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Inclusion of antibodies to cell culture media preserves the integrity of genes encoding RL13 and the pentameric complex components during fibroblast passage of human cytomegalovirus

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Abstract

Propagation of human cytomegalovirus (CMV) in cultured cells results in genetic adaptations that confer improved growth *in vitro* and significant attenuation *in vivo*. Mutations in *RL13* arise quickly during cell culture passage, while mutations in the *UL128-131A* locus emerge later during fibroblast passage and disrupt expression of a glycoprotein complex that is important for entry into epithelial and endothelial cells. As *in vivo* CMV replicates in the context of host antibodies, we reasoned that antibodies might mitigate the accumulation of adaptive mutations during cell culture passage. To test this, CMV in infant urine was used to infect replicate fibroblast cultures. One lineage was passaged in the absence of CMV-hyperimmuneglobulin (HIG) while the other was passaged with HIG in the culture medium. The former lost epithelial tropism and aquired mutations disrupting RL13 and UL131A expression, whereas the latter retained epithelial tropism and both gene loci remained intact after 22 passages. An epitheliotropic RL13+/ UL131A+ virus was isolated by limiting-dilution in the presence of HIG and expanded to produce a working stock sufficient to conduct cell tropism experiments. Thus, culture in the presence of antibodies may facilitate *in vitro* experiments using viruses that are genetically more authentic than has been previously possible.

Keywords: cytomegalovirus, cell culture, antibodies, adaptation

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Introduction

Human cytomegalovirus (CMV) causes a spectrum of diseases in immune compromised patients, including retinitis in HIV patients, pneumonitis in transplant patients, and serious birth defects characterized by sensorineural hearing loss and severe mental retardation when acquired during pregnancy. Available options for treating CMV infections are limited, and are hampered by dose-limiting toxicities and the development of resistance. These therapeutic limitations have fostered continued efforts toward the development of effective vaccines, the identification and exploitation of novel targets for antiviral interventions, and the development of passive polyclonal or monoclonal antibody therapeutics.

Since CMV was first isolated in 1957 from clinical samples using cultured human fibroblasts [1], cell culture models of viral propagation and experimentation have made major contributions to our understanding of CMV molecular biology, replication, immunology, and pathogenesis. Many of these contributions were achieved using virus strains that were extensively cell culture passaged, and are now known to contain substantial genetic deletions, rearrangements, and gene disruptions that were presumably aquired due to selective passage conditions, as well as stochastic genetic changes. Recent research has relied increasingly on the use of "clinical-like" strains that lack large deletions or rearrangements but retain certain changes associated with cell culture propagation.

Two such changes have been demonstrated to occur consistently during cell culture passage: mutations disrupting the *RL13* open reading frame (ORF) occur irrespective of the cell type used, while mutations in the *UL128-131A* locus emerge during passage in fibroblasts [2]. As the latter disrupt a complex that is necessary for infection of epithelial and endothelial cells, *UL128-131A* mutations do not occur during culture in epithelial or endothelial cells [2]. There

may also be additional adaptive mutations that occur less consistently, cause amino acid substitutions, or impact noncoding gene-regulatory regions.

Given that CMV replication *in vivo* generally occurs in the context of CMV-specific antibodies, we reasoned that cell culture virus propagation would more accurately model replication *in vivo* if CMV-specific antibodies were present in the culture medium. Consequently, the accumulation of certain adaptive mutations might also be mitigated. Here we report that a CMV clinical isolate serially passaged more than twenty times in fibroblasts cultured in the presence of CMV-hyperimmunoglobulin (HIG) retained epithelial tropism and lacked mutations disrupting *RL13* or genes in the *UL128-131A* locus. Moreover, a clonal virus retaining the genotypic and phenotypic properties of the parental stock was isolated by limiting-dilution and expanded to produce working stocks with titers sufficient to conduct cell tropism studies.

