Neutralizing antibodies are unable to inhibit direct viral cell-to-cell spread of human cytomegalovirus

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ABSTRACT

Infection with human cytomegalovirus (CMV) during pregnancy is the most common cause of congenital disorders, and can lead to severe life-long disabilities with associated high cost of care. Since there is no vaccine or effective treatment, current efforts are focused on identifying potent neutralizing antibodies. A panel of CMV monoclonal antibodies identified from patent applications, was synthesized and expressed in order to reproduce data from the literature showing that anti-glycoprotein B antibodies neutralized virus entry into all cell types and that anti-pentameric complex antibodies are highly potent in preventing virus entry into epithelial cells. It had not been established whether antibodies could prevent subsequent rounds of infection that are mediated primarily by direct cell-to-cell transmission. A thorough validation of a plaque reduction assay to monitor cell-to-cell spread led to the conclusion that neutralizing antibodies do not significantly inhibit plaque formation or reduce plaque size when they are added post-infection.

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Introduction

While human cytomegalovirus (CMV) infections in healthy individuals are generally asymptomatic, infection via perinatal transmission is a major cause of birth defects and infection in immunocompromised patients is associated with significant morbidity and mortality (Kenneson and Cannon, 2007; Griffiths, 2012). The current drugs to treat CMV infections, ganciclovir, valganciclovir, foscarnet, and cidofovir, all target the viral DNA polymerase and are associated with teratogenicity, toxicity, carcinogenicity and/or neutropenia (reviewed in Boeckh and Geballe, 2011). Due to these significant limitations, these drugs are not used to prevent the transmission of CMV from mother-to-infant during pregnancy and are only used off-label for short courses in symptomatic neonates (Kimberlin et al., 2003). Prior CMV seroconversion of mothers is associated with much lower rates of mother to child transmission (Kenneson and Cannon, 2007), giving indirect evidence that antibodies will prevent infection. Therefore, among other treatment possibilities such as vaccination, efforts are currently focused on identifying potent anti-CMV monoclonal antibodies that could fill this therapeutic void presently only partially addressed by experimental products such as CMV-specific hyperimmune globulins, Cytogam® or Cytotect®, that have low potency and require intravenous infusion (reviewed in Adler, 2012). The mechanism by which anti-CMV antibodies prevent transmission during pregnancy is not yet completely understood but may include direct neutralization of extracellular virus, antibody-dependent cell-mediated cytotoxicity, complement-dependent-cytotoxicity and perhaps inhibition of direct virus cell-to-cell spread (Navarro et al., 1993; Andreoni et al., 2001; Forthal et al., 2001; Burton, 2002; Frenzel et al., 2012).

Like herpes simplex virus and other well studied herpesviruses, cell-associated CMV is known to have the ability to spread to contiguous cells without having to transit via the extracellular space (Kinzler and Compton, 2005; Digel et al., 2006). This direct cell-to-cell spread of progeny virus to adjacent cells is thought to be an important immune evasion mechanism and may have significant clinical implications (reviewed in Sattentau, 2008). The identified viral proteins molecules required for this process often correspond to those required for viral entry. Among others, glycoprotein B (gB), glycoprotein H (gH) and glycoprotein L (gL) have been shown to be required for cell-to-cell spread as well as syncytia formation in many members of the herpesvirus family including CMV (Kinzler and Compton, 2005; Isaacson and Compton, 2009; Bowman et al., 2011). gH/gL are associated with

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other glycoproteins that influence the cell tropism, such as glycoprotein O and UL128-131a (the latter which with gH and gL form the pentameric complex (PC)) (Hahn et al., 2004; Wang and Shenk, 2005; Adler et al., 2006). These viral envelope proteins have been detected at the surface of the host cell where they likely play a role in cell-to-cell spread of the virus (Middeldorp et al., 1985; Jennings et al., 1987).

The importance of this dissemination mechanism is highlighted by the fact that despite a high tropism for many different cell types, the extracellular virus concentration is generally very low in CMV-infected individuals (Hamprecht et al., 1998; Kalil and Florescu, 2009). Although CMV laboratory prototypical strains like AD169 are highly cytopathic and rapidly shed into the supernatant of infected cells in culture, it has been shown that clinical isolates remain mostly cell-associated (Wang and Shenk, 2005; Sinzger et al., 2007). Further supporting the importance of cell-to-cell spread is a study showing that depleting blood of its peripheral leukocytes prevents lateral transmission of the virus (Ljungman et al., 2002).

