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, including microcephaly, brain malformations, hearing loss, vision loss, and cognitive impairment. The most common disability found in congenitally infected infants is sensorineural hearing loss, affecting about 36% of symptomatic and 12% of asymptomatic infants (1). Due to the high incidence of permanent sequelae from congenital CMV, the development of a CMV vaccine has been deemed a national priority by the Institute of Medicine (2).

Cytomegalovirus Virions Shed in Urine Have a Reversible Block to Epithelial Cell Entry and Are Highly Resistant to Antibody Neutralization

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ABSTRACT  Cytomegalovirus (CMV) causes sensorineural hearing loss and developmental disabilities in newborns when infections are acquired in utero. Pregnant women may acquire CMV from oral exposure to CMV in urine or saliva from young children. Neutralizing antibodies in maternal saliva have the potential to prevent maternal infection and, in turn, fetal infection. As CMV uses different viral glycoprotein complexes to enter different cell types, the first cells to be infected in the oral cavity could determine the type of antibodies needed to disrupt oral transmission. Antibodies targeting the pentameric complex (PC) should block CMV entry into epithelial cells but not into fibroblasts or Langerhans cells (which do not require the PC for entry), while antibodies targeting glycoprotein complexes gB or gH/gL would be needed to block entry into fibroblasts, Langerhans cells, or other cell types. To assess the potential for antibodies to disrupt oral acquisition, CMV from culture-positive urine samples (uCMV) was used to study cell tropisms and sensitivity to antibody neutralization. uCMV entered epithelial cells poorly compared with the entry into fibroblasts. CMV-hyperimmune globulin or monoclonal antibodies targeting gB, gH/gL, or the PC were incapable of blocking the entry of uCMV into either fibroblasts or epithelial cells. Both phenotypes were lost after one passage in cultured fibroblasts, suggestive of a nongenetic mechanism. These results suggest that uCMV virions have a reversible block to epithelial cell entry. Antibodies may be ineffective in preventing maternal oral CMV acquisition but may limit viral spread in blood or tissues, thereby reducing or preventing fetal infection and disease.

KEYWORDS  cytomegalovirus, neutralizing antibodies, congenital infection, vaccine

Received 24 January 2017  Returned for modification 20 February 2017  Accepted 4 April 2017
Accepted manuscript posted online 12 April 2017
Citation Cui X, Adler SP, Schleiss MR, Arav-Boger R, Demmler Harrison GJ, McVoy MA. 2017. Cytomegalovirus virions shed in urine have a reversible block to epithelial cell entry and are highly resistant to antibody neutralization. Clin Vaccine Immunol 24:e00024-17. https://doi.org/10.1128/CVI.00024-17.
Editor Marcela F. Pasetti, University of Maryland School of Medicine
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with virus in their urine or saliva are the main source of maternal infections. Vaccination could potentially prevent maternal acquisition through the induction of neutralizing antibodies in maternal saliva. However, because CMV uses different viral glycoprotein complexes to enter different cell types, the types of cells that are first infected by CMV within the oral cavity could dictate which antibodies are optimal for preventing oral acquisition. For example, antibodies to the viral glycoprotein B (gB) and a trimeric complex of glycoproteins H, L, and O (gH/gL/gO) neutralize CMV entry into a variety of cell types but are less potent than antibodies recognizing epitopes of the pentameric complex (PC), a five-subunit complex of gH/gL bound to UL128, UL130, and UL131A (4–8). However, as the PC is only required for entry into certain cell types (e.g., epithelial cells, endothelial cells, monocytes, and monocyte-derived dendritic cells) and is dispensable for entry into others (e.g., fibroblasts and Langerhans-type dendritic cells) (4, 9–15), the neutralizing activity of PC-specific antibodies is limited to those cell types that require the PC for viral infection.

The cells that are first infected in the new host are not known. Due to their abundance in the oral cavity, mucosal epithelial cells are likely candidates for the initial infection. If so, a vaccine that induces PC-specific neutralizing antibodies may prevent oral acquisition. On the other hand, if initial infection occurs in cells that use a PC-independent mechanism (e.g., fibroblasts or Langerhans-type dendritic cells), PC-specific antibodies would have no efficacy and gB- or gH/gL/gO-specific antibodies may prevent acquisition. However, this rationale is based on data from cell culture-based experiments in which established cell lines such as MRC-5 fibroblasts or ARPE-19 epithelial cells were used to generate virus stocks and to study viral infection and antibody neutralization. CMV passed in cell culture may behave differently from the CMV that is transmitted via urine (uCMV) or saliva. Indeed, it has been reported that uCMV has poor infectivity for endothelial cells (16) and is resistant to antibody neutralization (17).

