Neutralizing Activity of Saliva against Cytomegalovirus

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Congenital cytomegalovirus (CMV) disease is the leading cause of permanent disability in neonates in the United States. Neutralizing antibodies in saliva may protect against maternal CMV infection by blocking viral entry into oral epithelial cells, but the antibody response to CMV in the saliva following natural infection is not well characterized. Saliva specimens from naturally infected individuals were tested for CMV-neutralizing activity using epithelial and fibroblast cells. Saliva from seronegative adults had no inherent anti-CMV activity. Neutralizing activity of saliva from naturally infected adults was not detectable using fibroblast cells, and saliva from young children, adolescents, and Towne vaccine recipients did not have activity using either cell type. However, when using epithelial cells, neutralizing activity was present in saliva from 50% of seropositive adults, correlated with serum-neutralizing activity, and was more prevalent in mothers of children in day care than in non-day care-associated adults. Three day care mothers with high salivary neutralizing activities (>1:20) had exceptionally high serum-neutralizing titers (3- to 8-fold higher than typical seropositives) and were immunoblot positive for serum antibodies to the epithelial entry mediator UL130. These results suggest that salivary neutralizing activities are attainable by induction of high serum IgG levels and could be utilized to evaluate candidate cytomegalovirus vaccines.

Cytomegalovirus (CMV) is the leading cause of congenital abnormalities in the United States, causing serious permanent disabilities in greater than 5,500 children annually. Approximately 13% of congenitally infected infants are symptomatic at birth, and of those born infected but asymptomatic, 17 to 20% will later develop permanent sequelae, such as hearing loss and cognitive impairment. Sensorineural hearing loss is the most common disability found in congenitally infected infants, affecting about 36% of symptomatic and 12% of asymptomatic infants (6). Due to the high incidence of permanent sequelae from congenital CMV, development of a CMV vaccine has been deemed a national priority by the Institute of Medicine (20).

Two experimental vaccines have been evaluated for efficacy in humans. The Towne live attenuated vaccine has been used in nearly 1,000 volunteers with no serious side effects (4). The Towne vaccine induces neutralizing antibodies and T cell responses, but when used at a low dose failed to protect seronegative mothers of viruciric children from acquiring CMV (3). The glycoprotein B (gB)/MF59 vaccine, comprised of recombinant gB adjuvanted with MF59, induces gB-specific antibodies superior to those induced with natural infection, affecting about 36% of symptomatic and 12% of asymptomatic infants (6). Due to the high incidence of permanent sequelae from congenital CMV, development of a CMV vaccine has been deemed a national priority by the Institute of Medicine (20).

Neutralizing antibody is important for vaccine protection. CMV infection induces two neutralizing antibodies in serum. Antibodies directed mostly against gB impair viral entry into both fibroblasts and epithelial cells, whereas antibodies that target gH/gL/UL128-131, a complex comprised of gH, gL, UL128, UL130, and UL131 (originally known as UL131A) that is dispensable for fibroblast entry but critical for epithelial cell entry (16, 24), potently and selectively impair viral entry into epithelial cells (11). Following natural infection, the later activity is dominant, as serum-neutralizing titers measured with epithelial cells are on average 48-fold higher than those measured using fibroblasts (5). In contrast, responses to gB/MF59 or Towne immunization, while comparable to those for natural infection with respect to neutralization of fibroblast entry, are 15-fold (gB/MF59) and 28-fold (Towne) lower than those for natural infection with respect to neutralization of epithelial cell entry (5). For gB/MF59, this deficiency could be ascribed to its lack of epitopes from gH/gL/UL128-131, whereas Towne’s shortfall may be linked to a mutation that modifies the C-terminal end of UL130 (9), rendering it unstable and poorly expressed (15). This presumably also impacts presentation of conformational epitopes that require intact gH/gL/UL128-131 complexes (16). Hence, efficacy of these vaccines might be enhanced through strategies to induce epithelial entry-neutralizing antibodies.