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Materials and Methods

Human subjects and clinical sample collection. A CMV culture-positive urine sample designated KG was obtained from a congenitally infected newborn seen at the University of Minnesota Medical Center. Urine KG was clarified of cellular debris by centrifugation at 2600 x *g* for five minutes, then adjusted to 100 mM sucrose, aliquoted, and stored under liquid nitrogen. Informed consent was obtained from the guardian, and protocols were approved by the Committees for the Conduct of Human Research at Virginia Commonwealth University and University of Minnesota.

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/L penicillin, and ten mg/L streptomycin (Gibco-BRL) (medium). Normal oral keratinocytes (NOK cells) were a gift from Karl Munger [3] and were propagated using Keratinocyte-SFM medium supplemented with human epidermal growth factor and bovine pituitary extract (Invitrogen).

Virus. Two T25 flasks of confluent MRC-5 cells were inoculated with equal volumes of KG urine to establish parallel lineages passaged under different culture conditions. One lineage, designated ϕ-KG, was serially passaged using a conventional protocol [4]. The cultures were monitored visually for cytopathic effect (CPE) until large foci were observed. For the first two passages, cells were trypsinized and half were mixed with $2.5x10^5$ trypsinized uninfected cells and returned to the T25 flask. For subsequent passages the cells were trypsinized, sonicated on ice, recombined with the culture medium, then added in progressively decreasing amounts to T25 flasks containing uninfected confluent MRC-5 cells: five ml for five passages, two ml for

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two passages, and one ml thereafter. Culture times for each passage were approximately one week.

The second lineage, designated Ig-KG, was serially passaged by transferring one-half of the trypsinized infected cells or cell sonicates to T25 flasks containing uninfected confluent MRC-5 cells. One day after each passage the medium was replaced with medium containing two mg/ml HIG (CytoGam®). Culture media were not transferred, as they contained neutralizing antibodies and lacked detectable infectious virus. Culture times for each passage were two to three weeks.

Virus stocks were prepared from infected MRC-5 cultures as infected-cell sonicates or culture media supernatants that were clarified by centrifugation at 500 x *g* for ten minutes, adjusted to 100 mM sucrose, aliquoted, and stored under liquid nitrogen. Stocks were titrated on MRC-5 cells using a 32-well limiting-dilution method as previously described [5].

Single viruses were isolated from mixed stocks by limiting dilution. Ig-KG passage 22 and ϕ-KG passage 13 stocks were ten-fold serially diluted, then each dilution was inoculated into 96 replicate wells containing confluent MRC-5 cells in 96-well plates. After incubation for one day, the medium in wells inoculated with Ig-KG dilutions was replaced with medium containing two mg/ml HIG, whereas wells inoculated with ϕ-KG dilutions were maintained in medium lacking HIG. One CPE-positive well, designated ϕ-KG-B5, was selected from the plate that was inoculated with the highest dilution of the ϕ-KG stock that produced CPE-positive wells. ϕ-KG-B5 was expanded using MRC-5 cultures without HIG. A second CPE-positive well, designated Ig-KG-H2, was selected from the plate that was inoculated with the highest dilution of the Ig-KG

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stock that produced CPE-positive wells. Ig-KG-H2 was expanded using MRC-5 cultures containing HIG, as described above.

Detection of infected cells by Immunostaining. Urine and virus stocks derived from urine were inoculated onto MRC-5, ARPE-19, or NOK cell cultures in 96-well plates. CMV-infected cells were detected by staining for the CMV immediate-early proteins 1 and 2 (IE1/2) using previously described immunohistochemistry [6] or immunofluorescence [7] staining methods.

Genetic sequencing. One T75 flask of confluent MRC-5 cells was inoculated with 0.2 ml ϕ-KG passage 12 stock. After eight days, eleven ml of culture medium from the ϕ-KG-infected flask were transferred to a T225 flask of confluent MRC-5 cells. After nine days of incubation the culture medium was removed and clarified by centrifugation at 500 x *g* for ten minutes. One T75 flask of confluent MRC-5 cells was inoculated with two ml of Ig-KG passage 21 stock. After incubation for one day the medium of the Ig-KG-infected flask was replaced with medium containing two mg/ml HIG. Cells were trypsinized eleven days after inoculation and transferred to a T225 flask containing confluent MRC-5 cells. The next day the medium was replaced with medium containing two mg/ml HIG. Two weeks after infection the culture medium was removed and clarified by centrifugation at 500 x *g* for ten minutes. Virion-associated DNA was isolated by ultracentrifugation of virions from clarified culture supernatants followed by DNase I treatment, phenol/chloroform extraction, and ethanol precipitation, as described previously [8].

