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VIRUS-CELL INTERACTIONS

Pathogenesis of Wild-Type-Like Rhesus Cytomegalovirus Strains following Oral Exposure of Immune-Competent Rhesus Macaques

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ABSTRACT Rhesus cytomegalovirus (RhCMV) infection of rhesus macaques (*Macaca mulatta*) is a valuable nonhuman primate model of human CMV (HCMV) persistence and pathogenesis. *In vivo* studies predominantly use tissue culture-adapted variants of RhCMV that contain multiple genetic mutations compared to wild-type (WT) RhCMV. In many studies, animals have been inoculated by nonnatural routes (e.g., subcutaneous, intravenous) that do not recapitulate disease progression via the normative route of mucosal exposure. Accordingly, the natural history of RhCMV would be more accurately reproduced by infecting macaques with strains of RhCMV that reflect the WT genome using natural routes of mucosal transmission. Here, we tested two WT-like RhCMV strains, UCD52 and UCD59, and demonstrated that systemic infection and frequent, high-titer viral shedding in bodily fluids occurred following oral inoculation. RhCMV disseminated to a broad range of tissues, including the central nervous system and reproductive organs. Commonly infected tissues included the thymus, spleen, lymph nodes, kidneys, bladder, and salivary glands. Histological examination revealed prominent nodular hyperplasia in spleens and variable levels of lymphoid lymphofollicular hyperplasia in lymph nodes. One of six inoculated animals had limited viral dissemination and shedding, with commensurately weak antibody responses to RhCMV antigens. These data suggest that long-term RhCMV infection parameters might be restricted by local innate factors and/or *de novo* host immune responses in a minority of primary infections. Together, we have established an oral RhCMV infection model that mimics natural HCMV infection. The virological and immunological parameters characterized in this study will greatly inform HCMV vaccine designs for human immunization.

IMPORTANCE Human cytomegalovirus (HCMV) is globally ubiquitous with high seroprevalence rates in all communities. HCMV infections can occur vertically following mother-to-fetus transmission across the placenta and horizontally following shedding of virus in bodily fluids in HCMV-infected hosts and subsequent exposure of susceptible individuals to virus-laden fluids. Intrauterine HCMV has long been recognized as an infectious threat to fetal growth and development. Since vertical HCMV infections occur following horizontal HCMV transmission to the pregnant mother, the nonhuman primate model of HCMV pathogenesis was used to characterize the virological and immunological parameters of infection following primary mucosal exposures to rhesus cytomegalovirus.

KEYWORDS cytomegalovirus, mucosal pathogens, nonhuman primate, rhesus, shedding, viral immunity, viral pathogenesis

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Human cytomegalovirus (HCMV) infection is globally ubiquitous, and infection outcomes are usually either subclinical or mild and self-limiting in immunocompetent individuals (1). However, HCMV is a significant cause of morbidity and mortality in those without fully functional immune systems, including immunosuppressed transplant recipients and those coinfected with HIV who are not on antiretroviral chemotherapy (2–4). Notably, HCMV is also the most frequent congenital infection, with a global birth prevalence of 0.64% (5) and locale-specific frequencies ranging from 0.3 to 5.4% (6). Congenital HCMV can disrupt fetal growth and development (7), and postnatal sequelae, which can range from mild to severe, have been estimated to occur in 10 to 15% of congenital infections (8–11). Since the preponderance of congenital outcomes entail permanent neurological damage, including cognitive impairments and sensorineural hearing loss (12), the annual global burden of congenital HCMV infection is enormous. Based on an estimated 135 million global live births in 2019 (13), a global congenital infection rate of 0.64% (10) would equate to 864,000 congenital infections each year.

Although therapeutics are available for clinically apparent neonatal infections, expeditious use of a protective vaccine strategy remains the best prevention option. Clinical trials have shown that treatment of children, symptomatic at birth with congenital HCMV, with intravenous ganciclovir or oral valganciclovir can improve long-term hearing and neurological outcomes, although there can be drug-related adverse effects (14, 15). Development of a vaccine that protects fetuses from congenital HCMV infection and its sequalae has been a long-held health priority (7), and the quest for an effective HCMV vaccine has been ongoing for more than 4 decades (16). However, a licensed vaccine remains an unfulfilled objective, attributable in part to the complexity of HCMV pathogenesis, incompletely defined correlates of host protective immunity, HCMV immune evasion, and species-specific barriers to evaluating direct HCMV infection and immunology in tractable animal models (6, 8, 9, 17, 18). Moreover, gaps remain in the understanding of HCMV natural history that, if filled, could lead to optimized vaccine approaches. In particular, horizontal transmission of HCMV is mediated by shedding of infectious virions in bodily fluids (e.g., saliva, urine, breast milk) and subsequent exposure of mucosal surfaces to those virus-laden fluids (19). The primary goal of this study was to develop an animal model to characterize infection following exposure of the oral mucosa to infectious virus.

Infection of some animal species with cognate CMVs largely recapitulates patterns of HCMV persistence and pathogenesis, and these models are critical for providing a better understanding, treatment, and prevention of human CMV infection and disease. Given the extensive genomic and biological similarity between RhCMV and HCMV and between their primate hosts, RhCMV infection of rhesus macaques (RM) is a highly relevant nonhuman primate model for HCMV infection of humans (20–25). To date, cumulative studies have shown that RhCMV infection closely recapitulates HCMV infection. However, the tissue culture-adapted RhCMV strains 68-1 and 180.92 were used in many of these studies, along with nonnatural subcutaneous (s.c.) or intravenous (i.v.) routes of inoculation (26–32). RhCMV 68-1 and 180.92 have been extensively cultured in fibroblasts *in vitro* and have undergone multiple genetic alterations. Similar to fibroblast passage of clinical HCMV isolates, serial fibroblast propagation of RhCMV 68-1 and 180.92 has led to viral mutations (e.g., point mutations, deletions, genetic rearrangements) in both strains, most notably, at the right side of the unique long segment (UL/b′ region) (32–36). These collective genetic changes have resulted in the loss of epithelial tropism *in vitro* and *in vivo*, as well as virulence attenuation *in vivo* for 68-1 and 180.92 (21, 26, 27, 32, 37). Although some studies have used WT-like RhCMV strains UCD52 and UCD59 (both of which encode full-length ULb′ regions, exhibit epithelial cell tropism *in vitro* and *in vivo*, and are shed in bodily fluids of experimentally inoculated macaques), animals in these studies were inoculated i.v. or s.c., not mucosally (21, 32, 38–40).

A goal of HCMV vaccination of girls and women is to prevent the entry of virus at exposed mucosal surfaces and dissemination of progeny virions to distal sites, notably

the maternal-fetal interface. Oral exposure is believed to be the main transmission route for both primary and nonprimary HCMV to children prior to adolescence and from children to pregnant women (41–47). Oral exposure is also a natural transmission route for RhCMV (48). Bypassing mucosal inoculation obviates the physical and innate barriers of mucosal attachment and entry prior to systemic spread of progeny virions throughout the infected host (49). Natural history studies demonstrate that the magnitude and frequency of viral shedding in the bodily fluids that transmit HCMV and RhCMV via mucosal surfaces can vary broadly among infected individuals (50–55). Accordingly, a better understanding of the impacts of viral titers and frequencies of viral exposure at mucosal surfaces can inform hypotheses about which vaccine strategies are likely to be effective.

In individuals with extant antiviral immunity induced by prior HCMV infection, HCMV reinfection can occur. Indeed, severe adverse outcomes can occur in neonates born to mothers with nonprimary HCMV infection (56–62). Reinfection as a paramount, global clinical concern is highlighted in a recent review by Plotkin and Bopanna (8), "...it can be assumed that [>50%] of congenitally infected infants in high income countries are born to women with preexisting seroimmunity and in populations with high seroprevalence such as low-income minority women in the U.S. and the vast majority of women in [low-to-middle income countries], most infected infants are born to women with nonprimary CMV infection." Together with other mechanisms of immune evasion, such as genetic diversity and HCMV-encoded immunomodulators, mucosal entry is thought to contribute to the ability of HCMV to reinfect.

To develop a tool to study prevention of mucosal HCMV infections, this study establishes and characterizes an RhCMV oral infection model. We orally inoculated rhesus macaques with wild-type-like strains of RhCMV (UCD52 and UCD59) and temporally evaluated the effects of viral infectious dose level and inoculation frequency on seroconversion rate, viremia, virus dissemination, and shedding in bodily fluids. Oral inoculation established systemic RhCMV infection and shedding at a frequency comparable to that observed in animals naturally infected with RhCMV following exposures to endemic strains of RhCMV circulating in breeding cohorts of rhesus macaques. Rates of infection increased with inoculum size and the number of oral exposures to RhCMV. Large differences in the ability of sera from RhCMV-inoculated rhesus macaques to neutralize antigenically divergent strains in culture highlight a challenge for CMV vaccine development.

RESULTS

Effect of viral infectious dose and inoculation frequency on RhCMV seroconversion, DNAemia, and shedding. Three groups of animals (n = 4 per group) were inoculated *per os* (p.o.) with a single dose of 8×10^5 (group I), 8×10^4 (group 2), or 8×10^3 (group 3) PFU of UCD59 (Fig. 1A). This range of viral titers corresponds to the normative RhCMV viral loads in saliva (quantified by real-time PCR of DNA purified from saliva) in breeding cohorts of animals naturally exposed to endemic strains of circulating RhCMV (63). Longitudinal analysis of RhCMV-specific antibody responses in inoculated animals over 12 weeks postinoculation (p.i.) showed that, when seroconversion occurred, RhCMV-specific antibody responses were detected 2 to 5 weeks p.i. (Fig. 2A and B). The rates of seroconversion for groups 1, 2, and 3 were 75% (3/4; Group 1; animals 2 [G1-2], G1-3, and G1-4), 25% (1/4; G2-1), and 0% (0/4), respectively, suggesting an association between viral inoculation dose level and infection rate (log rank test for trend, P = 0.0382). However, neither the frequency nor the magnitude of viral DNA detection in plasma or saliva was associated with the titers of inoculating virus (Fig. 2C). No DNAemia was detected in any Group 1 animals, whereas frequent (G1-4) or sporadic (G1-2) oral viral shedding was detected in two of the three seroconverted Group 1 animals. The lone RhCMV antibody-positive Group 2 animal (G2-1) showed episodic plasma DNAemia and continuous viral shedding in saliva (Fig. 2B and C). These data indicate that a one-time oral exposure to $\geq 8 \times 10^4$ PFU of RhCMV UCD59 can sometimes establish infection in naive RM, as measured by seroconversion. However, seroconversion did not always correspond to detection of viral DNA in plasma and saliva.