The most evident *in vitro* manifestation of cell-to-cell spread is the observation of discrete plaques on monolayers of cells grown *in vitro*. Consequently, plaque reduction assays (PRA) are widely used to evaluate the ability of antiviral agents to inhibit cell-to-cell spread. While a standardized PRA has been employed for monitoring neutralization of extracellular virus (Landry et al., 2000), this is not the case for evaluating cell-to-cell spread of CMV. Studies investigating the efficacy of antibodies directed against CMV surface proteins have suggested that neutralizing antibodies can also prevent cell-to-cell spread in the PRA (Navarro et al., 1993; Andreoni et al., 2001; Frenzel et al., 2012), while other papers indicate that antibodies cannot prevent cell-to-cell spread of CMV (Sinzger et al., 2007; Schroer and Shenk, 2008).

Our goal was to clarify the role of antibodies in preventing the propagation of CMV in cell culture. We address the differences between the procedures for monitoring cell-to-cell spread as well as the conclusions drawn from our experiences and the literature.

Results

Genetically engineered monoclonal antibodies are functional and have neutralization potencies similar to those reported in the literature

A panel of seven tool monoclonal antibodies (mAb), based on patent applications, was synthesized and expressed in order to recapitulate neutralization data from the literature in parallel along with a CMV-specific hyperimmune globulin (CMV-IG) preparation (Cytogam®). It is important to note that this CMV-IG preparation was selected based on high binding titers to CMV and not for the ability to neutralize CMV. These mAb are directed against gB, gH and PC as described in Table 1. The CMV antibodies were tested in a microneutralization immunofocus assay with the detection of CMV Intermediate Early 1 protein (IE1) in MRC-5 fibroblasts infected either with the lab strain AD169 or the clinical isolate VR1814, or in ARPE-19 epithelial cells infected with VR1814. We confirmed that the tool anti-gB antibodies neutralize virus entry into all cell types at moderate potency, and that anti-PC antibodies are highly potent in preventing virus entry into epithelial cells but do not prevent entry into fibroblasts (Macagno et al., 2010). CMV-IG showed increased neutralization potency on VR1814-infected ARPE-19 cells as compared with MRC-5 cells, in agreement with the a study demonstrating the presence of anti-PC antibodies in CMV-IG preparations (Fouts et al., 2012).

CMV-IG was not able to inhibit cell-to-cell spread in a validated plaque reduction assay

PRAs were developed and CMV-IG was tested in parallel with the CMV entry inhibitor CFI02 (Jones et al., 2004) and the nucleotide analog ganciclovir (reviewed in Martin et al., 1983) when they were added at the time of infection or 3 h post-infection at 10x EC_{50}, followed by agarose overlay to limit extra-cellular spread of CMV. While all three were able to inhibit plaque formation when added at the time of infection (in the absence of agarose), only CFI02 and ganciclovir were able to inhibit plaque formation when added 3 h post-infection (Fig. 1A). This was our first indication that while CMV-IG could neutralize virus entry, it was not able to prevent cell-to-cell spread on subsequent rounds of infection even when tested at 10x EC_{50} for neutralization of extracellular virus.

Various measures were taken to ensure that the monitoring of plaque reduction reflected cell-to-cell spread inhibition and not inhibition of infection of contiguous cells via extracellular release of CMV. There was no shift in potency for GCV when it was added at the time of infection or 3 h post-infection (Fig. 1A, EC_{50} 2.2–6.8 μM against the different virus/cell combinations tested). CFI02 had very similar potency of 1–4 μM when added at the time of infection or 3 h post-infection on VR1814-infected MRC-5 and ARPE-19 cells. However CFI02 showed a 10-fold decrease in potency when added 3 h post-infection of AD169-infected MRC-5 cells ($t=0$ EC_{50} 0.2 μM; $t=3$ h EC_{50} 1.5 μM) (Fig. 1A, Table 2). This

Table 1
Antibodies can neutralize CMV in an immunofocus assay.