Virion DNA (100 ng) purified from ϕ-KG or Ig-KG cultures was sheared, ligated to barcoded adapters, and size-selected for products in the ~ 685 bp range, then PCR amplified 8-9 cycles and sequenced using an Ion S5 System. The two modal read lengths were 280-bp and 550-bp at > 5 Million reads per sample. Reads were *de novo* assembled using Newbler v2.9 and

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the resulting contigs mapped NC_006273/Merlin to yield a draft genome. All reads were re-mapped to the draft genome and a consensus derived using Geneious v10.1.3. Median coverage was > 500x. Differences between ϕ-KG and Ig-KG identified by whole genome sequencing were confirmed by targeted PCR amplification of affected regions and Sanger sequencing (Eurofins) of both strands.

Results

HIG in the culture medium prevents loss of epithelial tropism during fibroblast passage. Serial fibroblast passage of CMV clinical isolates results in viral mutants that have lost the capacity to infect epithelial and endothelial cells. This phenotypic change arises from mutations that disrupt expression of one of three proteins, UL128, UL130, and UL131A, which combine with glycoproteins H and L (gH and gL) to form a pentameric complex that is necessary for efficient entry into epithelial and endothelial cells, but dispensable for entry into fibroblasts [9-13]. Such mutants emerge at about passage 15 and become dominant within a few additional passages [2].

To determine if antibodies can mitigate the selective pressures that promote the emergence of these mutations, a CMV culture-positive urine from a congenitally infected infant was inoculated into replicate flasks of MRC-5 fibroblasts. One lineage (designated ϕ-KG), was serially passaged by a conventional protocol without CMV antibodies added to the culture medium. The other (designated Ig-KG) was also serially passaged, but after each passage the culture medium was replaced with medium containing two mg/ml of HIG. A decline in epithelial tropism of the ϕ-KG lineage was evident by passage seven, and by passage 13 epithelial cell entry was rare, while fibroblast entry was abundant (Fig. 1A, top). In contrast, even after 20 passages the Ig-KG lineage appeared to remain epitheliotropic (Fig. 1A, bottom). To directly compare fibroblast and epithelial cell entry efficiencies, the ϕ-KG and Ig-KG stocks were three-fold serially diluted, and matching amounts were added to MRC-5 and ARPE-19 cultures. The ϕ-KG stock displayed a profound deficiency in epithelial entry compared to fibroblast entry (Fig. 1B, top), while the Ig-KG stock entered both cell types with similar efficiencies at each dilution (Fig. 1B, bottom).

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Genetic analysis of ϕ-KG and Ig-KG. Loss of epithelial tropism in the ϕ-KG lineage is consistent with literature data reporting that viruses with mutations disrupting expression of pentameric complex components emerge, and eventually dominate, during fibroblast passage [2]. The fact that the Ig-KG lineage remained epitheliotropic after 22 passages suggest that viruses with pentamer mutations fail to emerge during passage in the presence of HIG. To confirm this assumption, DNA was isolated from the supernatants of cells infected with ϕ-KG passage 13 or Ig-KG passage 22 stocks, and subjected to Ion Torrent sequencing. Analysis of the ϕ-KG genome sequence identified a single nucleotide insertion in *UL131A,* frame-shifting the amino-acid sequence of the UL131A protein after amino acid 27. In the Ig-KG sequence *UL131A,* as well as ORFs encoding the other two pentamer subunits, UL128 and UL130, were intact and encoded full-length, wild type proteins. These results are consistent with the lack of epithelial tropism of ϕ-KG, as the mutation in *UL131A* should prevent expression of UL131A and, hence, assembly of a functional pentameric complex.