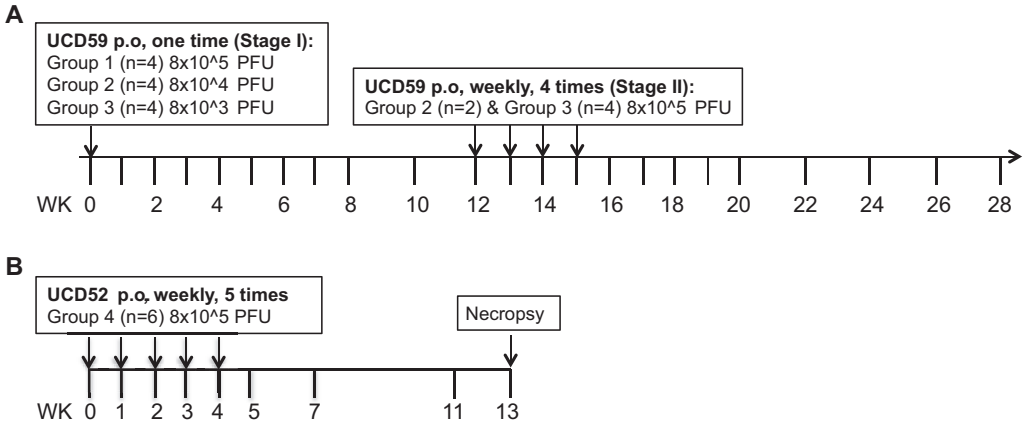

FIG 1 RhCMV oral inoculation and sample collection schedule. (A and B) The times of *per os* (p.o.) exposure to (A) UCD52 or (B) UCD59, and necropsy (UCD52 group only) are indicated by arrows. Sample collection times are indicated by vertical bars. Blood and saliva samples were collected from UCD59-exposed animals, whereas blood, saliva (except weeks [WK] 0 to 4), and urine samples were collected from UCD52-exposed animals.

The effect of repeated oral inoculation on RhCMV infection was evaluated in six of the animals that remained RhCMV seronegative at week 12 after the single inoculation (Group 2: G2-3, G2-4; Group 3: G3-1, G3-2, G3-3, G3-4) (Fig. 1A). Because the dose levels used for Group 1 (8×10^5 PFU) and Group 2 (8×10^4 PFU) bracketed the 50% seroconversion frequency, 8×10^5 PFU was chosen as the oral inoculum level for this experiment. The six animals were inoculated an additional four times at 1-week intervals (weeks 12 to 15; Fig. 1A). All animals seroconverted to RhCMV between week 15 and week 20, demonstrating that up to four additional p.o. exposures to 8×10^5 PFU of UCD59 infected 100% of the animals (Fig. 3A and B).

Virological parameters showed that three infected animals (50%) had plasma DNAemia (analyzed weekly through week 28), each with different kinetics and duration of detection (Fig. 3C). Initial detection of viral DNA in plasma occurred at week 14 (G2-4), week 20 (G2-3), and week 28 (G3-1). Detection of viral DNA in saliva was a more consistent consequence of RhCMV infection following oral exposure. RhCMV DNA was detected in the saliva of five animals (83%) after week 22, 7 weeks after the last inoculation p.o. (Fig. 3C). Interestingly, three animals showed intermittent or sporadic oral shedding prior to seroconversion (G2-3, G3-2, G3-4), consistent with the observation of transient oral infection with HCMV (64). Since weekly oral swabs were collected immediately prior to buccal exposure, detection of viral DNA in saliva at these time points likely represented *de novo* viral DNA and not residual viral DNA from the previous exposure to virus 7 days prior. These data showed that multiple oral exposures to 8×10^5 PFU of UCD59 resulted in 100% of the animals being infected and 83% shedding virus in saliva. These data highlight the impact of frequent mucosal oral exposure on overcoming innate and intrinsic barriers to infection.

To confirm that infection of naive macaques following repeated p.o. inoculation of RhCMV was not specific to the UCD59 strain, the experiment (5 p.o. inoculations of 8×10^5 PFU at 1-week intervals) was repeated with a second WT-like strain, UCD52. RhCMV strains UCD52 and UCD59 were isolated from the urine of different SIV-infected macaques and are distinguished by extensive genome and protein sequence differences (Table 1). A group of six naive animals (group 4; Fig. 1B) was exposed p.o. weekly for 5 weeks to 8×10^5 PFU of UCD52, and longitudinal blood, saliva, and urine samples were collected and evaluated for virological and immunological indicators of primary infection (Fig. 4). RhCMV-specific antibody responses demonstrated that 5 of 6 animals seroconverted by week 4 pi, contemporaneous with, or immediately prior to, the detection of RhCMV DNA in plasma (UCD52-R#1-5). There was variability both in the number (1 to 4 times with detectable DNA) and persistence of detectable RhCMV DNA

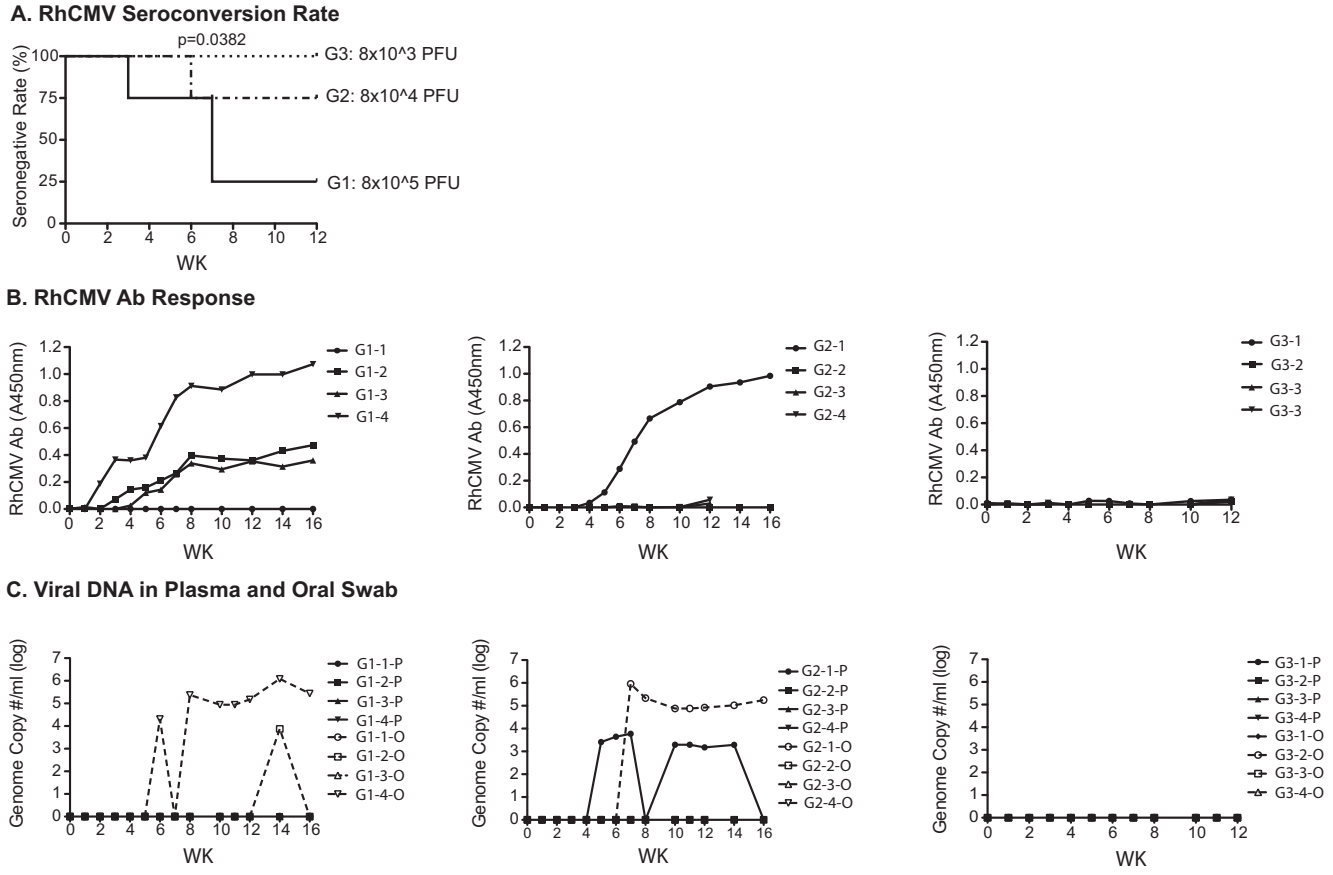

FIG 2 The effect of UCD59 inoculation titer on viral and immune parameters of infection. (A to C) Three groups of animals were p.o. exposed one time to different titers of UCD59 and longitudinally evaluated for (A) seroconversion to RhCMV-specific antigens post-p.o. exposure (WK, weeks), (B) development of RhCMV-specific binding antibody (Ab) responses, and (C) RhCMV genome copy numbers in plasma and oral swabs (#/ml). RhCMV Ab response and seroconversion rate were evaluated by RhCMV ELISA. RhCMV viral DNA was quantified by gB qPCR. G, group.

in plasma (Fig. 4B and C). RhCMV DNA was detected in the saliva and urine of all 5 animals with detectable plasma RhCMV DNA. The last animal (UCD-R#6) in group 4 that seroconverted (week 7) had the lowest RhCMV antibody response and no detectable RhCMV DNA in plasma, saliva, and urine. The association of late seroconversion, low RhCMV-binding antibody levels, and lack of detectable RhCMV DNA in plasma and saliva and/or urine was also noted in UCD59-inoculated animals G1-3 (Fig. 2B and C) and G3-3 (Fig. 3) (urine was not evaluated in UCD59-exposed animals), together amounting to ~20% of the animals (3/16) undergoing a primary mucosal infection after inoculation. Accordingly, RhCMV primary infection does not necessarily result in detectable DNAemia and virus shedding. Stated another way, some factors (e.g., local innate immunity or *de novo* immune responses) in these animals (3/16) may have restricted viral replication after infection. Nevertheless, the results from UCD52-inoculated animals confirmed that a high titer and repeated oral exposures resulted in viral DNA detection in plasma, saliva, and urine from most animals.

UCD52 histopathology and *in vivo* dissemination. To gain detailed insights into the profiles of RhCMV infection following oral inoculation, all UCD52-inoculated animals were euthanized at week 13 to assess the patterns of RhCMV dissemination and histopathological outcomes. Gross histopathological examination revealed prominent splenomegaly, multiple nodular lesions in the spleen, and enlarged mesenteric lymph nodes (LN) (2.0 to 4.0 cm maximum width) in all animals. Splenic lymphoid nodules ranged from <1 mm diameter (UCD52-R#1 and -6, represented by UCD52-R#6) in some animals to approximately 2 mm in others (UCD52-R#2 to -5, represented by UCD52-R#2 and -3) (Fig. 5A and B).