These observations led us to examine both the cell type-specific tropisms of uCMV and the ability of different antibodies to neutralize it. We found that uCMV enters and replicates poorly in epithelial cells and is profoundly resistant to neutralization by CMV hyperimmune globulin (HIG) or monoclonal antibodies. Remarkably, both of these properties were rapidly lost after one passage in fibroblasts, suggesting a nongenetic rather than genetic basis. However, once uCMV had entered epithelial cells, the spread to neighboring cells was restricted by both polyclonal and monoclonal antibodies. These findings have important implications for vaccine development, as they suggest that antibodies in saliva may have little impact on oral acquisition, although antibodies in saliva and submucosal tissues may serve to limit local replication and dissemination.

RESULTS

In comparison to fibroblasts, uCMV enters epithelial cells inefficiently. Historically, fibroblasts have been used for the detection and isolation of CMV from clinical samples. The fact that CMV from clinical samples infects human umbilical vein endothelial cells far less efficiently than fibroblasts (16) led us to ask whether this phenomenon extends also to epithelial cells. Monolayer cultures of MRC-5 fibroblasts and ARPE-19 epithelial cells were simultaneously inoculated with replicate dilutions of urine sample Uxc. After incubating for 3 days, the cultures were fixed and stained for CMV immediate early (IE) antigen expression as a marker for viral entry and the initiation of replication. At higher concentrations of urine, MRC-5 cultures were heavily infected, and IE antigen-positive cells remained abundant even when the urine was diluted 320-fold (Fig. 1A). By contrast, ARPE-19 cultures contained only a few IE antigen-positive cells at the highest concentration of urine (1:20). Based on IE antigen-positive cell counts at these dilutions (Fig. 1A), the approximate ratio of fibroblast to epithelial cell infectivity is 55:1.

This experiment was repeated using urine Uxc and two additional CMV-positive urine samples, Umn-2 and U2. To ensure that infected ARPE-19 cells had adequate time to express IE proteins and to provide a more compelling readout in the form of foci of
IE antigen-positive cells, MRC-5 cultures were stained 5 to 7 days postinfection (dpi) and ARPE-19 cultures were stained 13 dpi. The inoculation of MRC-5 cultures with urine samples Uxc, Umn-2, and U2 resulted in extensive infection at dilutions 1:16 (Fig. 1B). By contrast, the inoculation of ARPE-19 cultures with urine samples diluted 1:2 resulted in a few individual foci of antigen-positive cells, indicating that only a small number of infection events were successful and spread to form foci (Fig. 1B). To quantitatively compare infectivity between the two cell types, 11 CMV-positive urine samples were...
titrated by endpoint dilution on both cell types. Titers were 100- to 1,000-fold lower when assayed using epithelial cells versus fibroblasts (Fig. 1C). These results indicate that uCMV virions are significantly more capable of establishing productive infections in MRC-5 fibroblasts than in ARPE-19 epithelial cells.

**Epithelial entry efficiency of uCMV improves within one passage in fibroblasts.** Since all available laboratory strains of CMV were initially isolated from and passaged in fibroblasts, we produced a virus stock from urine Uxc that was passaged exclusively in ARPE-19 epithelial cells. After 14 passages, the culture supernatant was isolated and designated ARPE-19-adapted stock UxcAp14. An MRC-5-adapted stock, UxcMp14, was produced in parallel by passage in MRC-5 cells. The entry properties of the cell culture-passaged viruses were dramatically different from those of the parental Uxc-derived uCMV. IE antigen staining 3 dpi revealed that the ARPE-19-adapted stock UxcAp14 infected both cell types with similar efficiencies (Fig. 2A). Remarkably, the restriction to ARPE-19 entry observed for uCMV was lost following one or two passages in MRC-5 cells (Fig. 2B). ARPE-19 entry efficiency began to decline at passage four, presumably due to the increasing prevalence of a mutation disrupting UL130 that was observed in UxcMp14. Similar results were obtained for two additional urine samples, Umn-3 and Umn-4. MRC-5 and ARPE-19 infectivity were similar for the first two MRC-5 passages but thereafter, ARPE-19 infectivity declined and infected ARPE-19 cells were not detected after Umn-3 passage 9 or Umn-4 passage 5 (Fig. 2C).

ARPE-19 cells are derived from the retinal pigment epithelium and therefore may not accurately represent the presumed targets of uCMV during oral transmission, namely, the epithelial cells of the oral mucosa. To evaluate uCMV infectivity of mucosal epithelial cells, normal oral keratinocytes (NOKs) derived from human gingival tissue were used. Inoculation of MRC-5, ARPE-19, and NOK cultures with matching amounts of urine resulted in extensive antigen staining in MRC-5 cells, but no antigen-positive cells were detected in either the ARPE-19 or the NOK cultures (Fig. 2D). As with the ARPE-19 cells, NOK entry efficiency improved after limited MRC-5 passage, and while adaptation in ARPE-19 cells also improved virus entry efficiency in NOKs, ARPE-19-adapted virus exhibited significantly lower infectivity for NOKs than for ARPE-19 cells (Fig. 2D). Thus, to the extent that NOKs may be representative of oral mucosal epithelial cells, the restriction observed for uCMV entry into ARPE-19 cells appears to also extend to oral epithelial cells.