Examination of the *RL13* region in the ϕ-KG genome revealed the presence of a 10-bp deletion, causing a frameshift and consequent truncation of RL13 after amino acid 164. Surprisingly, the *RL13* ORF of Ig-KG lacked this deletion and was otherwise intact, suggesting that KG virus passaged in the presence of HIG did not acquire mutations disrupting RL13 expression.

Viruses isolated and expanded from mixed stocks retain parental genotypes and phenotypes. The above analyses used mixed stocks produced after 13 or 22 passages. To improve uniformity, individual viruses were isolated from the mixed stocks by limiting-dilution in 96-well MRC-5 cultures. Virus ϕ-KG-B5 was isolated from the ϕ-KG passage 14 stock and expanded in the absence of HIG. Virus Ig-KG-H5 was similarly isolated and expanded from the

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Ig-KG passage 22 stock in the presence of HIG. Resulting stocks, produced as clarified lysates of infected cells from one T75 flask of MRC-5 cells, had a final volume of two ml. The ϕ-KG-B5 stock had a titer on MRC-5 cells of 6.3 x 10^5 pfu/ml, while the Ig-KG-H5 stock had a titer of 6.9 x 10^4 pfu/ml.

The ϕ-KG-B5 and Ig-KG-H5 viruses recapitulated the epithelial tropisms of their mixed parental stocks. Virus ϕ-KG-B5 infected epithelial cells with very poor efficiency compared to fibroblasts, while Ig-KG-H5 infected both cell types with similar efficiencies (Fig. 2A). Ig-KG-H5 foci appeared larger on MRC-5 cells compared to ARPE-19 cells, and counting the number of IE1/2-positive nuclei per focus revealed that foci on MRC-5 cells were on average 1.35-fold larger than foci on ARPE-19 cells (Fig. 2B). In addition, ϕ-KG-B5 appeared to form larger foci on MRC-5 cells than Ig-KG-H5 (Fig. 2A) and counting IE1/2-positive nuclei confirmed that MRC-5 foci of ϕ-KG-B5 were on average 1.46-fold larger than those of Ig-KG-H5 (Fig. 2B). These results are consistent with improved replication efficiencies associated with mutations disrupting expression of RL13 or of pentamer components [14]. Sequencing of ϕ-KG-B5 virion DNA confirmed the presence of frame-shift mutations in *RL13* and *UL131A* identical to those identified in the ϕ-KG parental stock, while sequencing of Ig-KG-H5 virion DNA confirmed that both loci remained intact and wild type.

Experimental use of CMV stocks propagated with HIG. To show that stocks generated in the presence of HIG can be used experimentally, a two-ml 4 x 10^4 pfu/ml stock designated IgKG-mix was produced by combining and expanding several wells of virus isolated by limiting-dilution from the Ig-KG passage 22 stock. The IgKG-mix stock was three-fold serially diluted and added to replicate cultures of MRC-5 cells, ARPE-19 cells, and undifferentiated NOK cells, used to assess viral tropism for epithelial cells derived from the oral

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mucosa. Entry efficiency, as assessed by IE1/2 staining, was highest in MRC-5 cells, about four-fold lower in ARPE-19 cells, and eight-fold lower in NOK cells (Fig. 3A). Moreover, while the IgKG-mix virus formed foci ten days after infection of MRC-5 cells and ARPE-19 cells, no evidence of focus formation was observed in NOK cells (Fig. 3B).

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Discussion

For several decades, cell culture-based studies of CMV relied heavily on standard laboratory strains of CMV, such as Towne and AD169, which were adapted through extensive (> 100 times) serial passage in fibroblasts to replicate efficiently by releasing high levels (10^6 to 10^7 pfu/ml) of cell-free virus into the culture medium. In the 1990s it was discovered that AD169 and a common Towne variant had undergone substantial deletions in the *UL/b'* region: 15.2-kb from AD169 and 13.1-kb from Towne [15, 16]. Additional frame-shift mutations disrupting expression of the pentameric complex were later shown to drastically limit replication of these strains in endothelial, epithelial, and certain myeloid lineage cells [9-13, 17].