Antibody	Ag	AD169/MRC5		VR1814/MRC5		VR1814/ARPE19		Reported potency	Reference
		EC_{50}	EC_{90}	EC_{50}	EC_{90}	EC_{50}	EC_{90}		
Cytogam	Poly	40	140	20	150	1.2	5	40[a,c]	Planitzer et al. (2011)
EV2038	AD1 (gB)	0.05	0.58	0.19	1.6	0.2	1.2	0.027[a,c], 0.040[a,d]	Takada et al. (2010a)
7H3	AD1 (gB)	0.20	0.7	0.20	2	0.2	0.8	3[b,c], 0.6[b,d]	Macagno et al. (2010)
EV2045	AD2 (gB)	0.06	0.3	0.09	0.26	0.05	0.3	0.061[a,c], 0.053[a,d]	Takada et al. (2010b)
ITC-88	AD2 (gB)	0.7	3.8	0.23	1.3	0.16	0.67	1–2[a,c]	Ohlin et al. (1993)
MSL-109	gH	0.21	4	0.15	1.7	0.13	0.6	1.4[a,c]	Nokta et al. (1994)
1F11	PC	> 32	> 32	> 32	> 32	0.00024	0.0011	NN[b,c], 0.001[b,d]	Macagno et al. (2010)
4I22	PC	> 32	> 32	> 32	> 32	0.0003	0.0014	NN[b,c], 0.0015[b,d]	Macagno et al., 2010

All EC_{50} and EC_{90} reported as μg/ml and are the average of at least four experiments. NN: Non neutralizing.

[a] EC_{50} value (μg/ml).
[b] EC_{90} value (μg/ml).
[c] Tested on fibroblasts.
[d] Tested on epithelial cells.

Table 2
Antiviral potency of CFI02 and Ganciclovir in CMV plaque reduction assays with and without CMV-IG in the overlay.

EC_{50} (μM)	AD169/MRC-5		VR1814/MRC-5		VR1814/ARPE-19	
	No CMV-IG	CMV-IG $2xEC_{90}$	No CMV-IG	CMV-IG $2xEC_{90}$	No CMV-IG	CMV-IG $2xEC_{90}$
CFI02	1.5	1.5	1.3	2.4	3.9	5.2
Ganciclovir	2.2	2.8	6.8	4.2	6.4	4.0

EC_{50} are the average of two experiments.

Table 3
Number of plaques with and without CMV-IG in the agarose overlay in the plaque reduction assays.

Virus/Cell	7 day incubation		14 day incubation	
	No CMV-IG	CMV-IG $2xEC_{90}$	No CMV-IG	CMV-IG $2xEC_{90}$
AD169/MRC-5	38	39	ND	42
VR1814/MRC-5	38	35	36	40
VR1814/ARPE-19	39	38	39	40

Average plaque count for two wells from one experiment. This experiment was repeated 6 times ending at 7 days post-infection, and two times ending at 14 days post-infection.
ND: not determined. See Fig. 1B for micrograph.

could be attributed to the mechanism of action of CFI02 as an entry inhibitor where a decrease in potency was also seen in a cell-to-cell spread assay of AD169-infected fibroblasts (Jones et al., 2004), but it also could be due to transmission of extracellular virus in the PRA. It should be noted that VR1814 forms syncytia, while the lab strain AD169 does not (Wang and Shenk, 2005). It has also been shown that CMV entry into endothelial and epithelial cells requires UL128 to UL150, and that this is not required for entry of AD169 into fibroblasts (Ryckman et al., 2006). It is possible that CFI02 inhibits entry by binding to the pentameric complex in addition to gB. The addition of CMV-IG to the agarose overlay at a concentration sufficient to neutralize all extracellular virus ($2x$ EC_{90}) in liquid culture did not alter the EC_{50} of GCV and CFI02 obtained in the absence of CMV-IG (Table 2). This suggests that neutralization of extracellular virus, if any, does not influence the readout of the PRA at 7 days incubation.