**uCMVs are highly resistant to antibody neutralization.** To confirm a previous report that uCMVs are resistant to neutralizing antibodies (17), replicate aliquots of CMV-positive urine samples were incubated in medium alone or in medium containing a high concentration (1,280 μg/ml) of HIG. The mixtures were then added to MRC-5 or ARPE-19 monolayers and infectivity was assessed by IE antigen staining. Eleven urine samples were evaluated on MRC-5 cells but only seven had sufficient titers for evaluation on ARPE-19 cells. In all cases, 1,280 μg/ml HIG failed to neutralize CMV infectivity (Fig. 3A). However, an amniotic fluid sample was available from the same subject who, after birth, provided urine sample Ujh-1. MRC-5 infectivity of CMV in the amniotic fluid was sensitive to neutralization by HIG (Fig. 3A). Unfortunately, the viral titer of the amniotic fluid was too low to assess ARPE-19 infectivity.

Seven monoclonal antibodies with potent neutralizing activities were used to further assess the sensitivity of uCMVs to antibody neutralization. TRL345 is a human monoclonal antibody that recognizes the AD-2 epitope of gB and neutralizes both fibroblast and epithelial entry (18). TRL310 and 2-25 are human monoclonal antibodies that recognize PC epitopes, and both selectively neutralize epithelial entry (18, 19, 20). Rabbit monoclonal antibodies 70.7, 124.4, 270.7, and 316.2 recognize a discontinuous gH/gL epitope and neutralize both fibroblast and epithelial entry (19, 20).

Only the anti-gB antibody, TRL345, had activity in these assays. At 50 μg/ml, it partially inhibited the entry of uCMV from U2 into MRC-5 cells or the entry of uCMV from Uxc into both MRC-5 cells and ARPE-19 cells (Fig. 3B). However, 50 μg/ml is ~2,000-fold the neutralizing 50% inhibitory concentration (IC50) of TRL345 measured...
using cell culture-adapted viruses, and at 25 μg/ml, TRL345 had no effect (data not shown). The anti-gH/gL antibodies 70.7, 124.4, 270.7, and 316.2 had no effect on the MRC-5 infectivity of Uxc-derived virus, but given that they are 3- to 6-fold less potent than TRL345, this may be a reflection of their lower potency rather than antigen specificity.

These results confirm and extend previous findings that uCMVs are resistant to neutralizing antibodies (17). Eleven of 11 unique uCMVs were antibody resistant, and resistance extended to high concentrations of antibodies targeting a range of epitopes and viral entry mediators. The only exception was the anti-gB antibody TRL345, which, at a high concentration, partially inhibited uCMV entry into both fibroblasts and epithelial cells.

FIG 2 Inefficient epithelial entry of uCMV is lost after one passage in cell culture. (A) ARPE-19-adapted stock UxCp14 was serially diluted in medium and replicates were added to MRC-5 or ARPE-19 monolayers. After 3 days, the cells were fixed and stained for CMV IE antigen. (B) CMV in urine Uxc was serially passaged in MRC-5 cells. Urine Uxc or culture supernatants from each MRC-5 passage were added in replicates to MRC-5 or ARPE-19 monolayers. After 3 days, the cells were fixed and stained for CMV IE antigen. (C) CMV in urine samples Umn-3 and Umn-4 were serially passaged in MRC-5 cells, and culture supernatants from each passage were added in replicates to MRC-5 or ARPE-19 monolayers. Cells were fixed and stained for CMV IE antigen on days 4 to 7 (MRC-5) or day 13 (ARPE-19). (D) Cell monolayers were infected with matching amounts of urine Umn-4, Uxc passaged four times in MRC-5 cells (UxcMp4), or ARPE-19-adapted Uxc (UxCp14). Cells were fixed and stained for CMV IE antigen 7 days postinfection. Arrowhead indicates an IE antigen-positive NOK cell infected with UxcMp4 virus. Numbers within images indicate IE antigen-positive cell counts.
One passage in fibroblasts restores sensitivity to antibody neutralization. CMV that has been adapted for growth in cell culture is sensitive to antibody neutralization. To determine how rapidly this sensitivity to neutralization occurs upon passage, HIG neutralizing activity was assessed using Uxc-derived virus in culture supernatants after one or two passages in MRC-5 cells or after 16 passages in ARPE-19 cells. Only one passage in MRC-5 cells was sufficient to render the progeny virus sensitive to HIG neutralization using both MRC-5 cells and ARPE-19 cells as targets. The passage 16 ARPE-19-adapted virus was similarly sensitive to HIG neutralization (Fig. 4). One passage in MRC-5 cells was also sufficient to render CMV from urine samples U2, U3, Umn-3, and Umn-4 sensitive to neutralization by HIG (data not shown). This rapid loss of resistance implies a nongenetic mechanism of resistance, for example, the presence of novel factors associated with uCMV virions that are lost upon viral propagation in cell culture.