CMV-neutralizing responses have predominantly been studied in serum. However, the fact that most CMV infections are acquired via the oral route (2) suggests that neutralizing antibodies in saliva could potentially prevent initial host entry by blocking infection of epithelial cells lining the oral mucosa. Anti-CMV activities in saliva are not well studied. Salivary antibodies to gB are detectable by enzyme-linked immunosorbent assay (gB-ELISA) following natural infection or gB/MF59 vaccination, but the ability of these or other antibodies to neutralize CMV was not determined (26). Thus, we characterized the CMV-neutralizing potential of saliva from naturally infected adults, Towne vaccine recipients, teenagers, and children under 2 years of age.

MATERIALS AND METHODS
Study populations and sample collection. Serum and saliva samples were obtained from mothers of children at the Virginia Commonwealth University...
Medical Center day care and non-day care-associated adults from the University community. A total of 19 women with children in day care (n = 7 CMV seropositive; n = 12 CMV seronegative) and 11 non-day care-associated adults without young children in the home (n = 9 seropositive, 4 male and 5 female; n = 2 seronegative, both female) were enrolled in this study. Serum and saliva samples from eight Towne vaccine recipients (obtained 2 to 9 months postimmunization), 17 saliva samples from children in day care who were under 2 years old, and 8 saliva samples from adolescents were obtained during previous studies (3, 25, 28). Informed consent was obtained from all subjects or their guardians, and protocols were approved by the Virginia Commonwealth University Committee for the Conduct of Human Research.

**Antibody detection.** Adult sera were assayed for CMV seropositivity by gB-ELISA (10). Children and adolescents were evaluated for CMV positivity by detection of gB-specific IgG in saliva as described previously (25, 26). Avidity was determined using a commercial kit (Radim, Florence Italy) as described previously (12).

**Neutralization assays.** Virus, cell culture, and neutralization assays were conducted as described previously (5, 17). Briefly, samples were 2-fold or 10-fold diluted in cell culture medium (or in some samples, saliva) and then 2-fold serially diluted in cell culture medium. Serially diluted samples were incubated with an equal volume of culture medium containing 5,000 PFU of green fluorescent protein (GFP)-tagged virus BADrUL131-Y4 (23), incubated for 1 h at 37°C, and transferred to black-walled, clear-bottom 384-well plates containing confluent MRC-5 ATCC CCL-171 or ARPE-19 (ATCC CRL-2302) cells. Representative images were taken three (MRC-5) or four (ARPE-19) days postinfection using a Nikon Diaphot 300 microscope. GFP fluorescence was quantitated using a Victor3 1420 multilabel counter (PerkinElmer) at day 7 postinfection. Fifty percent inhibitory concentration (IC₅₀) values were determined using the nonlinear regression function in Prism 5 (GraphPad Software, Inc.) as previously described (17). All samples were assayed in triplicate to obtain geometric mean IC₅₀ ± standard errors.

**Immunoblotting.** Human serum antibodies to UL128, UL130, or UL131 were assayed by standard immunoblot methods (16) using transfected HEK-293T cells as antigens. Codon-optimized plasmid expression vectors encoding intact UL128, UL130, or UL131 proteins (strain Towne) in DNA vaccine vector VR10551 (Vical, Inc.) were synthesized by Blue Heron Biotechnology, Inc. GFP expression plasmid PM1A178b (27) was used as a control. HEK-293T cells were transfected with each plasmid using Effectene (Qiagen) according to the manufacturer’s instructions. After 48 h, cells were lysed in 0.5% NP-40, separated by SDS-PAGE, and transferred to 0.2-µm nitrocellulose membranes as described previously (16). Membranes were incubated overnight at 4°C with rabbit sera specific for UL128, UL130, or UL131 (a gift from David Johnson and Brent Ryckman) (16) or with 1 µl human sera. Reactivities were detected using horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-human IgG (Thermo), as appropriate, followed by use of SuperSignal chemiluminescent substrate (Pierce) and exposure of X-ray film.

**Statistical analyses.** Nonlinear regression, linear regression, paired Student’s t-test, Fisher’s exact test, and Pearson’s correlation were performed with Prism 5 (GraphPad Software, Inc.).