To better understand the contribution of extracellular virus to the number and size of the plaques, $10xEC_{90}$ CMV-IG was added in the overlay at 3 h post-infection, and the plates were incubated for 7 (as usual) or 14 days, and then fixed and stained (Fig. 1B). The plaques were then counted and their size was determined. The number of plaques counted with and without CMV-IG with every cell/virus combinations at 7 days incubation is similar (Table 3). As shown in Fig. 1C, the size of plaques after 7 days for AD169 and VR1814 were not different whether CMV-IG was added to the overlay or not. This was also true for VR1814 on both cell lines at 14 days incubation. However, wells infected with AD169 without CMV-IG at 14 days post-infection contained large and poorly defined plaques that merged, making counting impossible, whereas the plaques in the presence of CMV-IG were still well defined (Fig. 1B). These results indicate that AD169 and VR1814 were mainly transmitted from cell-to-cell in our PRA format for up to 7 days post-infection. While VR1814 was still transmitted cell-to-cell at 14 days post-infection, AD169 appeared to be spread both intracellularly and extracellulary as evidenced by the restriction of plaque growth in the presence of CMV-IG at this later time point. Moreover, this also demonstrated that antibodies are still functional in the agarose overlay at both 7 and 14 days

post-infection. Taken together, these data validate our assay format of examining plaque reduction at 7 days incubation in order to monitor cell-to-cell spread.

Monoclonal antibodies were not able to prevent cell-to-cell spread in PRA

Experiments were performed with tool mAb in the PRAs with AD169 and VR1814 on MRC-5 and ARPE-19 cells. While mAb were able to inhibit plaque formation completely when added at the time of infection, they were not able to inhibit at 3 h post-infection at 10x their EC_{50} (Fig. 2A). A time of addition study in the PRA was performed with CMV-IG, EV2045 (α-gB) and 4I22 (α-PC) where they were added at time 0, 1, 2 and 3 h after CMV infection at $2x$ their EC_{90}. Successively less inhibition of plaque formation was seen over the time course from 70 to 100% reduction at time zero, to 0% reduction at 3 h post-infection (Fig. 2B), suggesting that the extent of inhibition of plaque formation correlates with the inhibition of the initial viral entry at time zero. Therefore, both mAb and CMV-IG were not able to inhibit cell-to-cell spread of CMV.

Comparison of methods to monitor cell-to-cell viral spread

A cell-to-cell spread assay with the addition of antibody at different times post-infection was performed in order to replicate the observations found with EVEC2038 where 75–100% inhibition of plaque formation was observed with > 0.63 μg/ml when antibody is added 24 h post-infection (Takada et al., 2010a, 2012). The method used in this application relies on replenishment of the media containing the antibody every 24 or 48 h (Navarro et al., 1993). A condition in which the mAb was added at time zero was also tested. The addition of EV2038 at 24 h post-infection did not reduce plaque formation (Fig. 3A).

A PRA was also performed with CMV-IG (10x EC_{90}) and EV2038 and ITC-88 (10 μg/ml) using a method similar to that described previously (Frenzel et al., 2012) and compared to our in-house assay. In this method, cells were infected for 1 h by spinoculation and washed before adding the overlay with the antibody and incubating for 7 days. Modest to no reduction in plaque formation was seen when antibody was added at 1 h post-infection, whereas 90% inhibition was seen when antibody was added at $t=0$ during spinoculation (AD169: Fig. 3B, VR1814: data not shown). Overall examination of the various PRA methods led to the conclusion that the CMV antibodies were not able to prevent plaque formation when added post-infection.