HIG and monoclonal antibodies to gB and the PC can limit uCMV from spreading in epithelial cells but not in fibroblasts. HIG and monoclonal antibodies added to culture medium after infection of cells can limit the spread of CMV in ARPE-19 but not MRC-5 monolayers (19, 21). To determine if antibodies can similarly limit the spread of uCMV, MRC-5 or ARPE-19 monolayers were infected with uCMV from Uxc. After 24 h, the cultures were washed and incubated for 5 to 7 days (MRC-5) or 12 to 14 days (ARPE-19) in fresh medium alone or in medium containing HIG or monoclonal antibodies before IE antigen staining. As seen in Fig. 5A, neither 1,280 μg/ml HIG nor 25 μg/ml of monoclonal antibody TRL345 or 316.2 had any effect on the spread of
Uxc-derived virus in MRC-5 cultures. This dose is 1,042-fold the IC₅₀ of TRL345 and 187-fold the IC₅₀ of 316.2 for the neutralization of MRC-5 entry. By contrast, the spread of uCMV from Uxc in ARPE-19 monolayers was inhibited by HIG, the anti-gB antibody TRL345, and the anti-PC antibodies TRL310 and 2-25 (Fig. 5B). Moreover, at concentrations >1.5 μg/ml, the anti-PC antibodies prevented the virus from spreading beyond the initially infected cell (Fig. 5B). These results suggest that uCMV is similar to cell culture-produced CMV with respect to the ability of antibodies to limit its spread in epithelial cells but not fibroblasts. Thus, in epithelial cells, the ability of uCMV to evade neutralizing antibodies appears to be lost following the initial entry event.

Treatment with saliva, trypsin, or storage at 4°C does not render uCMV sensitive to neutralizing antibodies. A monoclonal antibody capture enzyme-linked immunosorbent assay (ELISA) failed to detect uCMV in fresh urine, but the detection improved significantly after incubating at 4°C for 9 to 14 days (22). The authors suggested that uCMV may be protected by an inhibitory substance that is gradually degraded during storage. Therefore, we hypothesized that uCMV may become sensitive to antibody neutralization after incubating at 4°C or in response to the exposure to trypsin or factors in saliva that might function to counteract this protective mechanism and render uCMV sensitive to neutralization. To test this, uCMV from U2 was tested for sensitivity to neutralization by HIG after storage at 4°C for 10 or 42 days, after incubating in the presence of 54% human saliva at 37°C for 1 h, or after incubating in

FIG 4 Sensitivity to neutralizing antibody is established after one passage in fibroblasts or with adaptation in ARPE-19 cells. uCMV from Uxc was passaged twice in MRC-5 cells. HIG was diluted in medium, and then each dilution was combined at the indicated final concentrations with a constant amount of MRC-5 passage 1 or passage 2 culture supernatants or with Uxc virus adapted by 16 passages in ARPE-19 cells. The mixtures were incubated for 1 h at 37°C and then added to MRC-5 or ARPE-19 monolayers. Cells were fixed and stained for CMV IE antigen after 3 days.

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the presence of 170 \( \mu g/ml \) trypsin at 37°C for 10 min. As shown in Fig. 6, uCMV from U2 remained insensitive to HIG neutralization regardless of these treatments. These results indicate that human saliva does not contain proteases or other factors that can overcome uCMV resistance to neutralizing antibodies, and that in contrast to previously reported ELISA reactivity (22), the resistance to antibody neutralization is stable at 4°C.

**Urine does not contain trans-acting factors that render CMV insensitive to neutralizing antibodies.** Urine might contain factors that inhibit the activity of neutralizing antibodies or act in trans to modify CMV virions and render them resistant to neutralization. To address this, the sensitivity to HIG neutralization was measured with
a virus produced in cell culture, called ABV. This virus was reconstituted from a bacterial artificial chromosome (BAC) clone that was derived from the UxcAp14 stock. A green fluorescent protein (GFP) marker cassette within the BAC origin of replication allowed assessment of ABV infectivity by the detection of GFP-positive cells. When incubated with HIG alone, ABV entry into MRC-5 cells was neutralized by HIG with an IC50 of approximately 10 μg/ml. When incubations also included urine U2 or urine U3 at concentrations matching those in the experiment in Fig. 3A in which uCMVs from U2 and U3 were insensitive to neutralization by HIG, the sensitivity of ABV to neutralization by HIG was unchanged (Fig. 7A). Therefore, urine samples U2 and U3 did not contain factors that, during the course of the 1 h incubation, were able to modify or render ABV virions resistant to neutralization.