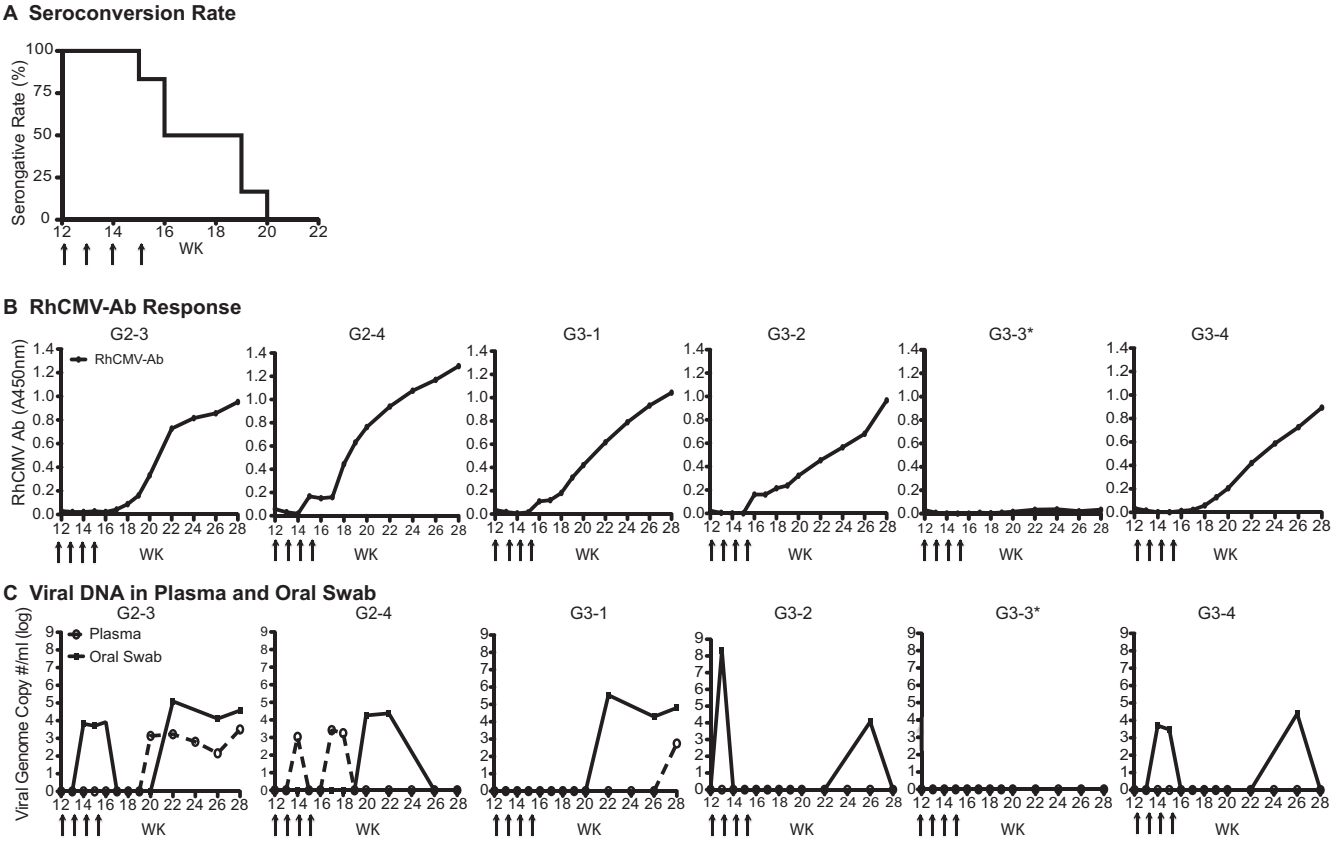

FIG 3 The effect of UCD59 exposure frequency on viral and immune parameters of infection. Six animals that did not become infected after a single p.o. exposure to UCD59 (week [WK] 0; Fig. 2) were p.o. exposed weekly to 8×10^5 PFU of UCD59 an additional four times starting at week 12 (arrows). (A to C) Animals were longitudinally evaluated for (A) seroconversion to RhCMV-specific antigens post-p.o. exposure (WK, weeks), (B) development of RhCMV-specific binding antibody (Ab) responses, and (C) RhCMV genome copy numbers in plasma and oral swabs. For the graphs in panel B, the *y* axis for each graph represents RhCMV Ab (A450nm) (absorbency measured at 450-nanometer wavelength). For the graphs in panel C, the *y* axis for each graph represents viral genome copy #/ml (log). *, Although G3-3 was seronegative on the RhCMV antigen ELISA (B), this animal was positive using RhCMV gB-specific ELISA (not shown).

Lymphoid tissue histology was analyzed in multiple sites, including spleen, tonsils, LNs (axillary, inguinal, submandibular, and mesenteric) and mucosa-associated lymphoid tissues (MALT) (sublingual gland and stomach, intestines, and genital mucosa). Lymphoid hyperplasia was observed in all animals and ranged from mild to severe (Fig. 6). Prominent changes included variable multifocal lymphofollicular hyperplasia, marginal zone (spleen) or paracortical expansion (LN), and some germinal centers coalesced into irregularly sized and shaped follicles in the spleen (Fig. 5B). In addition, for-

TABLE 1 Protein identities (% ID) between selected open reading frames (ORF) of UCD52 and UCD59[a]

		Accession no.	
ORF	% ID	UCD52	UCD59
gB (RhUL55)	88.9%	QQL11290.1	QQL11468.1
gO (RhUL74)	64.9%	QQL11298.1	QQL11476.1
gH (RhUL75)	87.5%	QQL11300.1	QQL11478.1
gL (RhUL115)	96.9%	QQL11333.1	QQL11511.1
RhUL128	72.5%	QQL11342.1	QQL11520.1
RhUL130	82.3%	QQL11343.1	QQL11521.1
RhUL131	82.5%	QQL11344.1	QQL11522.1
pp65 (RhUL83B)	100%	QQL11309.1	QQL11487.1

[a]The specific proteins of each strain were analyzed for sequence identity by BLASTP (3, 4) compared to the other strain using the GenBank accession numbers for each protein, based on the annotated genome sequences for UCD52 and UCD59 (95) (GenBank accession numbers MT157330 and MT157331, respectively).

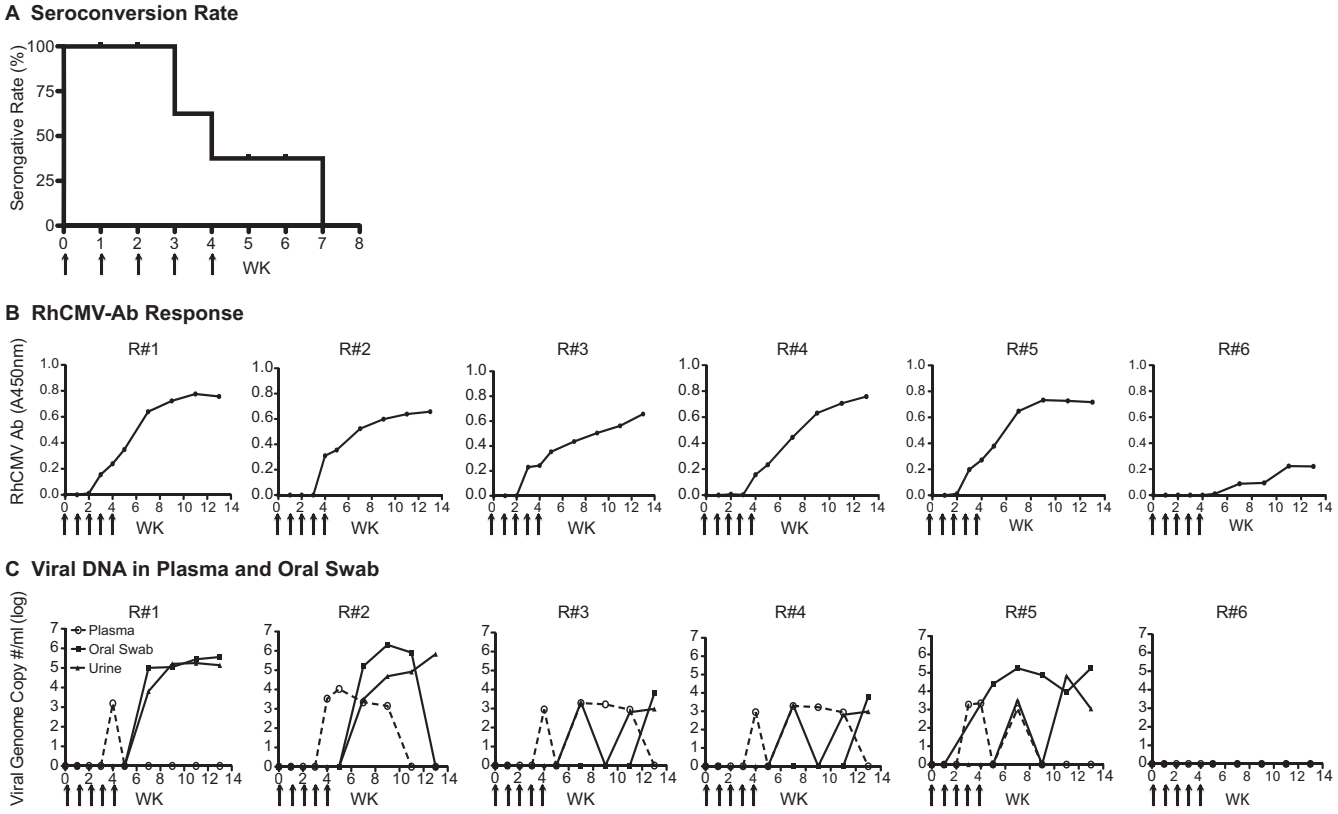

FIG 4 The effect of UCD59 exposure frequency on viral and immune parameters of infection. Six naive animals (R#1 to R#6)) were exposed p.o. to 8×10^5 PFU of UCD52 five times at weeks (WK) 0, 1, 2, 3, and 4 (arrows). (A to C) Animals were longitudinally evaluated for (A) seroconversion to RhCMV-specific antigens post-p.o. exposure, (B) development of RhCMV-specific binding antibody (Ab) responses, and (C) RhCMV genome copy numbers in plasma, oral swabs, and urine. For the graphs in panel B), the *y* axis for each graph represents RhCMV Ab (A450nm). For the graphs in panel C, the *y* axis for each graph represents viral genome copy #/ml (log).

mation of secondary lymphoid follicle with germinal centers was observed in the salivary gland and mucosa-associated lymphoid tissue (MALT; not shown). Other frequent microscopic changes (ranging from mild to moderate) included multifocal sialadenitis (parotid, 5/6; sublingual gland, 6/6), hepatitis (6/6), cholecystitis (5/6), and choroid plexitis (6/6), histologically distinguished by lymphocytic-histiocytic-plasmacytic infiltration (not shown). Incidental inflammation was also observed in the pancreas, vagina, prostate, epididymis, and meninges in some of animals. Despite the structural changes in lymphoid tissues, no pathognomonic RhCMV intranuclear and/or cytoplasmic inclusions, characteristic of fulminant RhCMV infection, were observed in any of these tissues.