More recent studies have trended toward the use of more genetically authentic CMV strains that retain expression of the pentameric complex and/or an intact *UL/b'* region. Low passage clinical isolates have also been employed, but pose significant technical challenges. In the initial passages clinical isolates are highly cell associated and produce low viral titers (10^3 to 10^4 pfu/ml) in culture supernatants. After five or six passages, replication and release of virus into the culture medium significantly improves [18]; however, because the properties of an isolate can change significantly from one passage to the next, it can be difficult to predict the degree of authenticity/extent of adaptation at any given passage.

While the mechanism remains unclear, mutations that disrupt RL13 expression appear to be a major contributor toward improved replication and increased release of cell-free virus during cell culture adaptation [2, 14]. Disruptive *RL13* mutations appear rapidly during passage and are not specific to one cell type but occur in fibroblast, epithelial, and endothelial cells [2, 14]. Mutations that disrupt expression of pentameric complex components also appear to

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enhance the release of cell-free virus, but only arise during serial passage in fibroblasts; viruses passed in epithelial or endothelial cells maintain an intact pentameric complex due to its required role for entry into these cell types [2]. Thus, while it is possible to preserve expression of pentameric complex components by propagating stocks of certain CMV strains in ARPE-19 epithelial cells, obtaining stocks of *RL13*+ CMV is challenging as even low passage isolates are likely to contain a population of *RL13*-mutant viruses and the prevalence of *RL13* mutants increases quickly with passage [14]. An elegant solution was to genetically modify the *RL13* promoter so that, in appropriate host cells, RL13 expression can be conditionally regulated. *RL13*+ stocks can thus be made under RL13-repressed conditions and phenotypes associated with RL13 expression can be assessed under RL13-induced conditions [14].

In the current study, we demonstrate that, by adding antibodies to the culture medium, CMV in clinical samples can be isolated, expanded, serially passaged, and sub-cloned in fibroblasts without accruing mutations disrupting expression of RL13 or assembly of the pentameric complex. Studies are in progress to determine if other adaptive mutations occur under these conditions or, conversely, whether viruses isolated and expanded in the presence of HIG maintain a fully wild type genome. While the mechanism by which HIG stabilizes CMV during serial passage remains uncertain, it is probable that neutralization by HIG of virus released into the culture medium forces virus amplification to occur exclusively by cell-to-cell spread, thereby alleviating selective pressures favoring disruption of *RL13* or *UL128-131A*, which promote release of cell-free virions. Analogous effects likely manifest *in vivo*.

Stocks produced from infected cell lysates reached moderate titers (~ 10^4-10^5 pfu/ml) but were sufficient for use in experiments that do not require high multiplicities of infection. Higher stock titers may be achievable through further optimization, and additional studies are needed to

266 determine the minimal concentration of HIG required and whether monoclonal antibodies can
267 substitute for HIG. It is possible that similar results can be achieved in the absence of antibodies
268 by passing only infected cells without any culture medium.

269 The culture conditions described herein should be amenable to extending current CMV
270 genetic systems for evaluation of CMV mutants in an *RL13*+ background. For example, it should
271 be feasible to derive bacterial artificial chromosome (BAC) clones of new *RL13*+ CMV isolates,
272 or to use *E. coli* genetics to repair *RL13* in established BAC-cloned CMV strains. Additional
273 mutants could then be constructed and reconstituted by transfection of fibroblasts in the presence
274 of antibodies. Alternatively, mutations in non-essential genes could be engineered directly using
275 CRISPR/Cas9 without the need for BAC cloning. Studies of this nature should help elucidate the
276 mechanisms underlying the repressive role(s) played by RL13 during CMV replication in
277 established cell culture systems such as fibroblasts or ARPE-19 cells, and may prove crucial for
278 identifying positive, perhaps essential roles for RL13 in other cell types or *in vivo* that are
279 currently in their nascent stages of investigation.