Ab could not inhibit plaque formation even when cells were not confluent

A PRA was performed to assess the influence of the density of cells seeded on the ability of EV2038 to inhibit plaque formation. In papers indicating that antibodies can inhibit plaque formation (Navarro et al., 1993; Frenzel et al., 2012) no specific detail was given on cell seeding density. It is possible that Ab could inhibit cell-to-cell spread if viral proteins are expressed and neutralized at the cell membrane before contacting the membranes of adjacent cells. Four different cell seeding densities were tested (10–85% confluency) and the antibody was added at the time of infection or 3 h post-infection. No inhibition was observed when the antibody was added at 3 h post-infection, independent of the cell density (Fig. 4). These results suggest that the cell confluency did not influence the ability of EV2038 to inhibit plaque formation.
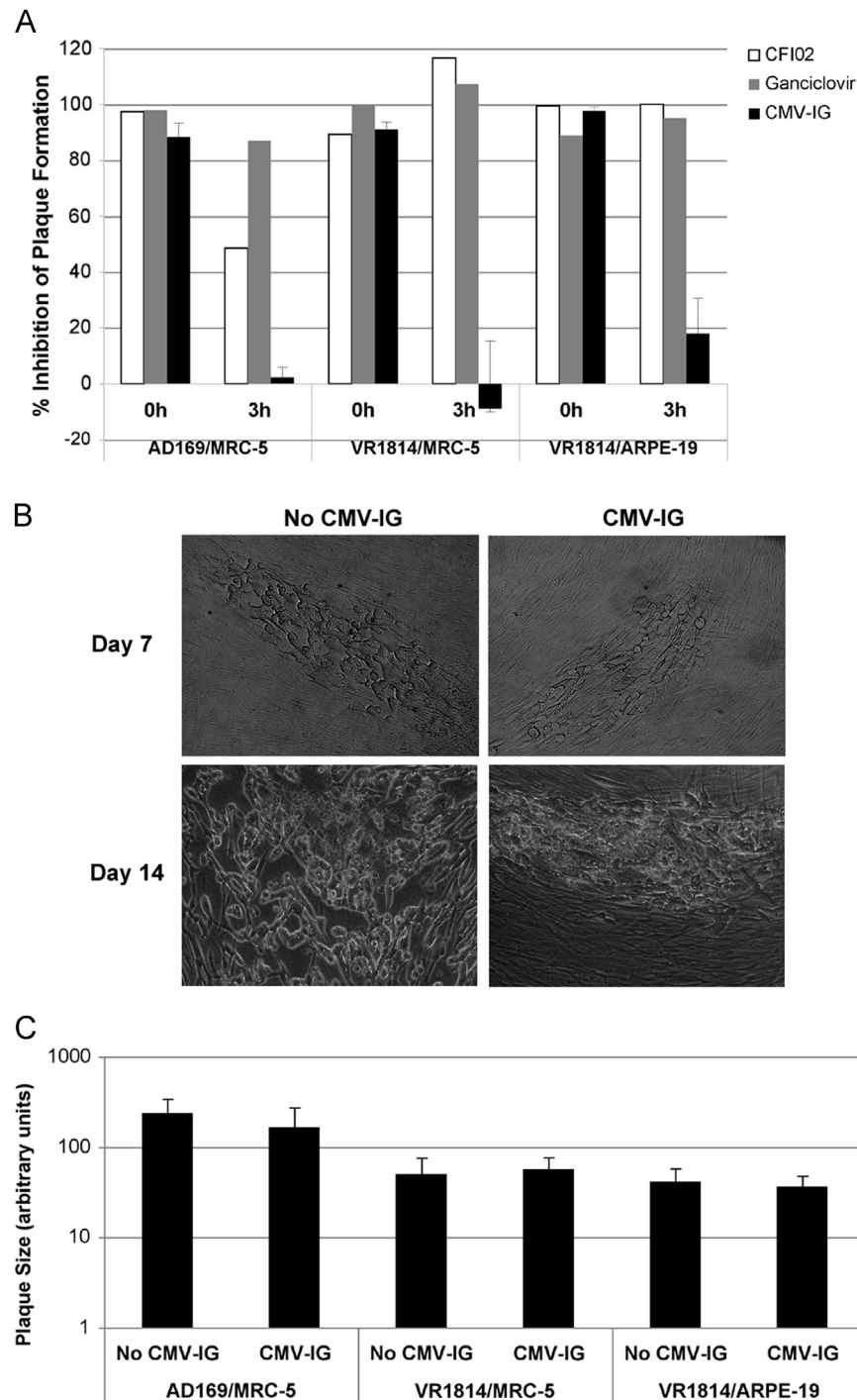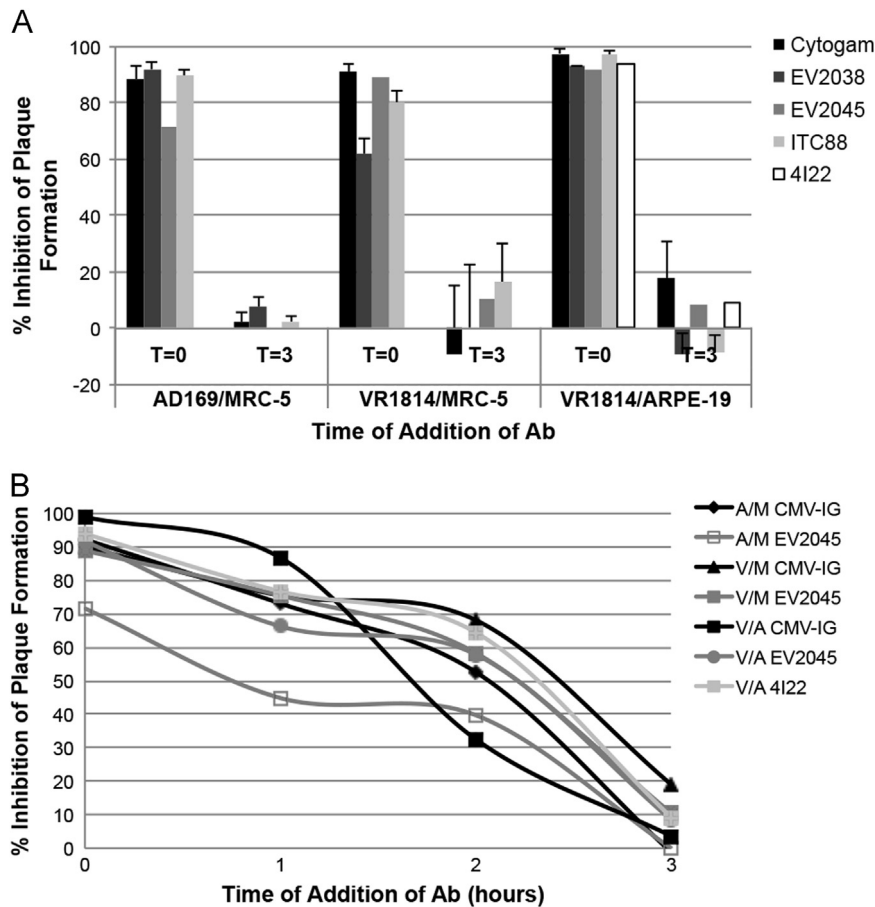
Fig. 1. CMV-IG does not prevent cell-to-cell spread in a plaque reduction when added after initial cycle of infection. (A) PRA was performed where CMV-IG, CFI02 and GCV at 10x EC_{50} were added at $t=0$, and/or $t=3$ h post-infection with agarose overlay. (B) Phase-contrast micrograph of AD169-infected MRC-5 cells at 7 and 14 days post-infection with and without CMV-IG at 2x EC_{90} added at 3 h post-infection with the agarose overlay. (C) No difference was seen in plaque size measured at 7 days with and without CMV-IG at 10x EC_{50}. PRA was performed as described and CMV-IG was added with overlay at 3 h post-infection and size of plaques was determined at 7 days post-infection by measuring the average size of 20 plaques using the Harmony software.