To assess the systemic distribution of RhCMV-bearing cells, the presence of RhCMV in tissues (*in vivo* dissemination) was systemically evaluated by a nested PCR (nPCR) of the RhCMV immediate early (IE2) gene together with immunohistochemical (IHC) staining of RhCMV IE1 expression. IE2 nPCR analyses of RhCMV genomic DNA demonstrated systemic spread of virus contemporaneous with the lymphoid structural changes. RhCMV DNA was detected in almost every tissue, with the exceptions of epididymis, uterus, and cervix (Table 2), emphasizing the efficiency with which RhCMV nucleic acid, presumably associated with progeny RhCMV virions, spread throughout a naive host. The spectrum of UCD52-infected tissues varied among individuals (Table 2, Fig. 7A and B), and the most frequently infected tissues (>50% of animals) included thymus, spleen, tonsils, LNs, kidneys, bladder, salivary glands, lungs, stomach, liver, pancreas, parietal lobe, spinal cord, and adrenals. The highest extent of tissue dissemination was observed in UCD52-#R1. Animal UCD52-R#6, with the lowest RhCMV antibody response, had no detectable RhCMV DNA in plasma, saliva, or urine (Fig. 4) and was

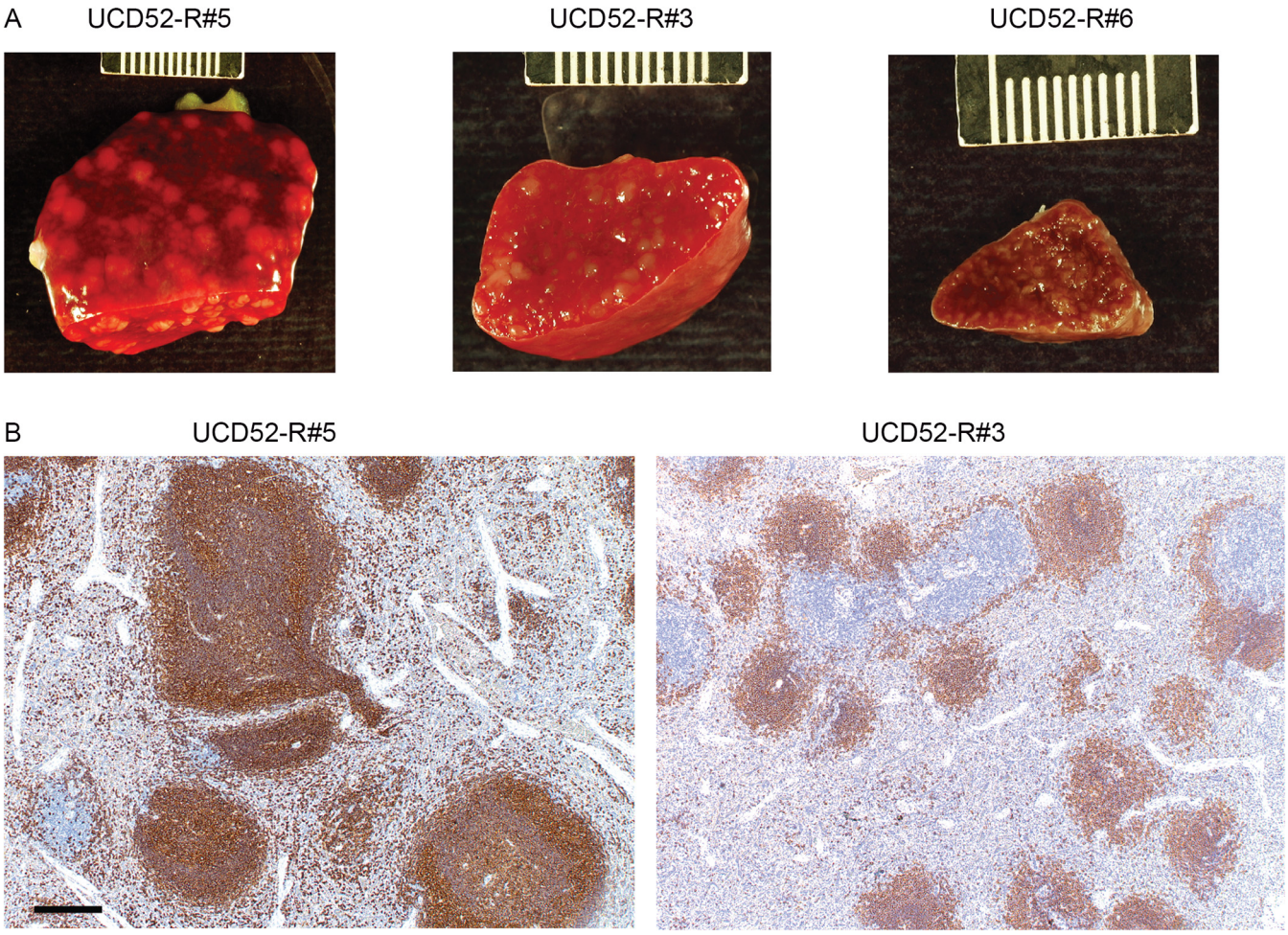

FIG 5 Spleen gross and microscopic pathological findings at week 13 after initial UCD52 p.o. inoculation. (A) A spleen multiple nodular lesion (1-cm scale) was observed at gross pathology examination. Splenic white pulp ranged in size from $<$1 mm in diameter (UCD52-R#4 and -R#6; only UCD52-#6 is shown on right) to 1- to 2-mm lymphoid nodules present on the capsular and cut surface (UCD52-R#1, -R#2, -R#3, and -R#5; only UCD52-R#5 on the left and -R#3 in the middle are shown). (B) Different levels of lymphofollicular hyperplasia were observed by histology and CD20 IHC staining. UCD-R#5 (left) and UCD-R #3 (right) are shown. Bar, 300- μm scale.

also the animal in this group with detectable RhCMV DNA in the fewest tissues (Fig. 7A). Further, in the subset of tissues in which RhCMV genomic loads were quantified by real-time PCR, animal UCD52-R#6 also exhibited the lowest tissue viral loads (Fig. 7B). These data strengthen the supposition that virus-host interactions following oral inoculation may have effectively controlled viral replication in ~20% of p.o.-exposed animals.

To determine if the presence of RhCMV DNA was accompanied by the presence of viral protein, the presence of RhCMV IE1-expressing cells in lymphoid, salivary gland, and kidney tissues was analyzed by IHC. The tissue distribution profiles (Table 2) were similar to those that have been observed following inoculation with RhCMV by other routes (i.e., intravenous, subcutaneous) (28, 32, 48, 65). In addition, the kinetics of RhCMV DNA detection in saliva (Fig. 2 and 4) recapitulate the temporal pattern of shedding following s.c. delivery of RhCMV (32).

It is important to consider whether the presence of RhCMV DNA in tissues reflects infection and replication in tissue parenchymal cells and/or passive carriage of RhCMV genomes by circulating blood cells, such as monocytes and $CD34^+$ myeloid progenitor cells, based on precedents with HCMV. The issue of infection versus quiescent carriage in blood cells is important since the animals were not perfused at necropsy prior to collection of tissues. HCMV IE1 expression is severely restricted in circulating monocytes

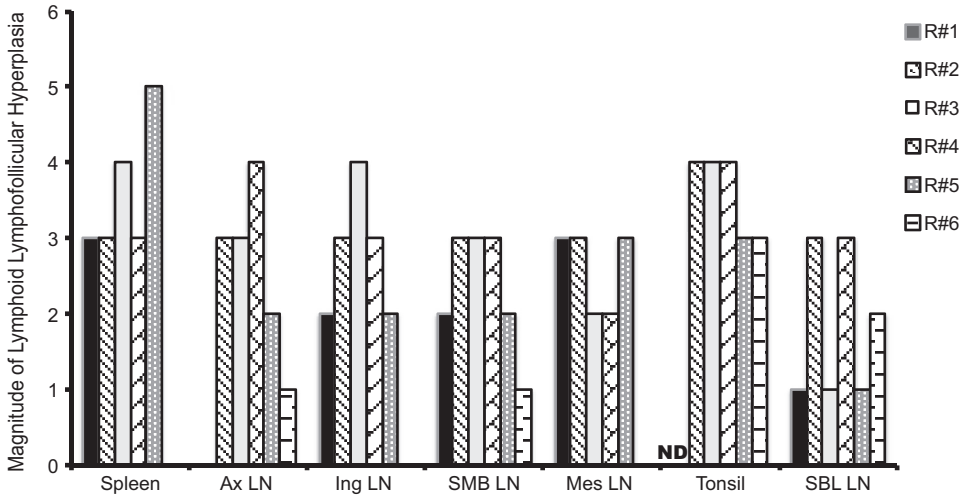

FIG 6 Lymphoid lymphofollicular hyperplasia in UCD52-inoculated animals. Six of the UCD52-inoculated animals (R#1 to R#6) were necropsied at week 13 after the initial p.o. exposure to UCD52. Lymphoid tissues were collected and sectioned for histological assessment. Different magnitudes of lymphofollicular hyperplasia were observed and scored as follows: 0, no change; 1, minimal change; 2, mild change; 3, moderate change; 4, marked change; 5, severe change. Ax LN, axillary lymph node (LN); Ing LN, inguinal LN; Mes LN, mesenteric LN; SMB LN, submandibular LN; SBL LN, sublingual LN; ND, Not Done (tonsil not collected for R#1).

and myeloid progenitor cells (66–68). The RhCMV IE1 IHC results in the analyzed tissues (n = 9) of the frequently infected tissues (Table 2) are in agreement with previous studies of RhCMV in immunocompetent macaques (23, 28) in which parenchymal cells from multiple tissues demonstrate RhCMV IE1 expression and/or exhibit the pathognomic cytomegaly of RhCMV-infected cells. Based on these prior studies, it is probable that in the frequently infected tissues (Table 2), at least, the PCR reflect parenchymal cells truly infected with RhCMV and expressing RhCMV IE1 antigen. However, in those tissues in which RhCMV DNA is infrequently detected (Table 2), it cannot be concluded whether the results reflect infected cells or passive carriage of RhCMV genomes within circulating blood cells.

Kinetics of neutralizing antibodies in UCD52-inoculated animals. Induction of neutralizing antibodies (NtAb) represents an important host defense against CMV infection. Therefore, the kinetics of antibody development elicited by p.o. inoculation with UCD52 that can neutralize virus in assays were characterized on fibroblasts (Telo-RF, to measure Telo-NtAb) and primary monkey kidney epithelial cells (MKE, to measure MKE-NtAb). Based on the kinetics of the RhCMV-binding antibody response (Fig. 4B), Telo-NtAb and MKE-NtAb to UCD52 (1:31 minimum dilution) were evaluated from plasma drawn on weeks 0, 2, 4, 7, 11, and 13. In five of six animals, oral exposure to UCD52 induced both Telo-NtAb and MKE-NtAb that were indistinguishable in kinetics and magnitude (Fig. 8A). Neutralizing titers in both cell types approached a plateau at week 7 post-initial inoculation, with smaller increases to either week 11 or 13. There was no significant difference between maximal MKE-NtAb and Telo-NtAb (Fig. 8B; area under the concentration-time curve [AUC] Wilcoxon test, P = 0.3125). Notably, the animal UCD52-R#6, with the lowest binding Ab titers and lowest viral loads in tissues (30%, especially in lymphoid tissues), did not develop any detectable NtAb titers in either cell type (Fig. 8A and B) (minimum dilution, 1:31).