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Figure Legends

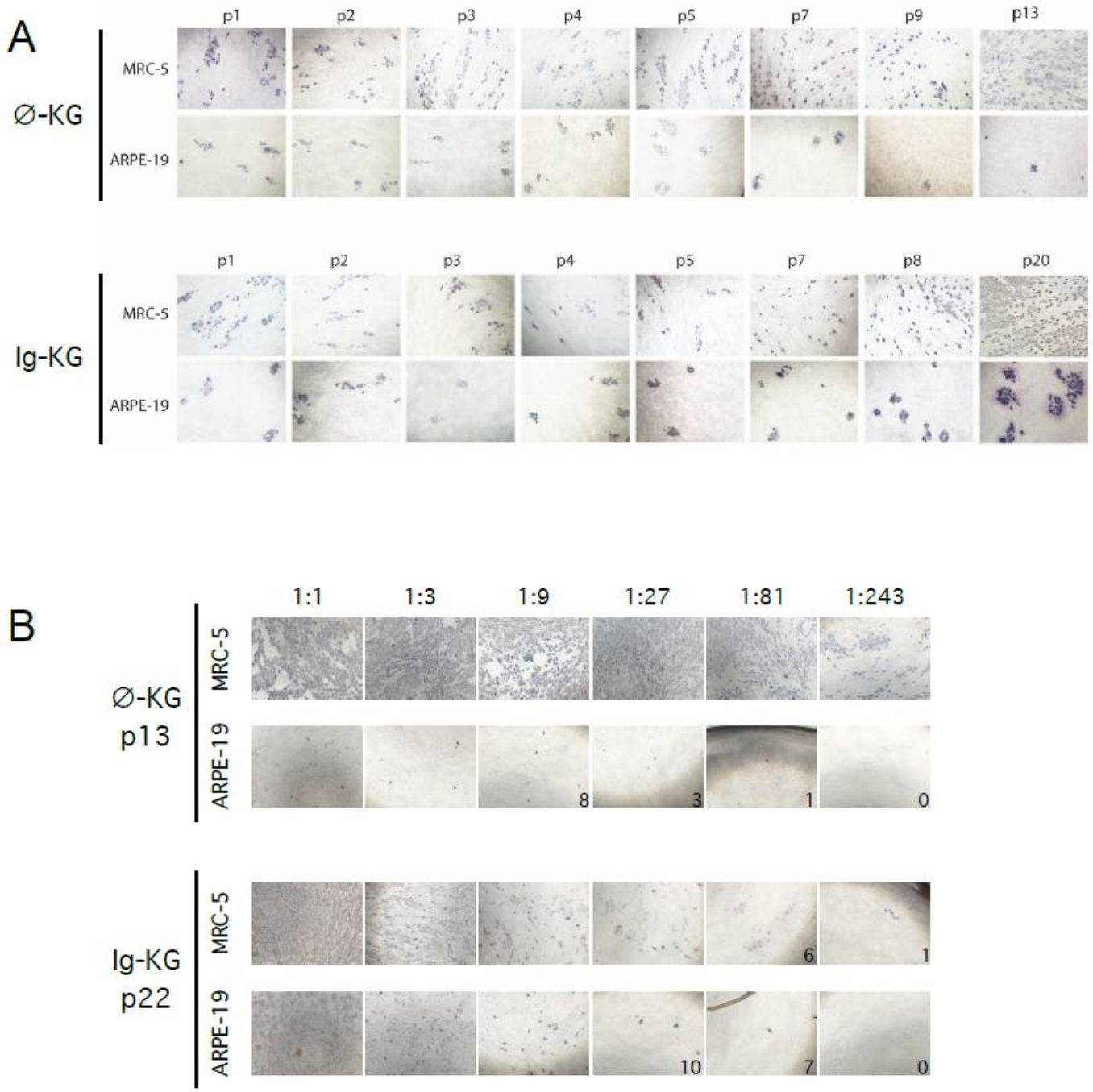

Fig. 1. A clinical CMV isolate passaged in the presence of antibodies retains epithelial entry tropism. (A) MRC-5 fibroblasts or ARPE-19 epithelial cells were infected with replicate virus stocks from the indicated passages of ϕ-KG or Ig-KG stocks and stained for CMV IE1/2 after 5 (MRC-5) or 12 (ARPE-19) days. (B) The passage 13 stock of ϕ-KG and passage 22 stock of Ig-KG were three-fold serially diluted,

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added to MRC-5 fibroblasts or ARPE-19 epithelial cells, and stained for CMV IE1/2 antigen after four days. The number of IE1/2-positive cells in photographic images was manually counted from enlarged prints and is reported in the bottom right corner of panels in (B).