**RESULTS**

**Saliva from some seropositive individuals has neutralizing activity.** Saliva samples from 16 seropositive adults, 17 day care children, 8 adolescents, and 8 Towne vaccine recipients were assayed for neutralizing activity (Fig. 1). To ascertain if saliva contains nonspecific CMV-neutralizing factors, saliva specimens from 14 CMV seronegative adults were tested. None had CMV-neutralizing activities, regardless of cell type, at dilutions as low as 1:2 (data not shown). To determine if saliva contains factors that inhibit neutralizing antibodies, epithelial entry-neutralizing activities were measured for a seropositive serum diluted 1:10 with culture medium or with saliva samples from two seronegative adults. The IC₅₀ for the serum diluted in culture medium (1:1,986) was no different from the IC₅₀ for the serum diluted in either saliva sample (1:2,011 and 1:2,022). Representative data for one saliva sample are shown in Fig. 2A. These results demonstrate that in the absence of CMV-specific antibodies, saliva contains no factors that nonspecifically inactivate virus. Moreover, that serum-neutralizing activity was unaffected by saliva suggests that saliva lacks factors that impair antibody-neutralizing activity.

All saliva specimens from children and adolescents contained gB-specific IgG by ELISA but lacked detectable neutralizing activity using either cell type. Towne vaccinees were also negative for salivary neutralizing activity using both cell types. Table 1 summarizes immunological data for the 16 naturally seropositive adults. All lacked detectable salivary neutralizing activity using fibroblasts, but saliva samples from eight adults neutralized epithelial cell entry, with IC₅₀ titers ranging from 1:6 to 1:28 (representative data are shown in Fig. 2B). Serum titers for adults with salivary activity were statistically higher than for those without (Student’s t test, P = 0.04, r² = 0.5), and a linear correlation was observed between serum and salivary neutralizing titers (linear regression, r = 0.67, P = 0.005, R² = 0.44) (Fig. 3). Seven of the adults were mothers of children in day care, while the rest were non-day care-associated adults. Salivary neutralizing activity was more frequent among day care mothers than non-day care-associated adults (6/7 versus 2/9; Fisher’s exact test P value, 0.04) (Table 1), and when only day care mothers were analyzed (Fig. 3, squares), the correlation between serum and salivary-neutralizing titers was more apparent (linear regression, r = 0.97, P = 0.0003, R² = 0.94).

Three subjects (M2, M4, and M6; shaded in Table 1) with the highest salivary neutralizing titers (>1:20) had unusually high serum-neutralizing titers (Fig. 2B and 3). Indeed, their serum IC₅₀ (1:15,094, 1:16,117, and 1:7,122) were 3- to 8-fold higher than the geometric means for other adults in this study (1:1,900; range, 1:181 to 1:5,031) or for six seropositive adults evaluated in a previous study (1:2,070; range, 1:1,007 to 1:3,118) (17).

To determine if the presence of salivary neutralizing activity corresponds to recent infection, the avidities of CMV-specific serum IgG in the adult group were determined (Table 1). The three subjects with high salivary neutralizing activity had high serum IgG avidity, suggesting that these subjects were not...
infected recently; however, the fact that three subjects with low avidity also had salivary neutralizing activity (A4, M1, and M3; Table 1) suggests that salivary neutralizing activity can occur shortly after infection.

Subjects with high salivary neutralizing activities have antibodies to UL130. Although human epithelial entry-neutralizing responses are believed to target the gH/gL/UL128-131 complex, immunoblot detection of antibodies to UL128, UL130, or UL131 in sera from naturally infected subjects has not been reported. To detect serum antibodies reactive with UL128, UL130, or UL131, lysates of cells transfected with plasmids engineered to express each protein or a GFP control were used as antigens for immunoblot assays. Expression of each protein was confirmed using rabbit antisera (Fig. 4A). For all 16 adult seropositive sera, no reactivities to UL128 or UL131 were detected (Fig. 4B and C); however, serum antibodies to UL130 were detected in the three day care mothers with high serum and salivary neutralizing activities (M2, M4, and M6) and in subject A8, a non-day care-associated adult with salivary epithelial neutralizing activity (Fig. 4B). All other adults lacked detectable UL130 antibodies (Fig. 4C).