Strain- and cross-strain-specific gB and gH-pentamer complex-binding IgG and neutralizing antibodies. Five of six (83%) UCD52-infected monkeys or UCD59-infected monkeys produced both strain-specific and cross-strain-specific, glycoprotein B (gB)- and gH-anchored pentamer complex (gH-PC)-binding IgG (Fig. 9). Cross-strain-specific gB- and gH-PC-binding antibodies were not detected in the animal in each group that had low levels of strain-specific gB- and gH-PC-specific antibodies (Fig. 9A and B). Moreover, higher levels and faster kinetics of antibody responses were observed using

TABLE 2 RhCMV IE2 nested PCR (nPCR) and RhCMV IE1 immunohistochemistry (IHC) summary

Tissue (no.)[a]	Assay[b]	Data for:[c] R#1	R#2	R#3	R#4	R#5	R#6
Tissues in which RhCMV DNA was frequently detected (>50% of the animals)							
Thymus (1)	IE2 nPCR	+	+	+	−	−	+
	IE1 IHC	ND	ND	ND	ND	ND	ND
Spleen (1)	IE2 nPCR	+	+	+	+	+	+
	IE1 IHC	+	+	+	+	+	−
Tonsil (R&L)	IE2 nPCR	+	−	+	−	+	+
	IE1 IHC	−	+	−	−	−	−
Ax LN (R&L)	IE2 nPCR	+	+	+	+	+	+
	IE1 IHC	+	+	−	+	+	−
Ing LN (R&L)	IE2 nPCR	+	+	+	+	+	+
	IE1 IHC	−	+	+	+	−	−
SMD LN (R&L)	IE2 nPCR	+	+	+	+	+	+
	IE1 IHC	+	−	+	+	−	−
Mes LN (R&L)	IE2 nPCR	+	+	+	+	+	+
	IE1 IHC	+	−	−	−	−	−
Kidney (R&L)	IE2 nPCR	+	+	+	+	+	+
	IE1 IHC	−	+	+	−	−	−
Bladder (1)	IE2 nPCR	+	+	+	−	+	−
	IE1 IHC	ND	ND	ND	ND	ND	ND
PSG (R&L)	IE2 nPCR	+	+	+	+	+	+
	IE1 IHC	−	+	+	+	−	−
SLSG (R&L)	IE2 nPCR	+	+	+	−	+	+
	IE1 IHC	−	+	−	−	−	−
Lung (6)	IE2 nPCR	+	+	+	+	+	+
	IE1 IHC	ND	ND	ND	ND	ND	ND
Stomach (3)	IE2 nPCR	+ + + + + +	+ + + + + +	+ + + + + +	+ + + − − −	+ + + + + +	+ + + − − −
	IE1 IHC	ND	ND	ND	ND	ND	ND
Liver (3)	IE2 nPCR	+ + +	+ + +	+ + +	+ + +	+ + −	+ + −
	IE1 IHC	ND	ND	ND	ND	ND	ND
Pancreas (1)	IE2 nPCR	+ + + + −	+ − − − −	+ + + + +	+ − − − −	+ − − − −	− − − − −
	IE1 IHC	ND	ND	ND	ND	ND	ND
Brain parietal (1)	IE2 nPCR	+	+	+	+	+	−
	IE1 IHC	ND	ND	ND	ND	ND	ND
Spinal cord (3)	IE2 nPCR	+ − −	+ + −	+ + −	− − −	+ − −	+ − −
	IE1 IHC	ND	ND	ND	ND	ND	ND
Adrenal gland (R&L)	IE2 nPCR	+	+	+	−	+	+
	IE1 IHC	ND	ND	ND	ND	ND	ND
Vagina (1)	IE2 nPCR	+			+		−
	IE1 IHC	ND	ND	ND	ND	ND	ND
Testes (R&L)	IE2 nPCR		+/−	+/−		−	
	IE1 IHC	ND	ND	ND	ND	ND	ND
Sem. vesicle (R&L)	IE2 nPCR		+/+	+/−		+/−	
	IE1 IHC	ND	ND	ND	ND	ND	ND
Tissues in which RhCMV DNA was infrequently detected (<50% of animals) (IE2 nPCR only)							
Duodenum (1)	IE2 nPCR	+	−	+	−	+	−
Jejunum (1)	IE2 nPCR	−	−	−	−	+	−
Ileum (1)	IE2 nPCR	+	+	−	−	−	−
Cecum (1)	IE2 nPCR	−	+	−	−	−	+
Colon (2)	IE2 nPCR	+/+	+/+	+/−	−/−	−/−	−/−
Rectum (1)	IE2 nPCR	+	−	−	+	−	−
Gall bladder (1)	IE2 nPCR	+	−	−	−	−	−
Brain cerebellum (1)	IE2 nPCR	+	−	−	+	+	−
Brain temporal lobe (1)	IE2 nPCR	+	−	−	−	−	−
Brain frontal lobe (1)	IE2 nPCR	+	−	−	−	−	−
Brain occipital lobe (1)	IE2 nPCR	+	−	−	−	−	−
Brain choroid plexus (1)	IE2 nPCR	+	−	−	−	−	−
Pituitary (1)	IE2 nPCR	+	−	−	−	−	−
Trigeminal ganglia (1)	IE2 nPCR	+	−	−	−	−	−
Dorsal root ganglia (1)	IE2 nPCR	+	−	−	ND	+	−
Epididymis (R&L)	IE2 nPCR		−/−	−/−		−/−	

(Continued on next page)

TABLE 2 (Continued)

Tissue (no.)[a]	Assay[b]	Data for:[c] R#1	R#2	R#3	R#4	R#5	R#6
Prostate (1)	IE2 nPCR		+	−		−	
Fallopian tube (R&L)	IE2 nPCR	+/+			−/−		−/−
Ovary (R&L)	IE2 nPCR	+/+			−/−		−/−
Cervix (1)	IE2 nPCR	−			−		−
Uterus (1)	IE2 nPCR	−			−		−
Aorta (1)	IE2 nPCR	+	−	−	−	+	−
Bone marrow (1)	IE2 nPCR	+	−	−	−	−	−

[a]The number of samples collected per tissue. R&L, 1 sample was collected from the right (R) half of the pair, and 1 sample was collected from the left (L) half of the pair. Ax LN, axillary lymph node; Ing LN, inguinal lymph node; Mes LN, mesenteric lymph node; PSG, parotid salivary gland; SLSG, sublingual salivary gland; sem., seminal vesicle.
[b]IE1 IHC, a single sample from some tissues was analyzed for RhCMV IE1 expression by immunohistochemistry (IHC).
[c]ND, Not Done; +, tissue with detectable RhCMV DNA (by nPCR) or detectable RhCMV IE1 expression (by IHC); −, tissue with undetectable RhCMV DNA (by nPCR) or undetectable RhCMV IE1 expression (by IHC).

strain-specific gB and gH-PC proteins in the enzyme-linked immunosorbent assay (ELISA) than using cross-strain proteins, indicating that cross-reactive binding antibodies can be induced at inferior magnitude or slower kinetics. Put another way, the results demonstrate that higher responses were observed when there was autologous

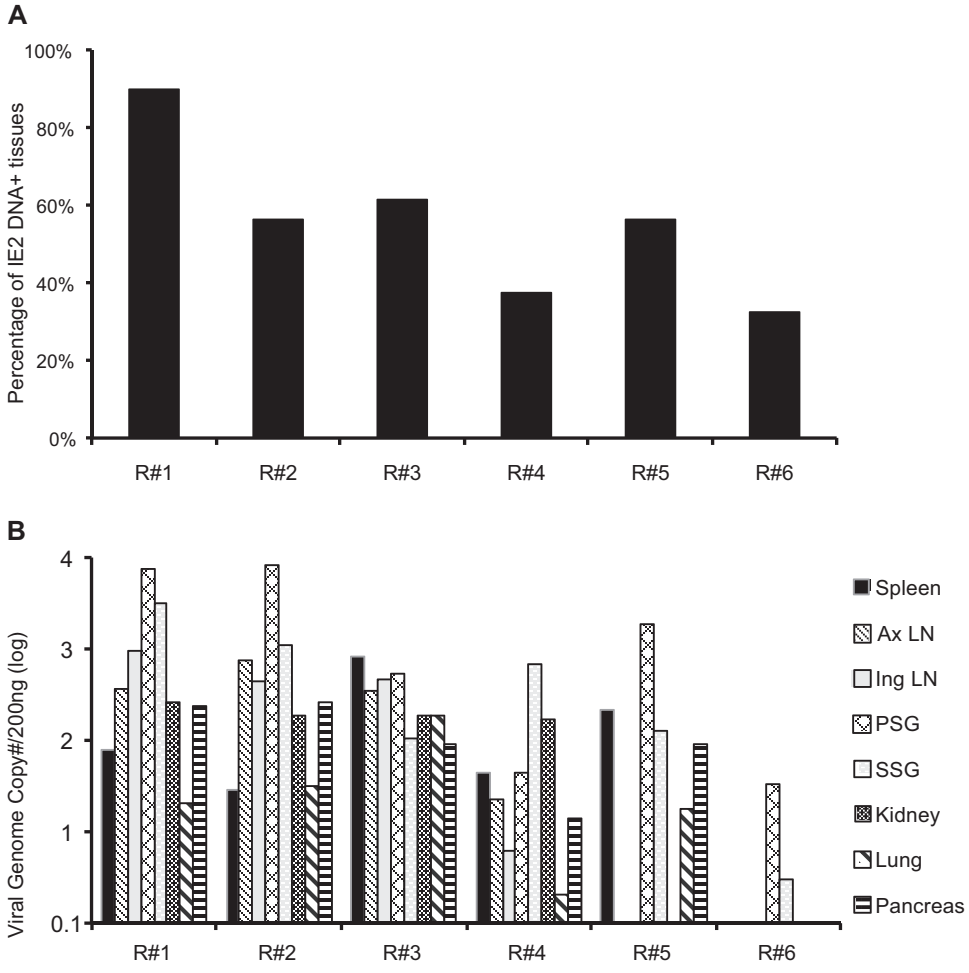

FIG 7 UCD52 dissemination after p.o. exposure. Six UCD52-inoculated animals (R#1 to R#6) were necropsied at week 13 post-initial exposure to UCD52. A total of 66 tissues per animal were collected for DNA extraction. (A) The presence of RhCMV DNA in different tissues was evaluated by the frequency of IE2 DNA-positive (+) tissues (using nested IE2 PCR; nPCR) in each animal. The results are presented as the percentage of IE2 DNA+ tissues over the total number of collected tissues (*y* axis) for animals R#1 to R#6 (*x* axis). (B) The viral load in the most frequently infected tissues of each animal (Table 2) was quantified by RhUL132-specific qPCR. RhCMV genome copy numbers (*y* axis) are presented (in log scale) versus the tissue assayed (*x* axis). The limit of detection was 1 copy of RhCMV DNA; tissues of animals R#5 and R#6 with undetectable RhCMV DNA were left blank. Ax LN, axillary lymph node (LN); Ing LN, inguinal LN; PSG, parotid salivary gland (SG); SSG, sublingual SG.