**Passage in the presence of HIG does not render CMV resistant to neutralizing antibodies.** CMV propagated in the presence of a gH-specific neutralizing murine monoclonal antibody becomes resistant to neutralization by a rapidly reversible (presumably nongenetic) mechanism (23). Subsequent studies found that the addition of the gH-specific human monoclonal antibody, MSL-109, to the culture medium of CMV-infected cells results in progeny virions containing MSL-109, and that somehow this provides an alternative entry pathway that is insensitive to MSL-109 neutralization (24). Given that people who shed CMV are CMV-seropositive, the resistance to antibody neutralization observed for uCMV may arise in vivo from CMV replication in an extracellular environment that includes high levels of CMV-specific IgG.

To test this potential mechanism, MRC-5 cells were infected with uCMV from Umn-4, and after 24 h, the medium was replaced with either medium alone or medium containing 2 mg/ml HIG. After an extensive cytopathic effect (CPE) was observed 5 to 7 dpi, cell-associated virus or virus in the culture medium was evaluated for sensitivity to neutralization by HIG. When grown in the absence of HIG, both cell-associated and cell-free Umn-4-derived virus was sensitive to HIG neutralization (Fig. 7B). Cell-associated Umn-4-derived virus produced in the presence of HIG was also sensitive, while no infectious virus was detected in the culture medium, presumably because any virus released was sensitive and neutralized by the HIG in the culture medium. To determine if long-term culture in the presence of HIG results in resistance, Umn-3- and Umn-4-derived viruses, as well as the BAC-derived virus, ABV, were passaged extensively in the

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**FIG 6** uCMV resistance to antibody neutralization is not lost following storage at 4°C or exposure to saliva or trypsin. Urine U2 was pretreated as follows: incubation for 1 h at 37°C with medium (U2), incubation for 1 h at 37°C in the presence of 54% saliva from a healthy adult human subject (+saliva), incubation for 10 min at 37°C in the presence of 170 μg/ml trypsin (+trypsin), or incubation undiluted at 4°C for 10 (4°C 10 d) or 42 (4°C 42 d) days. Each sample was then combined with medium (Ø) or with HIG at a final concentration of 640 μg/ml, was incubated for 1 h at 37°C, and then was added to MRC-5 or ARPE-19 monolayers. Cells were fixed and stained for CMV IE antigen after 5 days (MRC-5) or 15 days (ARPE-19).
presence of HIG as described above. Cell-associated Umn-3- and Umn-4-derived viruses passaged in the presence of HIG remained sensitive to neutralization by HIG (Fig. 7B), while the absence of detectable virus in the culture medium (not shown) implied the absence of resistant cell-free virus. Similarly, cell-free ABV virus passaged in the presence of HIG and then passaged once without HIG was also sensitive to neutralization by HIG (Fig. 7B). Thus, virus propagation in the presence of polyclonal antibodies does not result in antibody-resistant virus.

FIG 7 uCMV resistance to antibody neutralization is not conferred by soluble factors in urine or by propagation in the presence of CMV-specific antibodies. (A) HIG was diluted in medium and then each dilution was combined at the indicated final concentrations with a constant amount of GFP-tagged virus ABV with or without a constant amount of CMV-positive urine sample U2 or U3. The mixtures were incubated for 1 h at 37°C and then added to MRC-5 monolayers. Micrographs were taken with an inverted UV microscope 5 days after infection. (B) uCMV from Umn-4 was passaged once in MRC-5 cells cultured in medium (Umn-4 p1+Ø) or in medium containing 2 mg/ml HIG (Umn-4 p1+HIG). Cell-associated (CA) or cell-free (CF) viruses were prepared 7 dpi. Additionally, cell-associated virus stocks with HIG (Umn-4 p17+HIG and Umn-3 p16+HIG) were prepared after 17 (Umn-4) or 16 (Umn-3) passages in MRC-5 cells in the presence of 2 mg/ml HIG, and cell-free ABV virus was produced by passage of virus ABV for 6 months in the presence of 2 mg/ml HIG (ABV +HIG) and then passaged once without HIG to produce a culture supernatant containing ABV virus but lacking HiG. Samples were combined with medium (Ø) or with HIG at a concentration of 1,280 µg/ml, were incubated for 1 h at 37°C, and then were added to MRC-5 or ARPE-19 monolayers. Cells infected with uCMVs derived from Umn-3 or Umn-4 were fixed and stained for CMV IE antigen after 5 days (MRC-5) or 12 days (ARPE-19); ABV-infected cells were photographed with an inverted UV microscope 3 to 4 days after infection.
DISCUSSION

The discovery of the PC and elucidation of its role as a target for potent neutralizing antibodies specific for epithelial/endothelial cell entry have inspired new avenues in CMV vaccine development. In particular, vaccines that induce PC-specific salivary antibodies could block inoculum virus from infecting the epithelial cells of the oral mucosa. In support of this, saliva samples from a subset of naturally infected subjects neutralized the entry of cell culture-passaged CMV into epithelial cells (25). However, this assumes that mucosal epithelial cells are the first cells infected during oral acquisition. If this is not the case, PC-specific antibodies may have little effect. These considerations led us to evaluate the entry tropisms of CMV found in clinical specimens for fibroblasts and for epithelial cells, and to assess the sensitivity of these infection events to neutralizing antibodies. Three key observations were made.