**DISCUSSION**

Despite its presumed importance in protection against primary infection, salivary humoral immunity to CMV remains poorly characterized. One study from 1980 measured salivary neutralizing activity in children age 1 to 4 years who were hospitalized or attending an outpatient clinic in Sapporo, Japan (21). Using a fibroblast-based plaque reduction assay, salivary neutralizing activities were found in 76% of CMV-positive subjects. The activity cofractionated with secretory IgA (sIgA), and while most titers were quite low (mean of 1:7), a few reached as high as 1:32. Our results contrast with these findings in that we did not detect neutralizing activity in saliva samples from any subjects using fibroblasts, and using epithelial cells, we did not detect neutralizing activities in saliva specimens from children of a similar age attending day care. Differences in demographics, subject’s health, sample collection and preparation, or perhaps the sensitivities of the assays may account for the discrepancies.

Wang et al. detected anti-gB IgG by ELISA in saliva samples from 100% of adults that were naturally infected or gB/MF59 immunized, but the neutralizing activity of these gB-specific
antibodies was not determined (26). Levels of anti-gB IgG in saliva strongly correlated with serum levels, but were approximately 1,000-fold lower. This is consistent with salivary IgG being passively transferred into oral secretions. Salivary anti-gB sIgA was detected in only 50% of subjects in both groups, implying that transudated IgG may be the CMV-reactive immunoglobulin most consistently present in saliva. Our data on neutralizing activities are consistent with these results. We observed a correlation between saliva- and serum-neutralizing titers, and the slope of the linear regression line was 1:627, observed a correlation between saliva- and serum-neutralizing neutralizing activities are consistent with these results. We determined by immunoblotting. +, antibodies detected; −, antibodies not detected. Reciprocal titers. Ranges are inverse logs of the mean log(IC50) ± standard error of the mean log(IC50). NA, not applicable. Shading indicates results for individuals with high immunity to CMV.

FIG. 3. Salivary-neutralizing titers correlate with serum-neutralizing titers. Salivary and serum-neutralizing titers from 16 CMV seropositive adult subjects were compared using linear regression analysis (r = 0.67, P = 0.005, R2 = 0.44). Squares symbolize day-care-associated subjects and circles symbolize non-day-care-associated subjects. Dashed lines indicate 95% confidence limits.

**TABLE 1. Immunologic characteristics of the adult study population**

<table>
<thead>
<tr>
<th>Subject</th>
<th>gB-ELISA</th>
<th>Avidity</th>
<th>UL139</th>
<th>Serum Saliva Serum</th>
<th>Epithelial neutralization</th>
<th>Fibroblast neutralization</th>
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<tr>
<td>A1</td>
<td>102,400</td>
<td>37</td>
<td>−</td>
<td>1,124</td>
<td>962–1,314</td>
<td>&lt;2</td>
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<tr>
<td>A2</td>
<td>6,400</td>
<td>47</td>
<td>−</td>
<td>303</td>
<td>223–293</td>
<td>&lt;2</td>
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<tr>
<td>A3</td>
<td>25,600</td>
<td>46</td>
<td>−</td>
<td>534</td>
<td>441–648</td>
<td>&lt;2</td>
</tr>
<tr>
<td>A4</td>
<td>102,400</td>
<td>33</td>
<td>−</td>
<td>1,777</td>
<td>1,578–2,004</td>
<td>6.8</td>
</tr>
<tr>
<td>A5</td>
<td>25,600</td>
<td>41</td>
<td>−</td>
<td>1,233</td>
<td>1,081–1,407</td>
<td>&lt;2</td>
</tr>
<tr>
<td>A6</td>
<td>25,600</td>
<td>49</td>
<td>−</td>
<td>3,686</td>
<td>3,405–3,998</td>
<td>&lt;2</td>
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<tr>
<td>A7</td>
<td>25,600</td>
<td>49</td>
<td>+</td>
<td>5,031</td>
<td>4,401–5,760</td>
<td>&lt;2</td>
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<tr>
<td>A8</td>
<td>25,600</td>
<td>35</td>
<td>−</td>
<td>3,630</td>
<td>3,307–3,986</td>
<td>5.7</td>
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<tr>
<td>A9</td>
<td>102,400</td>
<td>35</td>
<td>−</td>
<td>3,072</td>
<td>2,742–3,435</td>
<td>&lt;2</td>
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<tr>
<td>M1</td>
<td>25,600</td>
<td>31</td>
<td>−</td>
<td>2,261</td>
<td>1,863–2,740</td>
<td>10.0</td>
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<td>M2</td>
<td>256,200</td>
<td>51</td>
<td>−</td>
<td>15,094</td>
<td>13,233–17,232</td>
<td>21.4</td>
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<td>M3</td>
<td>102,400</td>
<td>21</td>
<td>−</td>
<td>3,139</td>
<td>2,642–3,733</td>
<td>13.2</td>
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<tr>
<td>M4</td>
<td>256,200</td>
<td>56</td>
<td>+</td>
<td>16,117</td>
<td>14,861–17,456</td>
<td>27.9</td>
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<tr>
<td>M5</td>
<td>25,600</td>
<td>38</td>
<td>−</td>
<td>1,808</td>
<td>1,371–2,382</td>
<td>8.8</td>
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<tr>
<td>M6</td>
<td>102,400</td>
<td>45</td>
<td>+</td>
<td>7,122</td>
<td>5,985–8,491</td>
<td>21.0</td>
</tr>
<tr>
<td>M7</td>
<td>25,600</td>
<td>39</td>
<td>−</td>
<td>181</td>
<td>151–218</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