Discussion

There are multiple steps where congenital infection could be blocked during the process from primary infection of the mother to transmission to the fetus. There are three main ways that viral spread could be prevented: direct neutralization of extracellular virus, prevention of cell-to-cell spread, and, the destruction of infected cells that express the viral antigens at their surface through antibody effector function. Data from this present study and the literature demonstrate that CMV-specific antibodies

against gB, gH and PC can neutralize extracellular virus and prevent viral entry in relevant cell lines using both immunofocus neutralization assays and plaque reduction assays (PRA) when antibody is added during the first cycle of infection (Ohlin et al., 1993; Nokta et al., 1994; Macagno et al., 2010; Takada et al., 2010a, 2010b, 2012; Planitzer et al., 2011).

However, it was not clear from previous studies if antibodies could prevent subsequent rounds of infection that are mediated primarily by direct cell-to-cell transmission. While on first glance it did not appear possible for antibodies, due to their size and

Fig. 2. CMV monoclonal antibodies do not prevent cell-to-cell spread in PRA when added 3 h post-infection. (A) Comparison of antibodies ($10xEC_{50}$) added at $t=0$, and/or $t=3$ h post-infection with agarose overlay in PRA. (B) Addition of CMV-IG and EV2045 ($2xEC_{90}$) at $t=0$, 1, 2 and/or $t=3$ h post-infection with agarose overlay in PRA.

nature, to be able to get to the site of cell-to-cell spread, examples in the literature indicated that CMV antibodies were capable of preventing this mode of transmission in cultured fibroblasts (Navarro et al., 1993; Frenzel et al., 2012; Takada et al., 2012). This is in contrast to other studies where CMV-IG was specifically used in media to prevent extracellular spread without affecting cell-to-cell transmission (Silva et al., 2005; Ryckman et al., 2006; Schroer and Shenk, 2008; O'Connor and Shenk, 2011). Moreover, sera from CMV-infected patients was used to demonstrate that while lab isolates of CMV show both intracellular and extracellular spread *in vitro*, CMV clinical isolates are strictly cell-associated and therefore inaccessible to neutralizing antibodies (Sinzger et al., 2007).

In our hands, CMV-IG and a panel of neutralizing mAb to CMV envelope proteins were not able to inhibit plaque formation when added 3 h post-infection with both a lab and a clinical isolate on fibroblasts and epithelial cells. Concentrations up to 1.5 mg/ml CMV-IG and 10 µg/ml mAb were tested. The latter concentration corresponds to the range of IgG seen in gB-specific antibodies *in vivo* (Ohlin et al., 1997). We validated our PRA by testing small molecule inhibitors with and without CMV-IG and monitoring plaque count and size over the duration of the assay (7 days) and beyond (14 days).

It was reported that CMV-IG could inhibit plaque formation of lab and most clinical isolates on fibroblasts at 2 mg/ml when it was added 1 h after spinoculation (Frenzel et al., 2012). EV2038, a gB mAb, has been shown to inhibit plaque formation of AD169 on

fibroblasts at concentrations as low as 0.63 µg/ml when added 24 h post-infection (Takada et al., 2012). Our data is in contrast to what has been reported, therefore we sought to compare multiple methods for monitoring cell-to-cell spread of CMV. We saw no inhibition of plaque formation by 10 µg/ml EV2038 when added 24 h post-infection and replenished every 24 or 48-h as per the published protocol (Navarro et al., 1993). We also saw modest to no reduction of plaque formation when antibody was added at 1 h post-infection by spinoculation as per published protocol (Frenzel et al., 2012), whereas 90% inhibition was seen when antibody was added at the same time as infection. The inhibition of plaque formation was not enhanced by a 30 min incubation with antibodies at room temperature prior to the application of the agarose overlay (data not shown), suggesting that antibodies only inhibit the initial entry of virus. It should be noted that in the latter published method, Avicel® was used and not agarose, although this is unlikely to explain the differences seen in the results. Furthermore, we have demonstrated that antibodies are still functional when added in the agarose overlay as evidenced by discrete plaques being seen after 14 days of infection of MRC-5 cells with AD169 in the presence of CMV-IG, versus cytopathic effect being observed in the absence of CMV-IG.