First, uCMV is profoundly deficient for entry into ARPE-19 epithelial cells, which in recent years have been used extensively to model CMV epithelial tropism. How accurately these cells represent CMV’s interactions with epithelial cells from other tissues remains largely unknown. Using cell culture-passaged CMV, we confirmed that the PC is necessary for efficient entry into epithelial cells derived from a variety of mucosal tissues, including of the tonsil, bronchus, cervix, foreskin, and vagina (6), while in the current work, we extended these studies to include NOK cells derived from gingiva. Surprisingly, uCMV failed to infect NOK cells, but as for ARPE-19 cells, NOK infectivity was quickly acquired upon passage in fibroblasts. Gerna et al. observed very similar defects in entry of uCMV or CMV in throat washes into human umbilical vein endothelial cells (16). Taken together, these data suggest that CMV shed in urine and saliva may have a general deficiency in the ability to enter cells by the PC-mediated pathway. The molecular basis of this deficiency remains to be determined but could include low levels of functional PC in virions caused by an intrinsic property of the cells that produce CMV in vivo or the degradation or inactivation of the PC during or after virion release by factors in urine or saliva. That an alternative entry pathway may be utilized that is inefficient for epithelial or endothelial cell entry is discussed further below.

Second, the entry of uCMV into both fibroblasts and epithelial cells is profoundly resistant to neutralization by polyclonal antibodies in HIG and to monoclonal antibodies specific for gB, gH/gL, or the PC. However, at high concentrations, the gB-specific antibody TRL345 had a partially inhibitory effect (Fig. 3B). The fact that TRL345 is less potent than the PC-specific antibodies TRL310 and 2-25 at neutralizing cell culture-passaged virus may suggest that the mechanism of antibody resistance is less effective against antibodies that neutralize by targeting gB versus those that target the PC. Given that a recombinant gB vaccine provided partial protection against CMV acquisition (26), it is possible that, in the context of the oral cavity, vaccine-induced gB-specific antibodies can provide some efficacy by neutralizing inoculum CMV in urine or saliva.

As for the defect in epithelial cell entry, a sensitivity to antibody neutralization is acquired within one passage in fibroblasts. This implies that the two phenomena may be linked, i.e., that the mechanism that protects the virus from neutralizing antibodies also impairs its ability to enter epithelial cells. If so, the observation that CMV in throat washes is deficient in endothelial cell entry (16) may further imply that CMV in saliva may also be resistant to antibody neutralization.

The mechanism of antibody resistance is unknown. In contrast to cell culture-propagated virus, the envelope fraction of uCMV contains significant amounts of β₂ microglobulin (β₂m), and it has been proposed that this β₂m serves to prevent antibodies from binding to surface glycoproteins, thereby protecting uCMV virions from antibody neutralization and preventing their detection by an antibody capture ELISA (17). This hypothesis was supported by two experimental observations. First, the ELISA signal from uCMV in fresh culture-positive urine samples became positive after storage at 4°C for 9 to 14 days, suggesting that uCMV glycoprotein epitopes became accessible to the monoclonal antibody as the inhibitory factors (possibly β₂m) gradually degraded (22). Second, the ELISA signal from cell culture-propagated virus could be
inhibited by the addition of soluble β₂m (27). It remains uncertain whether β₂m simply occludes antibody access to the established entry mediators (gB, gH/gL/gO, or PC) without impairing their ability to engage receptors and mediate entry or, alternatively, if it inhibits the established entry pathways while perhaps facilitating a novel entry pathway that does not require gB, gH/gL/gO, or the PC. Such an alternative entry pathway could explain both resistance to antibody neutralization and the deficient infectivity of uCMV for epithelial cells if, for example, epithelial cells have low levels of the required receptor(s).

The idea that β₂m associated with uCMV might function to mediate an alternative entry pathway has been proposed and was supported by evidence that free β₂m can compete with CMV virions for binding to fibroblasts and that β₂m-coated virions bind to HLA class I-positive cells at significantly higher levels than to cells devoid of HLA class I expression (28). Evidence that antibodies to β₂m can neutralize uCMV would greatly support this model, but McKeating et al. found that uCMV was not neutralized by four monoclonal antibodies or a polyclonal rabbit antiserum raised against β₂m (17). Moreover, while the addition of β₂m to cell culture-propagated virus increases its infectivity (28), this has not been demonstrated to confer resistance to antibody neutralization, and in our hands, prolonged incubation at 4°C, shown previously to permit access of monoclonal antibodies to uCMV epitopes (22), did not render uCMV sensitive to antibody neutralization (Fig. 6). Thus, the proposed role for β₂m in uCMV antibody resistance and/or an alternative entry pathway requires further study.

