microcentrifuged at 13,000 × *g* for 20 s at room temperature. The PBS containing the swab was diluted 1:4 in PBST with 3% BSA and centrifuged at 17,500 × *g* at 4°C. A 100-μl aliquot of supernatant or diluted saliva was added to microtiter plate wells precoated with either gB or control antigen. After 90 min at 37°C, the microtiter plate wells were washed four times with PBST, and then 100 μl of a 1:250 dilution of alkaline phosphatase-conjugated goat anti-human IgG (Tago, Inc., Burlingame, Calif.) in PBST with 3% BSA was added to each well. After 90 min at 37°C, the wells were washed four times with PBST, and then 100 μl of a 1-mg/ml solution of *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) in 10% diethanolamine (pH 9.6) was added to each well. The plates were incubated at room temperature until an optical density (OD) of 0.200 at 405 nm had developed with a 1:6,400 dilution of a CMV-positive serum (between 30 and 60 min). The difference in A_{405} and A_{490} values 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, Calif.). All assays were run at least twice.

* Corresponding author. Mailing address: Medical College of Virginia, Virginia Commonwealth University, P.O. Box 980163, Richmond, VA 23298-0163. Phone: (804) 828-1807. Fax: (804) 828-6455. Electronic mail address: SADLER@GEMS.VCU.EDU.

CMV DNA was detected by a nested PCR which had been modified from a previously described method (6). Urine samples were first clarified by centrifugation at 1,500 × *g* for 5 min. A 0.2-ml aliquot of urine supernatant and 0.8 ml of sterile distilled water were placed in a Centricon-100 tube and filtered by centrifugation at 1,000 × *g* for 30 min in 4°C. To the filtrate was added 0.2 ml of sterile distilled water, and the samples were heated at 100°C for 15 min. Mucosal samples were also heated at 100°C for 15 min before PCR.

The first oligonucleotide primers, located in the *Eco*RI J fragment of strain AD169, consisted of 5′ TGA GGA TAA GCG GGA GAT GT 3′ and 5′ ACT GAG GCA AGT TCT GCA GT 3′. The nested pair of primers consisted of 5′ AGC TGC ATG ATG TGA GCA AG 3′ and 5′ GAA GGC TGA GTT CTT GGT AA 3′. The reaction mixture consisted of 10 mM Tris-HCl (pH 9.6), 10 mM $MgCl_2$, 50 mM NaCl, 0.24 mM each deoxynucleotide triphosphate, 0.2 μg of BSA per ml, 0.15 μM primers, and 1 U of *Taq* DNA polymerase (Promega, Madison, Wis.). Samples (2.5 μl) of treated urine or mucosal samples were diluted to a final volume of 50 μl. After covering each sample with 50 μl of paraffin oil, the amplification reaction was performed in a DNA thermal cycler (Perkin-Elmer, Foster City, Calif.): 20 incubation cycles consisting of 30 s at 92°C, 30 s at 57°C, 30 s at 72°C, and finally 2 min at 72°C. The initial cycling was followed by a second set of 40 cycles. Products (2.5 μl) from the first amplification were added to a new reaction mixture consisting of the nested primers and the buffer system. Controls included for each run included purified viral DNA (AD169) and sterile distilled water in place of target DNA.

To detect CMV-specific reaction products, Southern blot hybridization was performed. Ten microliters of the amplification product was applied to a nylon membrane (Schleicher & Schuell Corp., Keene, N.H.). The filter was prehybridized with 10 ml of buffer containing 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2 PO_4, and 1 mM EDTA [pH 7.7]), 1% sodium dodecyl sulfate (SDS), 10× Denhardt's reagent, 20 μg of yeast RNA per ml, and 50 μg of salmon sperm single-stranded DNA per ml for 3 h at 42°C and then hybridized by using ^{32}P labeling of oligonucleotides located within the amplification product of the PCR in 10 ml of hybridization solution (6× SSPE, 1% SDS) at 42°C overnight. The filter was washed three times (15 min each) in hybridization solution at room temperature and then for 15 min at 42°C, followed by exposure to X-ray film for 2 h at −70°C.

Children were considered CMV infected if they shed CMV in one or more sets of specimens for viral isolation (19 children) or were PCR positive (3 children) in either urine or saliva obtained simultaneously with mucosal specimens for antibody determination. Children were considered uninfected if they lacked CMV in any set of specimens and were PCR negative (37 children) or were found to be CMV seronegative when serum samples were obtained simultaneously with saliva specimens (2 children). Ten children ceased shedding CMV prior to procurement of mucosal specimens for antibody testing, and 12 children were found to be infected when mucosal specimens were obtained for antibody testing.