a Subjects A1 to A9 are non-daycare-associated adults; subjects M1 to M7 are daycare-associated mothers.
b <35, low; 35 to 45, medium; ≥45, high.
c Determined by immunoblotting. +, antibodies detected; −, antibodies not detected.
d Reciprocal titers.
e IC50, titers. f IC50, Range. IC50, Range.

Assuming that salivary neutralizing activity is primarily IgG mediated and that IgG transudated from plasma is present in saliva at concentrations ~1,000-fold lower than serum, no neutralizing activities should be detected in saliva when serum titers are below ~1:2,000, as the predicted salivary titers (~1:2) would be below the assay’s limit of detection. Indeed, all adults with serum epithelial entry-neutralizing titers below 1:1,777 lacked epithelial entry-neutralizing activity in their saliva samples, while fibroblast entry-neutralizing activities were never detected in saliva, presumably because fibroblast-based titers in serum never exceeded 1:835. Similarly, saliva samples from Towne recipients were all negative for neutralizing activity, presumably because Towne recipients have low serum epithelial entry-neutralizing activities (5).

Conversely, salivary neutralizing titers correlated with serum titers, and mean serum titers for subjects with salivary activity were statistically higher than for those without. Three subjects, however, were inconsistent with this trend, as they had serum epithelial entry-neutralizing titers greater than 1:1,777 yet lacked salivary neutralizing activity. This could be due to the sampling variability inherent to saliva collection or to fluctuations in total salivary IgG levels (8). Thus, while it appears that salivary neutralizing activity may be predominantly IgG mediated, additional studies are needed to determine if actively secreted (sIgA) antibodies play a role in some subjects.

Saliva samples from 17 children and 8 adolescents were negative for neutralizing activity yet positive for gB-specific IgG (indicating that all were CMV infected). In contrast, 50% of seropositive adults were positive for salivary neutralizing activity. This suggests either that the natural history of CMV infection in children and adolescents differs from that in adults, or that immunological immaturity of children or adolescents attenuates the salivary humoral response. Children may be more efficient transmitters of CMV because they shed virus in
Three subjects in the adult group were clearly unique. They had the highest salivary neutralizing activities, their serum-neutralizing and gB-ELISA titers were well above ranges typical for other seropositives, and they had detectable serum IgG to UL130. These observations suggest that something unique about the viral exposure in these subjects resulted in hyperstimulated humoral responses. One possibility is that these subjects were undergoing active CMV infections subsequent to primary infection. If so, the high IgG avidity for all three subjects suggests that infection occurred at least 3 months prior. Alternatively, that all three subjects had young children (4 to 32 months) in day care suggests the possibility that elevated humoral immunity may arise from frequent CMV exposure. Although urine and saliva samples from their children were not cultured, in the past prevalence of viruria in children under age 2 at this day care center has been as high as 70%. Consistent with this hypothesis, positivity for salivary neutralizing activity was more frequent among day care mothers than non-day care-associated adults. However, these findings should be considered preliminary due to the small size and demographic diversity of our study populations. A larger study is needed to confirm that mothers of children in day care have elevated humoral responses to CMV.