The ability of CMV to evade antibody neutralization by the incorporation of antibodies into progeny virions, as described for MSL-109 (24), suggests an intriguing mechanism for antibody resistance of uCMV, given that uCMV is likely derived from an environment rich in CMV-specific IgG. Moreover, similar to uCMV antibody resistance, MSL-109 resistance results in impaired PC-mediated entry and is lost upon one passage in medium lacking MSL-109 (24). Unlike uCMV, MSL-109-resistant virus is specifically resistant to MSL-109 and is not cross-resistant to antibodies recognizing gB or other epitopes in gH (24). Even so, if the mechanism of MSL-109 resistance extends to other glycoprotein targets and epitopes, it is possible that the incorporation of a polyclonal mixture of antibodies into CMV virions could result in resistance to antibodies targeting a broad spectrum of epitopes. However, our failure to maintain or reconstitute resistance to antibody neutralization by propagating uCMV or cell culture-adapted CMV in the presence of polyclonal CMV-specific antibodies suggests that in vivo replication of CMV in the presence of CMV-specific antibodies is probably not the mechanism by which uCMV attains resistance.

Third, while neutralizing antibodies had little effect on entry, they were effective at blocking the spread of virus in ARPE-19 epithelial cell monolayers. We previously reported that HIG can inhibit the spread of cell culture-passaged and uCMV in ARPE-19 cells (21) and have more recently extended these studies to quantitate the inhibition of cell culture-passaged CMV spreading by these and other monoclonal antibodies. We observed a close correlation between spread inhibition and neutralizing potencies, suggesting that in cultured ARPE-19 cells, CMV spread likely requires the transient release of infectious virions into the extracellular compartment (19). By contrast, cell-to-cell spread between fibroblasts uses a distinct mechanism that is highly resistant to antibody inhibition.

Our findings have two major implications. First, CMV in the form that is responsible for oral transmission appears to be optimized for infecting fibroblasts, while entry into epithelial cells (this study) and endothelial cells (16) is profoundly impaired. The fact that this impairment is rapidly lost upon propagation in vitro suggests a nongenetic rather than genetic cause, for example, a deficiency of the PC in the uCMV virion envelope, posttranslational modifications that impair PC function, or the interaction of the PC with inhibitory factors. These observations further suggest that CMV may have evolved mechanisms to actively block uCMV from infecting many of the cells that it initially encounters upon first entering the oral cavity, perhaps to prevent unproductive infections at sites from which dissemination may be difficult and to promote infections
at sites where access to the lymphatics may be less constricted (e.g., the tonsil). If so, this may in part explain why CMV has evolved multiple entry mechanisms, as having multiple pathways allows one to be temporarily blocked while others remain fully functional.

While the cells initially targeted by uCMV remain uncertain, the data thus far suggest that they may be fibroblasts or other cells that can be infected by a PC-independent (fibroblast-like) mechanism. CMV could potentially gain access to fibroblasts through microbreaks that permit viral access to submucosal tissues, as is thought to occur during the oral transmission of human papillomaviruses, which initially infect the undifferentiated basal cell layers of mucosae (29). Alternatively, CMV infection of Langerhans-type dendritic cells does not require the PC (15), and while these cells reside in the submucosa, their dendrites can extend to the mucosal surface. Thus, CMV could initially infect Langerhans-type dendritic cells and subsequently spread to adjacent epithelial cells and/or fibroblasts. This pathway is similar to that of the measles virus, which does not initially infect mucosal epithelial cells but first infects and replicates in dendritic cells before spreading to infect adjacent pulmonary epithelial cells (30). It is also possible that uCMV selectively targets certain epithelial cells that are unique to a specific site within the oral cavity, or that uCMV entry into oral mucosal cells in vivo involves cellular factors that are not expressed by cells grown in culture. The development of assays that more accurately model the mucosal environment (e.g., organotypic keratinocyte cultures representing stratified/differentiated epithelium) may be useful for elucidating the initial interactions of CMV with the oral mucosa.

Second, regardless of which cell types are initially infected and which entry pathway is involved, the profound resistance of CMV in the inoculum to antibody neutralization suggests that salivary antibodies may not be highly effective at interrupting CMV acquisition. While we have shown that saliva samples from some seropositive subjects can neutralize CMV propagated in cell culture (25), additional studies are needed to determine if these saliva samples also have the capability of neutralizing CMV in urine. In the event that saliva fails to fully neutralize CMV in the inoculum, our in vitro findings further suggest that CMV released from the initially infected cells or spreading to adjacent epithelial cells should be sensitive to the effects of antibody inhibition. Intervention at this stage may be crucial for limiting replication in local tissues and thereby reducing or preventing dissemination that prefaces fetal infection.