For the enzyme immunoassay, the negative-positive cutoff was set at the upper limit of two times the standard deviation for the uninfected children. For the aspirated saliva, the positive-negative cutoff was an OD of 0.072, and for mouth swabs it was 0.079. Table 1 shows the association between CMV infection and salivary IgG antibodies against gB. Detection of IgG antibodies in the aspirated saliva was associated with a CMV infection with 100% specificity and sensitivity. There were no false-positive or false-negative reactions. In contrast, however, when antibodies eluted from swabs were tested, there was a 55% rate of false negatives but no false positives. This lack of sensitivity was due to a failure of the mucosal swabs to adsorb and release a sufficient quantity of IgG. The OD values produced by the antibody eluted from the mucosal swabs were significantly lower ($P < 0.001$, Student's *t* test) than the OD values obtained when assaying undiluted aspirated saliva (Fig. 1).

TABLE 1. Association between CMV infection and mucosal antibodies to CMV gB among preschool children by type of mucosal specimen

CMV infected[a]	No. of children by type of mucosal specimen[b]					
	Saliva			Mucosal swab		
	With IgG to gB	Without IgG to gB	Total tested	With IgG to gB	Without IgG to gB	Total tested
Yes	20	0	20	9	11	20
No	0	38	38	0	38	38
Total	20	38	58	9	49	58

[a] Yes indicates evidence of CMV infection as determined by viral shedding, PCR, or serum antibodies (please see text).

[b] A total of 61 children were tested; 55 children had both saliva and mucosal swabs tested, 3 children had only saliva tested, and 3 children had only mucosal swabs tested.

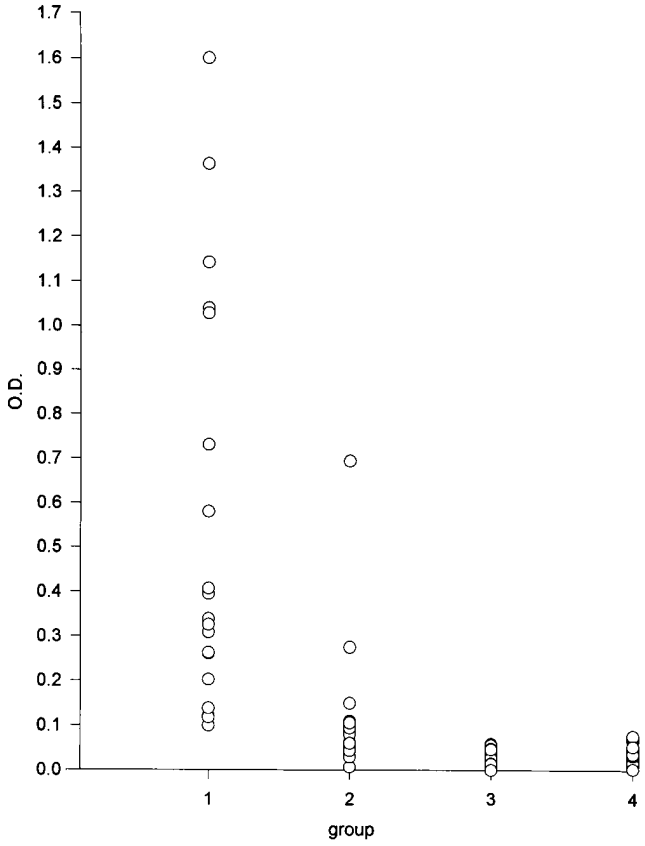

FIG. 1. The distribution, by group, of OD values obtained in the enzyme immunoassay. Group 1, aspirated saliva from 20 CMV-infected children; group 2, mucosal swabs from 20 CMV-infected children; group 3, aspirated saliva from 38 uninfected children; group 4, mucosal swabs from 38 uninfected children. Many samples had similar or identical OD values, which are shown as overlapping circles.

These results show that aspirated saliva from CMV-infected children contains IgG antibodies to CMV, specifically CMV gB. This observation extends our previous findings from adults. In adults we found that 10 of 10 naturally seropositive individuals had IgG antibodies to CMV gB in either nasal washes or parotid saliva, whereas 10 of 10 seronegative adults lacked these antibodies in the same samples (11). IgG antibodies to gB in saliva are most likely a passive transudate of serum antibodies because their concentration in saliva is linearly proportional to their concentration in serum (11). Presumably all IgG antibodies against CMV proteins in serum are also present in saliva. We chose purified gB antigen for our enzyme immunoassay because, unlike CMV antigens extracted from infected cells, gB produced a low background with saliva.

Compared with obtaining serum, obtaining saliva from preschool children is rapid, easy, and painless. Drawing blood from some children is difficult. Many children need to be restrained, and most experience real or perceived discomfort. For these reasons, many parents are reluctant to have blood drawn from their children, particularly for study purposes. Obtaining saliva with a plastic aspirator is simple, rapid, painless, and nontraumatic for children of any age. A sensitive and specific salivary assay for CMV infection in preschool children and children attending day care should facilitate either providing identification of CMV-positive children to pregnant caregivers or identifying them during studies of CMV transmission or CMV vaccine evaluation. For these purposes, testing saliva provides a sensitive and specific alternative to obtaining serum.

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