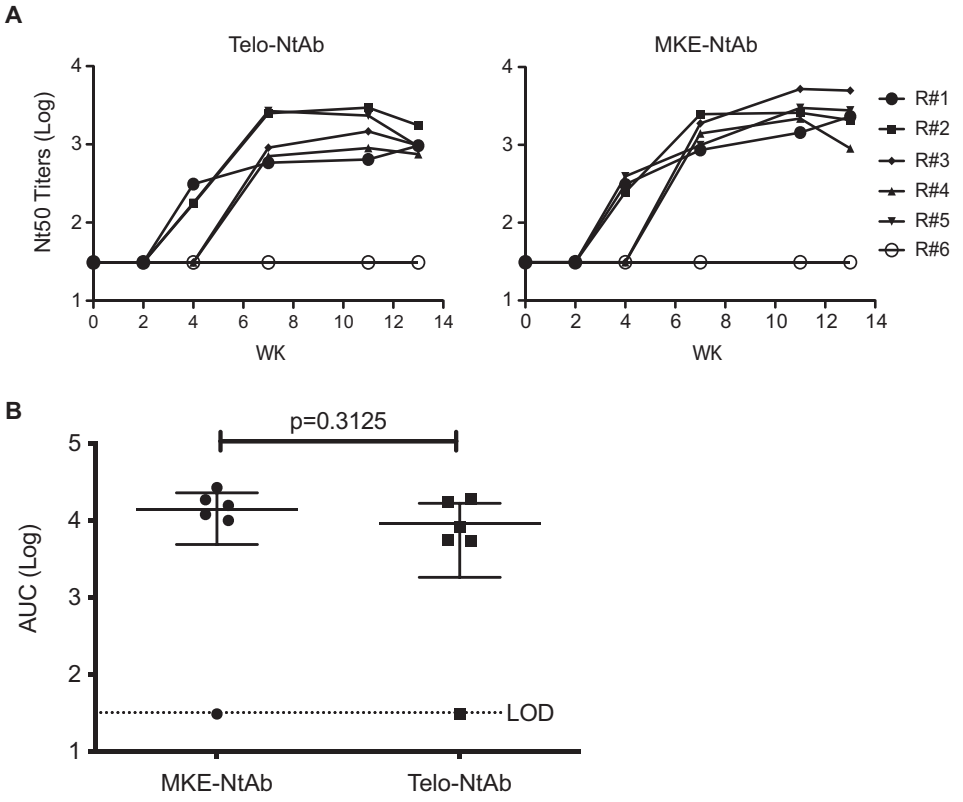

FIG 8 The kinetics of neutralizing antibodies (NtAb) in UCD52-inoculated animals. (A and B) The kinetics of neutralization antibodies (NtAb) in UCD52-exposed (p.o.) animals assayed on (A) telomerized rhesus fibroblasts (Telo) and (B) primary monkey kidney epithelial (MKE) cells by serial dilution of plasma samples starting from 1:31 (1.5 logs). (C) The difference between UCD52 MKE-NtAb and Telo-NtAb was analyzed by area under curve (AUC) Wilcoxon test (P = 0.3125). WK, weeks. The AUC for R#6 was zero for both Telo and MKE (A and B), and these AUC results are plotted at the limit of detection (LOD; 1.5 logs; 1:31 dilution).

plasma and autologous antigen, rather than heterologous plasma and antigen. That is, UCD52-inoculated animals exhibited higher ELISA responses using UCD52-specific gB (Fig. 9A, left) or PC (Fig. 9B, left). In contrast, UCD59-inoculated animals exhibited higher ELISA responses using UCD59-specific gB (Fig. 9A, right) or PC (Fig. 9B, right). This result suggests that genetic drift of the glycoproteins between UCD52 and UCD59 involved in attachment/entry in fibroblasts (Telos) and epithelial cells (MKE) (Table 1) could affect the magnitude and specificity of the immune responses. To assess the extent of genetic drift of these proteins between these two RhCMV strains, BLASTP analyses (69, 70) were performed. With the exception of gL (96.9% protein identity), there was considerable genetic drift between the two strains for gB, gO, gH, UL128, UL130, and UL131, ranging from 64.9% (gO) to 88.9% (gB) protein identities (Table 1). Notably, there was absolute sequence conservation of pp65 (100% protein identity), an immunodominant $CD8^+$ T cell target following RhCMV infection in macaques (71).

Plasma samples from five UCD52-infected monkeys and five UCD59-infected monkeys, all of which contained binding antibodies to gB- and PC from both RhCMV strains, were cross-analyzed for neutralization titers to each strain at the time points when anti-gB and PC antibodies reached or approached peak responses (Fig. 10A and B). In contrast to the high binding activities, only weak or undetectable cross-reactive neutralization activities were detected (1:100 minimum dilution). More low-level cross-reactive neutralization was detected at the late time point than the early time point.

DISCUSSION

Mucosal acquisition of infectious HCMV and RhCMV, particularly via the oral mucosa, is believed to be a major route for horizontal viral transmission. In this study,

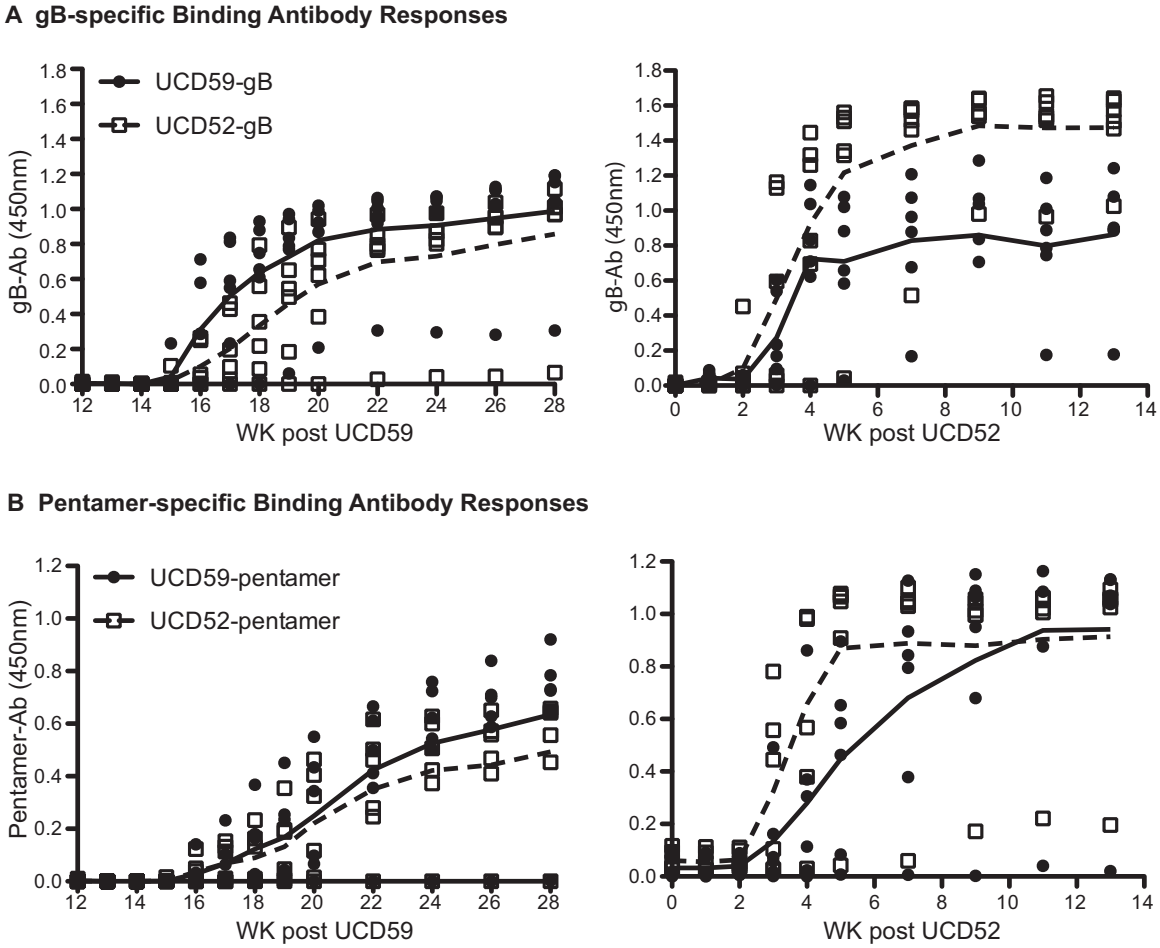

FIG 9 (A and B) Binding antibody (Ab) responses to homologous and heterologous strain (A) gB and (B) pentamer post-UCD59 and -UCD52 inoculation. The plasma samples collected at different time points from UCD59 p.o.-exposed animals (left half) and UCD52 p.o.-exposed animals (right half) were tested by ELISA for homologous and heterologous strain-specific gB and pentamer-specific binding antibodies. WK, weeks post-p.o. exposure.

using WT-like RhCMV strains, an oral RhCMV infection model that mimics natural infection in naive animals was established. As with natural acquisition of endemic strains of RhCMV in nonhuman primate facilities (72–75), oral inoculation of WT-RhCMV led to (i) disseminated, systemic infection and frequent shedding in bodily fluids; (ii) a positive association between the probability of detecting plasma anti-RhCMV antibodies and the magnitude of oral exposure (i.e., inoculation dose level and the number of inoculations); (iii) preponderant detection of RhCMV DNA in particular tissues (thymus, spleen, lymph nodes, kidneys, bladder, lungs, and salivary glands) and occasional putative detection in other tissues (central nervous system and reproductive organs); (iv) induction of high levels of NtAb specific to the inoculated strain of RhCMV; and (v) minimal or undetectable NtAb to a second, genetically divergent strain of RhCMV.