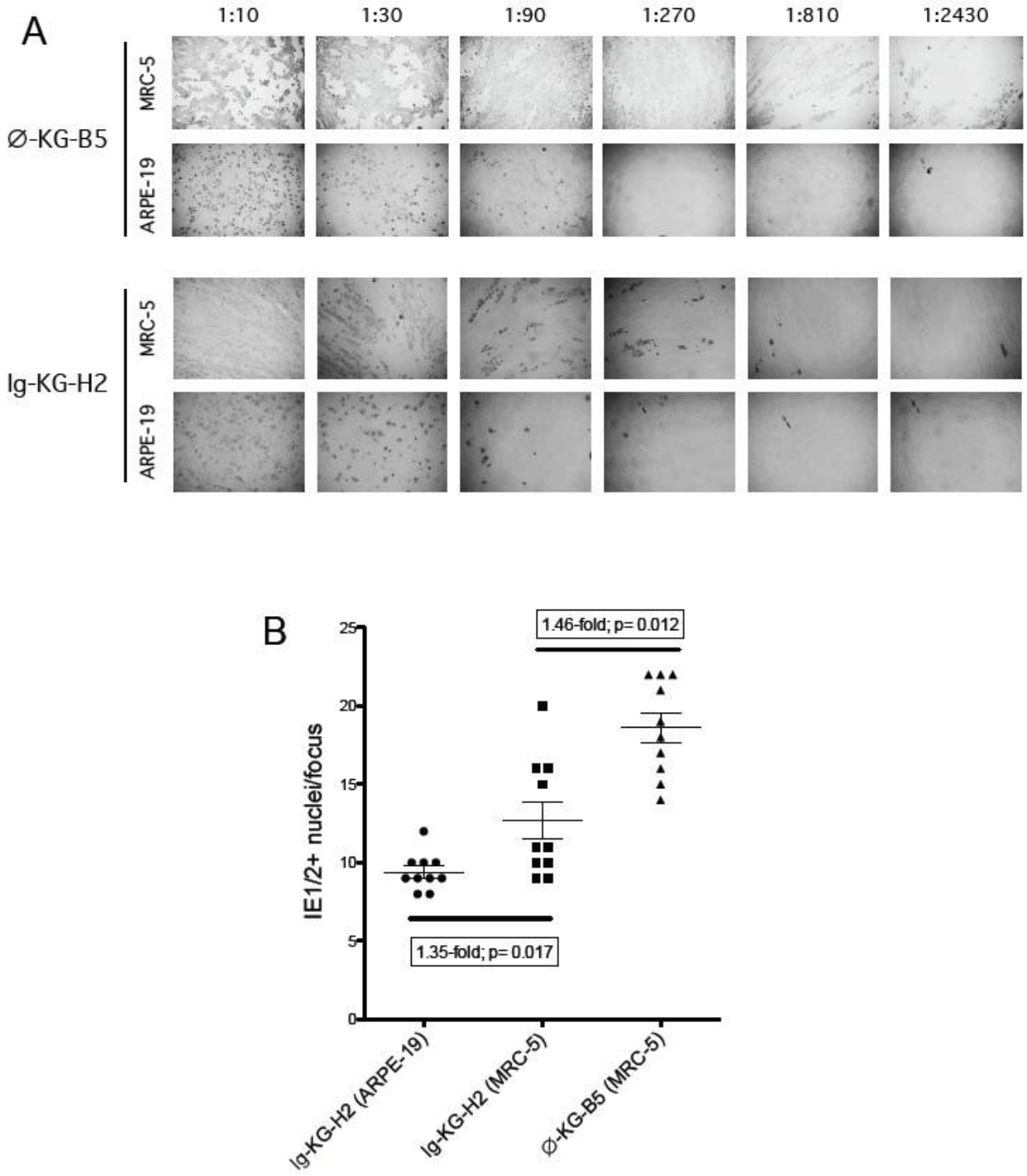

Fig. 2. Subcloned viruses retain the tropism of the parental stocks. (A) Viruses ϕ-KG-B5 and Ig-KG-H2 were isolated from ϕ-KG or Ig-KG stocks, respectively, then evaluated for entry into MRC-5 or ARPE-19