While antibodies to various subcomponents of the gH/gL/UL128-131 complex can neutralize epithelial entry (1, 7, 11, 24), and even peptide epitopes from UL130 or UL131 can induce high titer epithelial entry-neutralizing activity in rabbits (17), the UL128-131 epitopes that dominate human responses to CMV infection remain poorly defined. In immunocompetent seropositive subjects, ELISA or indirect immunofluorescence...
cence assay (IFA) reactivities to individually expressed UL128 or UL130 proteins are uncommon (7), while immunoblot detection of antibodies to UL128, UL130, or UL131 in seropositive human sera has not been previously reported. Consistent with this, we found that most seropositive adults lacked immunoblot-reactive antibodies to UL128-131 proteins, although four subjects with unusually high neutralizing titers had detectable antibodies to UL130. These results suggest that natural infection produces weak humoral responses to the individual proteins such that antibodies are either absent or present at levels below the limits of detection for these assays, but in a few subjects humoral responses are sufficiently robust that antibodies to UL130 can be detected. While this may imply that in humans UL130 is the better immunogen, it is also possible that for technical reasons (e.g., better expression or membrane transfer of UL130), the UL130 assay is simply more sensitive. It is also not known whether the UL130 antibodies detected by immunoblot are neutralizing.

The apparent lack of antibodies to individual UL128-131 proteins in most seropositive suggests that the robust epithelial entry-neutralizing responses induced by natural infections may target primarily conformational or multisubunit-dependent epitopes that are not adequately represented in the immunoblot assay, ELISA, and IFA discussed above. Consistent with this, of 17 monoclonal antibodies with potent epithelial entry-neutralizing activities isolated from seropositive donors, only one reacted with an individual subunit; the others recognized epitopes formed by two or more subunits (11). However, that these antibodies are exceptionally potent for virus neutralization (11) suggests another possibility—that regardless of the nature of their epitopes, antibodies present below our limits of detection could, due to high potency, confer robust neutralizing activities.

While the potency of antibodies targeting gH/gL/UL128-131 makes epitopes of this complex attractive as vaccine immunogens, antibodies targeting this complex will not neutralize fibroblast entry (1, 7, 11, 17, 24). Epitopes in other viral glycoproteins, such as gB and gM/gN, should also be considered, as they may have the advantage of inducing antibodies that neutralize entry into a broader range of cell types (fibroblast, epithelial, and endothelial cells and others). For example, antibodies induced by the gB/MF59 vaccine are more potent against epithelial entry than fibroblast entry (5), and while fibroblast entry-neutralizing activity of antibodies to gM/gN have been described (18, 19), the observation that a gM/gN-reactive human monoclonal antibody neutralizes both fibroblast and epithelial cell entry (11) suggests that like gB, gM/gN epitopes are not cell type specific.

The salivary neutralizing activities observed in this study (1:6 to 1:28) may have biological relevance. In our studies, saliva samples with the highest neutralizing activities (1:21 to 1:28) diluted 1:4 (the lowest tested) nearly eliminated infectivity of 5,000 PFU of CMV (only a few cells became infected) (Fig. 2B). Undiluted, these saliva samples should be capable of fully neutralizing a 100 PFU inoculum (a conservative estimate based on the authors’ unpublished observations that urine samples from viruric day care children generally contain less than 10^7 PFU/ml and assuming a 10-μl inoculum). However, this prediction is speculative, as it is based on in vitro data. Further studies will be needed to establish the ability of salivary antibodies to prevent infection in vivo. With respect to CMV vaccine development, our results suggest that measurable salivary neutralizing activities may be achieved when serum epithelial entry-neutralizing titers exceed ~1:2,000, while higher titers in the 1:20 to 1:30 range may require serum titers of 1:7,000 to 1:16,000. These predictions, however, are based on a small and diverse study population and should be considered preliminary. Future vaccine studies may provide the means to test these predictions and to determine if neutralizing activity in saliva correlates with protection.

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