The parameters of oral acquisition of RhCMV in this model appear to recapitulate the natural history of HCMV acquisition. The exceedingly high global seroprevalence to HCMV, 86% in women of reproductive age (1) highlights that HCMV is well adapted for dissemination throughout the major histocompatibility complex (MHC)-diverse human population. Based on natural history studies of infectious virus in bodily fluids, the high seroprevalence of HCMV most likely results from mucosal exposure to infectious virus particles frequently shed in multiple bodily fluids (e.g., breast milk, saliva, urine). Although HCMV is almost universally shed in the breast milk of seropositive, lactating mothers, not all milk-exposed infants acquire primary HCMV infection (47, 76–83). A

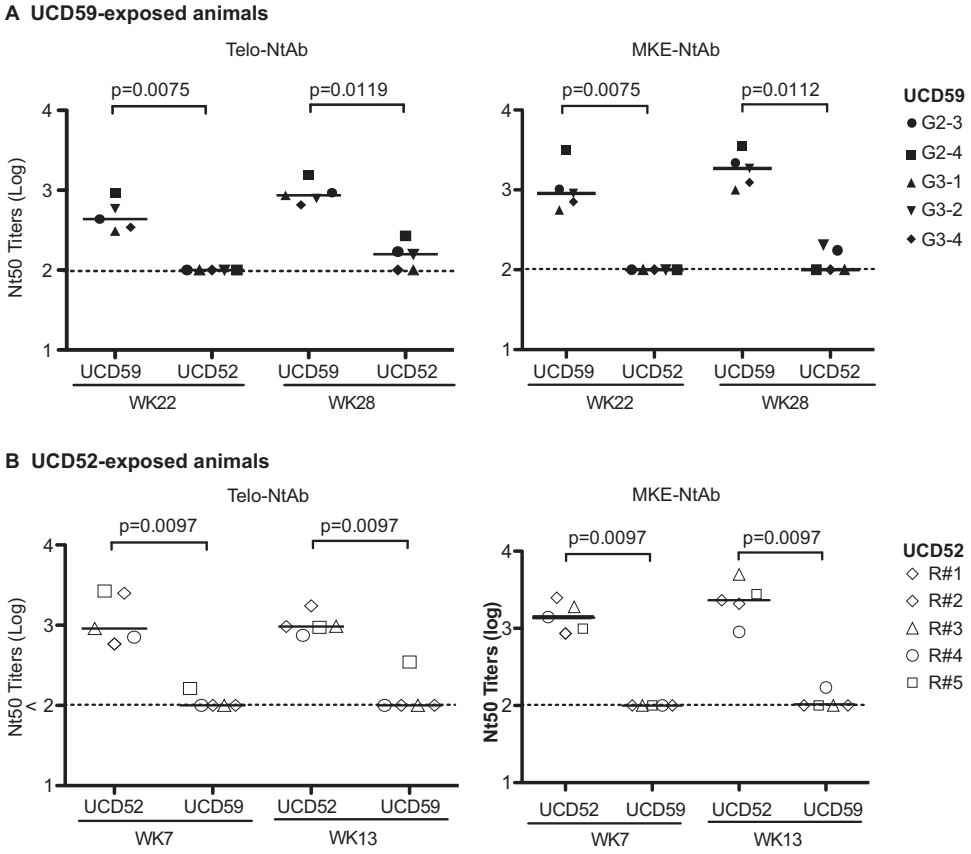

FIG 10 Neutralizing antibodies (NtAb) to homologous and heterologous strains post-UCD59 and -UCD52 p.o. exposure in Telo-RF (Telo-NtAb) (left half) and MKE (MKE-NtAb) (right half). (A) The plasma samples collected at week (WK) 22 and week 28 for UCD59-exposed animals (filled symbols) were assayed for homologous and heterologous neutralization using UCD59 and UCD52 as target viruses, respectively, for neutralization. (B) Week 7 and week 13 plasma samples for UCD52-exposed animals (open symbols) were tested for homologous and heterologous neutralization using UCD52 and UCD59 as target viruses, respectively, for neutralization. The minimum dilution for neutralization was 2 logs (1:100) (dotted line). The median value for each group is presented (solid line).

high HCMV load and prolonged virus excretion in breast milk are maternal risk factors for viral transmission to infants with very low birth weight (77). Mayer et al. observed that oral exposure to HCMV in infants mostly results in transient, localized infection, defined as self-limited, coincident with a low level of virus shedding at the oral cavity and an absence of systemic spread (64). This same study also indicated that repeated exposure is likely a prerequisite to establish a successful primary infection in the infant. Supporting the hypothesis that repeated oral exposures to HCMV are critical for establishing systemic human infection, a small natural history study of RhCMV transmission involving mother-to-infant transmission has shown that exposure to frequent and low levels of RhCMV shedding in breast milk only induces transient oral RhCMV shedding in infants (84).

This current study of UCD59 p.o. inoculation via exposure of the buccal mucosa to infectious virus provides support for a linkage between viral inoculation dose and frequency of mucosal exposures with infection rate (as defined by seroconversion), viral DNAemia, and shedding in saliva (Fig. 3). The observations in humans and rhesus macaques (64, 84) that mucosal infections can apparently be contained locally without systemic infection implies that CMV evolved to overcome this local restriction by frequent shedding in bodily fluids to enable repeated mucosal exposures in susceptible hosts.

Notably, a single, relatively low titer (8×10^3 PFU) p.o. exposure to UCD59 failed to initiate RhCMV systemic infection consistently. It is generally believed that CMV must amplify locally within the epithelium of mucosal sites and overcome the local immune

response prior to crossing the mucosal membrane and gaining access to the rest of the body (49, 85–87). In our previous studies, we have shown that UCD59 is capable of replicating in multiple types of cells, including epithelial and endothelial cells and polymorphonuclear lymphocytes (PMN) (21, 27, 32). Furthermore, subcutaneous inoculation of 1×10^3 PFU of UCD59 resulted in 100% efficiency of DNAemia and frequent shedding in saliva and urine (63). Plausibly, the failure to initiate primary infection by 8×10^3 PFU UCD59 p.o. may have been the result of local innate restriction or *de novo* immune responses, implying the importance of local mucosal immunity. These data in p.o.-exposed monkeys suggest that global prevalence of HCMV is facilitated by frequent shedding of virus in bodily fluids and repeated mucosal exposures of susceptible individuals to these bodily fluids.

In search of factors associated with virus shedding, clinical studies have observed that the levels of HCMV-binding IgG are inversely correlated with virus shedding during primary infection (88–90), whereas studies in the murine CMV (MCMV) model have found that the degree of antibody response is directly associated with the amount of initial inoculated virus and the length of viral persistence (91). In this study, the 3 of 16 p.o.-infected animals that had the lowest RhCMV-specific antibody responses also lacked detectable RhCMV DNA in plasma, saliva, and urine (Fig. 2 and 4). Moreover, in the single animal (UCD52-R#6) of this subgroup analyzed in detail, there were marked reductions in (i) the number of RhCMV DNA-positive tissues (Fig. 7), (ii) RhCMV genome copies in infected tissues (Fig. 7), and (iii) the magnitude of lymphofollicular hyperplasia (Fig. 6) compared to other virus-shedding animals. These results suggest greatly restricted virus infection and a reduction in overall RhCMV burden.

The observation that ~20% of orally exposed/infected animals had no detectable RhCMV DNA in saliva or urine is comparable to observations from a natural history study of corral-housed, RhCMV-seropositive animals naturally exposed to circulating strains of RhCMV endemic within large breeding cohorts of rhesus macaques (92). In a cross-sectional analysis of 45 genetically outbred, adolescent-to-young adult (3 to 5 years) RhCMV-infected macaques, RhCMV DNA was detected in the saliva of 78% ($n = 35$) of the animals. Typically, rhesus macaques housed in outdoor corrals acquire RhCMV before their first year of age (72). Therefore, it is likely that the 3- to 5-year-old animals in the corral survey (92) had been infected for 2 to 4 years. Since only saliva was analyzed, it is not known whether there was shedding of RhCMV DNA in other bodily fluids (e.g., urine) in any of the 10 saliva-negative animals. However, a weak association was observed between NtAb titers and oral RhCMV shedding (Pearson's correlation), although no association was noted when a nonparametric (Spearman) test was used. Analyses of oral shedding in aged adult animals (≥14 years) in this same study suggested that changes in host antiviral immune parameters are implicated in greater control of persistent viral reservoirs (92).

Moreover, this same cross-sectional study showed that oral shedding was detected at significantly higher frequency and magnitude in adolescent animals (78%, 5.5×10^4 copies/mL) than in cohoused adults (28%, 2.3×10^3 copies/mL) (92). Reductions in oral shedding in the adult animals of this previous study were coincident with increased cytokine production in peripheral blood mononuclear cells (PBMCs) of adult animals following nonspecific (ConA), RhCMV antigen-specific, and T cell receptor stimulation *in vitro* compared to those in adolescent/young adult animals. Since shedding of infectious virus from infected hosts to those at risk for primary and nonprimary infections is clinically relevant, the results of this earlier study suggest that progressive changes in virus-specific T cell responses lead to greater control of viral shedding, thereby reducing the potential for horizontal transmission.

HCMV and RhCMV exist as multiple strains distinguished by genetic and protein-coding polymorphisms. Genetic diversity can be quite extensive among circulating clinical strains. A critical element in vaccine design is that immunization with an immunogen can broadly protect against antigenically divergent strains that the vaccinated individual could encounter. Indeed, reinfection with genetically divergent strains, in

the case of congenital HCMV infection, is often linked to adverse clinical outcomes in the offspring of individuals with preexisting HCMV-specific immunity resulting from preconceptional HCMV immunity. Studying the neutralizing capacity of antibodies induced by single-strain infections against heterologous strains is clinically relevant to elucidating the underlying mechanism of reinfection and directing the development of broadly protective HCMV vaccines (93, 94).

Viral envelope glycoprotein B (gB) and the glycoprotein H (gH)-anchored pentamer complex (gH/L/UL128/UL130/UL131, gH-PC) are major HCMV targets of NtAb as well as prominent targets following RhCMV infection. gB and gH-PC induce prominent NtAb responses that restrict infection of fibroblast and epithelial/endothelial cells, respectively (95–102). There is considerable protein sequence diversity between the RhCMV gB and gH-PC proteins (UL128, UL130, and UL131) of UCD52 and UCD59 (Table 1). A fundamental concern of vaccine development is whether immunization with an antigen of one strain will cross-protect against other strains. In the RhCMV model, we interrogated IgG antibody responses to strain-specific and cross-reactive gB and gH-PC antigens after UCD52 or UCD59 oral inoculation. Though the cross-reactive binding activities did exist, the absence of cross-neutralization between samples from animals inoculated with UCD52 and a second genetically divergent UCD59 strain emphasize that induction of broad NtAb responses should be analyzed in human vaccine trials. Given that HCMV strains are genetically diverse, and adverse clinical consequences are usually related to reinfection (57–62, 103–105), the finding implies that, for HCMV vaccines, vaccination with an antigen representing only a single clade may limit the efficacy of such an approach in regions where antigenically divergent strains may predominate or where multiple antigenic variant strains are in circulation.