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cells as described in the legend for Figure 1. (B) The numbers of IE1/2-positive nuclei in ten foci from ϕ-KG-B5-infected or Ig-KG-H2-infected MRC-5 or ARPE-19 cultures were manually counted. Fold differences in the means and p values (two-tailed t test) are indicated.

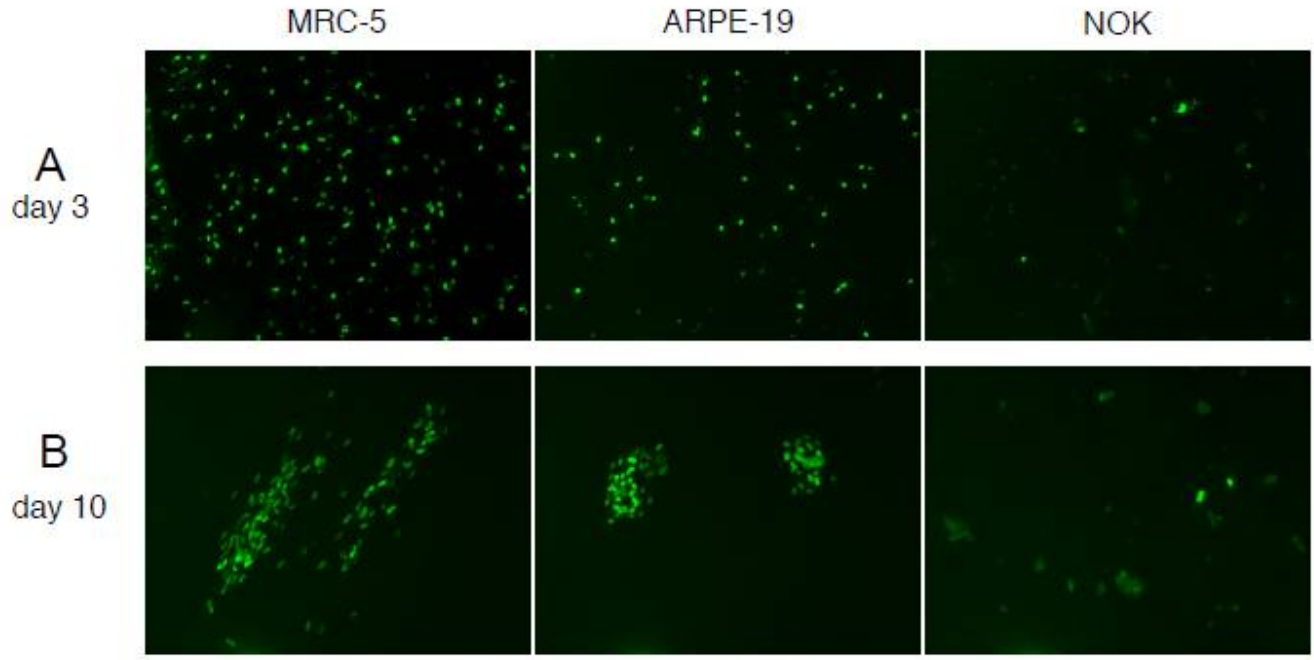

Fig. 3. Using Ig-KG to evaluate CMV tropisms for different cell types. (A) Replicate cultures of MRC-5, ARPE-19, or NOK cells in 96-well plates were infected with 1000 pfu of Ig-KG-mix (A) or with 28, 83, or 250 pfu Ig-KG-mix stock, respectively (B), and stained for IE1/2 proteins at the indicated times post-infection. Images were taken using a 5x (A) or 10x (B) objective and were false colored in green.