MATERIALS AND METHODS

Study populations and sample collection. CMV culture-positive urine samples were obtained from 10 congenitally infected newborns seen at Virginia Commonwealth University Medical Center, University of Minnesota Medical Center, Johns Hopkins Hospital, Baylor College of Medicine, or Texas Children’s Hospital. Amniotic fluid AFjh-1 was obtained at 21.5 weeks gestation from the same subject that provided urine Ujh-1. Urine U3 was obtained from a normal healthy child attending day care in Richmond, Virginia. Urine and amniotic fluid samples were clarified of cellular debris by centrifugation at 2,600 × g for 5 min, and then were adjusted to 100 mM sucrose, aliquotted, and stored under liquid nitrogen. Saliva and sera were obtained from normal healthy adults. Informed consent was obtained from all subjects or their guardians, and protocols were approved by the committees for the conduct of human research at Virginia Commonwealth University, University of Minnesota, Johns Hopkins Hospital, and Baylor College of Medicine.

Antibodies. HIG (CytoGam; CSL Behring, King of Prussia, PA) was purchased from the manufacturer. TRL345 (Trellis Bioscience) is a human monoclonal antibody specific for the AD-2 (site I) epitope of gB (31). TRL310 (Trellis Bioscience) (18) is a human monoclonal antibody reconstructed from published sequences of antibody 1F11 specific for a discontinuous epitope formed by UL130 and UL131A (5). Human monoclonal 2-25 was isolated and cloned from cultured memory B cells of a healthy CMV-seropositive donor (20). Rabbit monoclonal antibodies 70.7, 124.4, 270.7, and 316.2 are described in reference 4 and recognize a discontinuous epitope formed by gH and gL (19, 20).

Cells. Human MRC-5 fetal lung fibroblasts (ATCC CCL-171) and ARPE-19 retinal pigment epithelium cells (ATCC CRL-2302) were obtained from ATCC and propagated in high glucose Dulbecco’s modified Eagle medium (Gibco-BRL) supplemented with 10% fetal calf serum (HyClone Laboratories), 10,000 IU/liter penicillin, and 10 mg/liter streptomycin (Gibco-BRL). NOKs were a gift from Karl Munger (32) and were propagated using Keratinocyte-SFM supplemented with human epidermal growth factor and bovine pituitary extract (Invitrogen).
Virus adaptation for growth in ARPE-19 or MRC-5 cells. Urine Uxc was inoculated onto ARPE-19 or MRC-5 cells and serially passaged until extensive cytopathic effects were observed. The adaptation for growth in ARPE-19 cells required 10 to 12 passages over a period of 12 months. Passage 14 and passage 16 ARPE-19-adapted stocks were designated UxcAp14 and UxcAp16. Adaptation on MRC-5 cells took 10 passages over 3 months. A passage 14 fibroblast-adapted stock was designated Uxcm14. Umn-3 and Umn-4 uCMVs were serially passaged in MRC-5 cells as described above with or without 2 mg/ml HIG in the culture medium. A viral genome designated ABV was cloned as a BAC from the UxcAp14 stock using methods described previously (33). The ABV BAC contains a BAC origin of replication with GFP marker cassette inserted between US28 and US29 and a spontaneous deletion in the UL146 to UL150 region. ABV virus was reconstituted by the transfection of ABV BAC DNA into ARPE-19 cells and was amplified in ARPE-19 cultures as described previously (33). Frozen stocks were prepared from culture supernatants and the titers were determined as described previously (33).

Detection and determination of viral titers by IE antigen staining. CMV-infected cells were detected by immunohistochemical staining of IE antigen 3 to 18 dpi as described previously (34). IE antigen-positive cells in photographic images were counted manually from enlarged prints. Urine samples and virus stocks derived from urine samples were titrated by serial dilution in medium, were inoculated onto ARPE-19 or MRC-5 cultures in 96-well plates, and were stained for IE antigen on days 4 and 5.

Neutralization assays and photomicroscopy. Neutralization and spread inhibition assays were conducted as described previously (6, 21, 34). For neutralizing assays, antibodies were serially diluted in medium, were mixed with an equal volume of medium containing virus and incubated for 1 h at 37°C, and then were transferred to 96-well plates containing confluent MRC-5 or ARPE-19 cells. For spread inhibition assays, monolayers in 96-well plates were infected at a low multiplicity (50 to 150 PFU/well) and then were transferred to 96-well plates containing confluent MRC-5 or ARPE-19 cells. For spread inhibition assays, monolayers in 96-well plates were infected at a low multiplicity (50 to 150 PFU/well) and incubated for 24 h. The culture medium was then removed and replaced with medium containing serial dilutions of antibodies. Representative bright field and fluorescence images were taken using a Nikon Diaphot 300 UV microscope and 10× objective. GFP-based determination of antibody neutralizing IC50 using virus ABV is described elsewhere (19).

ACKNOWLEDGMENTS

We thank Cindy Gandaria for help with processing and shipping urine samples, Karl Munger for NOK cells, Tong-Ming Fu and Merck and Co., Inc., for rabbit monoclonal antibodies and human monoclonal antibody 2-25, and Lawrence Kauvar and Trellis Bioscience for monoclonal antibodies TRL310 and TRL345.

This work was supported by grants R01AI088750 and R21AI073615 (to M.A.M.) from the National Institutes of Health.

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