This study suggests a role for nonhematologic dissemination of the virus, based on relatively early spread to lymph nodes. Evidence from MCMV studies support that, following viral entry at the mucosa, MCMV replicates locally and then sequentially spreads to draining lymph nodes, blood, and salivary glands (106, 107). The current study showed that RhCMV DNA (Fig. 3 and 4) was not always detected in plasma, or, at least, was not detectable on the days of weekly sampling. Even for those UCD52 orally infected animals, primary viral DNAemia (Fig. 4) was delayed 1 to 2 weeks compared to DNAemia in animals inoculated with RhCMV by the subcutaneous and intravenous routes (28, 32). Nevertheless, viral DNA systemically disseminated to multiple tissues, including tonsils, spleen, and regional lymph nodes in all cases, possibly due to dissemination via the lymphatic system.

In sum, the results of this study characterize for the first time the virologic and immunologic consequences of oral RhCMV exposure in RhCMV-seronegative rhesus macaques. The results of this defined inoculation protocol appear to strongly recapitulate features of natural animal-to-animal transmission that occurs in large breeding cohorts of macaques in which multiple sequence variants of RhCMV are endemic. Accordingly, the rhesus macaque model of HCMV persistence and pathogenesis may be used to interrogate clinically relevant aspects of HCMV natural history and vaccine design more rigorously.

MATERIALS AND METHODS

Cells and viruses. Rhesus macaque kidney epithelial cells (MKE), primary rhesus skin fibroblasts (RF), and telomerized-RF (Telo-RF) were routinely cultured for virus preparation and cellular assays (21, 108, 109). WT-like RhCMV strains UCD52 and UCD59 were originally isolated from the urine of two SIV-infected monkeys. Following passage of both strains initially in human fibroblasts and subsequently in RF, the viruses were serially maintained in MKE. For inoculation, UCD59 virus stocks were prepared from infected MKE, whereas UCD52 stocks were prepared from one round of infection in RF, according to our published protocol (21). Both UCD59 and UCD52 are epithelial-tropic and have a virulent phenotype *in vivo* (21, 29, 32). For unknown reasons, the RhUL128/130/131 genetic locus exhibits greater lability in UCD59 than in UCD52 even after serial culture in MKE. This is evidenced by a fraction of virions within the virus stock that have rearrangements in the RhUL128/130/UL131 locus. The GenBank accession numbers for the complete genomes of UCD52 and UCD59 are MT157330 and MT157331, respectively (110).

Animals. Eighteen genetically outbred, healthy, and specific-pathogen-free (SPF) rhesus macaques (*Macaca mulatta*, age 1 to 3 years) were used in this study (111). The SPF animals were free of infection by RhCMV, *Macacine alphaherpesvirus 1* (herpes B), simian foamy virus, simian T-lymphotropic virus, and

simian immunodeficiency virus (111). The animals were housed and maintained according to the standards of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Seronegativity of study animals to RhCMV antigens was confirmed by sequential screening with an RhCMV-specific ELISA (22). All animal protocols were approved in advance by the UC Davis Institutional Animal Care and Use Committee, which is fully accredited by the AAALAC.

RhCMV oral inoculation and sample collection. Juvenile macaques were inoculated with either UCD59 (n = 12) or UCD52 (n = 6) via an oral mucosal exposure procedure. A total volume of 0.5 or 1.0 mL RhCMV in sterile saline containing different titers of virus (details below) was administered to both sides of the buccal mucosa (0.25 or 0.5 mL per side). Anesthetized animals were placed in either a left or right lateral decubitus position. To maximize absorption of the virus to the oral mucosa and prevent the risk of aspiration, RhCMV was applied dropwise onto the corresponding side of buccal mucosa and allowed to remain in place for ~5 min. After one side was exposed to virus, the animal was placed in the contralateral decubitus position and was inoculated with the remainder of the virus inoculum.

The study of UCD59 oral inoculation was initiated first and carried out in two stages (Fig. 1A). For stage I, 12 rhesus macaques were randomly divided into three groups (n = 4 per group), and each animal in each group received one *per os* (p.o.) exposure to either 8×10^3, 8×10^4, or 8×10^5 PFU of UCD59 at week 0. For stage II, six of the animals that did not seroconvert after RhCMV exposure in stage I were administered 8×10^5 PFU of UCD59 p.o. weekly for an additional four exposures. Subsequently, the study of UCD52 p.o. inoculation was performed by administering 8×10^5 PFU of UCD52 weekly for a total of five exposures (Fig. 1B). Blood and saliva from UCD59-inoculated and UCD52-inoculated animals and urine from UCD52-inoculated animals, were collected longitudinally and processed as previously described (32). All UCD52-inoculated animals were necropsied at week 13 post-initial p.o. exposure (week 0) for histopathological and virological assessment of primary infection (Fig. 1B). The necropsied animals were not perfused prior to collection of tissues. A total of 66 tissue samples per animal were harvested for histology, immunohistochemical (IHC) examination, and detection of RhCMV tissue dissemination.

Real-time PCR and nested conventional PCR. DNA was extracted from plasma, saliva, urine, and frozen tissue samples as previously described (32). Due to the sequence variation between different RhCMV strains, UCD59 viral DNA was detected using a previously described real-time quantitative PCR targeting the RhCMV 68-1 gB gene (gB qPCR) (112), whereas UCD52 viral DNA was measured by a newly developed real-time quantitative PCR targeting the UCD52 RhUL132 gene (RhUL132 qPCR). For RhUL132 qPCR, the primer-probe set consisted of the following pair of primers: forward primer 5′-TTGTCGCAGTAAGAACGGTGGTGA-3′ and reverse primer 5′-TTAATACCGACGCGGGTTCGCAGC-3′. The primers spanned a 193-bp region corresponding to nucleotides 3224 to 3416 of the UCD52 RhUL132 gene and the introns between the RhUL132 and RhUL148 genes (GenBank accession no. GU552457). The probe (5′-6FAM-AGTGCTGTTGGCAGTCGTGGTATTGT-TAMRA-3′) corresponds to positions 3393 to 3416 of UCD52 RhUL132. A plasmid containing the RhUL132 amplicon was constructed for generation of a standard curve for RhUL132 qPCR. Each PCR mixture contained 6.25 μL of TaqMan universal PCR master mix, 1.25 pmol of the probe, 6 pmol of each primer, and 5 μL of purified DNA (isolated from plasma, saliva, or urine) for a total volume of 12.5 μL. For DNA isolated from tissues, 200 ng of DNA (in 5 μL; quantified by absorbency at 260 nM) was used as the template. Both gB and RhUL132 qPCR assays were performed using an ABI Prism 7700 sequence detection system with one incubation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A standard curve was generated from wells containing 10-fold serial dilutions of a gB or RhUL132 plasmid (from 10^6 to 10^0 copies per 5 μL). All samples were run in triplicate, and results were reported as the average RhCMV genome copy numbers per mL (of plasma, saliva, or urine) or the average RhCMV genome copy numbers per 200 ng of genomic DNA. The limit of RhCMV DNA detection was 200 RhCMV genome copies per mL of plasma, saliva, or urine, as determined by the reaction mixtures containing the control plasmids.

A conventional, nested PCR targeting exon 5 of the immediate early 2 protein (IE2 nPCR) was performed to detect the presence of UCD52 DNA in tissues following a previously described protocol (28). Using 500 ng of extracted tissue DNA as the template, the first round of PCR was carried out with primers PAB196 (5′-GCCAATGCATCCTCTGGATGTATTGTGA-3′) and PAB249 (5′-TGCTTGGGGAATCTCTGCAC-3′), yielding a 1,068-bp amplicon. Subsequently, 5 μL of the first-round PCR product was used as the template for the second round of PCR (2nd PCR), in which primers PAB201 (5′-CCCTTCCTGACTACTAATGTAC-3′) and PAB248 (5′-TTGGGGAATCTCTGCACAAG-3′) were used to produce a nested amplicon of 407 bp. The running conditions for both the primary and nested PCR were an incubation at 95°C for 3 min, followed by 40 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by an incubation at 72°C for 15 min. After the nested PCR, 10 μL of the 2nd PCR was electrophoresed (1.5% agarose with ethidium bromide staining) for the detection of the 407-bp IE2 amplicon. Positive and negative controls were included for each PCR assay.

Enzyme-linked immunosorbent assays (ELISA) and plaque reduction neutralization assays. RhCMV-specific binding antibodies were evaluated by ELISA using an infected-cell lysate prepared from 68-1-infected primary RM fibroblasts exhibiting 100% cytopathic effect (CPE) (22). Baculovirus-expressed UCD52 gB and UCD52 and UCD59 pentameric complexes (gH/gL/RhUL128/RhUL130/RhUL131) were used to detect antigen-specific antibodies. Due to the high identity between 68-1 gB and UCD59 gB (99%), baculovirus-expressed 68-1 gB protein (a gift from Mark Walter at University of Alabama, Birmingham) was used to detect UCD59 gB-specific antibodies. Optimal concentrations of coating antigens and horseradish peroxidase conjugated goat anti-monkey IgG (KPL, Inc.) were determined, and each antigen-specific ELISA was performed following previously published protocols (22, 65, 71). All the plasma samples were heat-inactivated at 56°C for 30 min prior to the assay and tested at a dilution of 1:100. A sample was considered positive for a specific antigen if the absorbance at a wavelength of

450 nm (OD 450 nm) was ≥0.1 unit greater than that of a pool of RhCMV-seronegative plasma (collected from 20 Western blot-confirmed RhCMV-seronegative rhesus macaques).

UCD52 and UCD59 neutralizing activities of monkey plasma were measured in Telo-RF and MKE cells by a plaque reduction neutralization assay with or without the presence of 10% guinea pig complement (Sigma) as previously described to measure "Rf-NtAb" and "Ep-NtAb" titers (21, 65).

Immunohistochemistry (IHC). The distribution of RhCMV in tissues was determined by immunohistochemical staining for RhCMV IE1 (28). The level of RhCMV infection in each tissue was based on the number of IE1-positive (IE1$^+$) cells observed per section, as follows: −, 0 IE1$^+$ cells; +, 1 to 19 IE1$^+$ cells; ++, 20 to 50 IE1$^+$ cells; +++, >50 IE1$^+$ cells. In addition, thin sections of spleen were analyzed by IHC for cellular expression of CD20 (i.e., B cells).

Statistics. GraphPad Prism 8 software was used for statistical analysis. The association between initial inoculation dose of UCD59 and infection rate was tested by the log rank test for trend. Ep-NtAb and Rf-NtAb elicited by UCD52 inoculation were measured by the area under curve (AUC), and the difference between Ep-NtAb and Rf-NtAb was assessed by the Wilcoxon test.

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We declare no conflict of interest.

Y.Y., W.L.W.C., J.L., P.R.D., X.Y., and P.A.B. designed research; Y.Y., W.L.W.C., N.N., and K.A.S. performed research; Y.Y., W.L.W.C., N.N., K.A.S., J.L., P.R.D., X.Y., and P.A.B. analyzed data; and Y.Y., P.R.D., and P.A.B. wrote the paper.

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