The *in vivo* situation is more complicated than cell culture models of monolayers of fibroblasts or epithelial cells. Studies have been conducted with polarized cells to bridge this gap. It has been reported that CMV-specific mAbs failed to inhibit CMV transmission across lateral membranes of polarized ARPE-19 epithelial cells
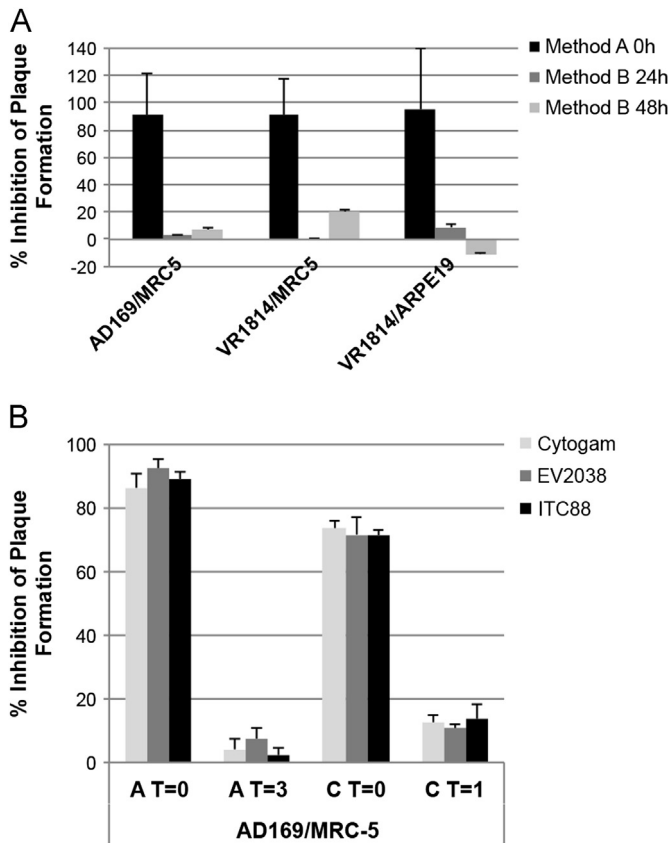
Fig. 3. CMV antibodies are not active in various PRA methodologies when added after initial cycle of infection. (A) EV2038 (10 μg/ml) was added at $t=0$ (Method A 0 h), $t=24$ h and replenished every 24 h (Method B 24 h), or at $t=24$ h and replenished every 48 h (Method B 48 h) in cell-to-cell spread assay (without agarose). (B) CMV-IG ($10 \times EC_{90}$) or CMV monoclonal antibodies (10 μg/ml) were added at $t=0$ or $t=3$ post-infection, agarose was overlaid at $t=3$ h and plaques were detected at 7 days post-infection (Method A). The CMV antibodies were also added at $t=0$ or after 1 h spinoculation at room temperature followed by the application of agarose overlay containing CMV-specific antibodies as above (Method C). Plaques were visualized at 7 days post-infection as described in Materials and Methods.
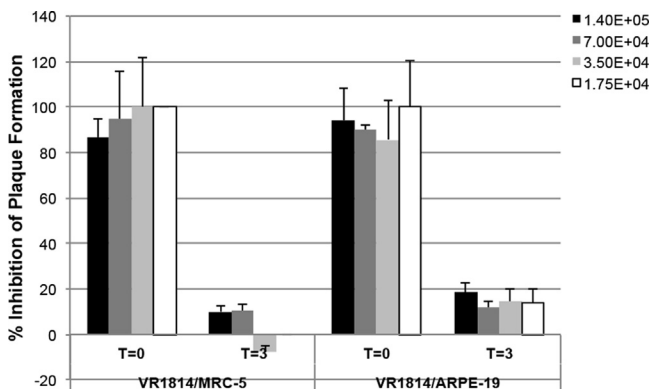
Fig. 4. Activity of CMV monoclonal antibody is not affected by confluency of cells. EV2038 (10 μg/ml) was added at $t=0$ and/or $t=3$ h post-infection with agarose overlay on cells seeded at different densities (cells/well) in PRA.

as measured by plaque development (Tugizov et al., 1996). While no data was presented on cell confluency in the monolayer cultures where inhibition of cell-to-cell spread was reported for CMV antibodies (Navarro et al., 1993; Frenzel et al., 2012; Takada et al., 2012), we found that cell confluency did not influence the inability of a CMV mAb to inhibit plaque formation.

Overall, we performed a thorough validation of our CMV PRA to monitor inhibition of cell-to-cell viral spread and an in-depth comparison to two other methods. Our data confirms several publications in the literature and leads to the conclusion that CMV-specific neutralizing antibodies do not significantly inhibit plaque formation or reduce plaque size when they are added post-infection.

Materials and methods

Cells and viruses

MRC-5 (lung fibroblast) and ARPE-19 (retinal pigmented epithelium) were obtained from ATCC (CCL-171 and CRL-2302, respectively). MRC-5 cells were used between passages 18 and 29 and were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin–Streptomycin (DMEM: Invitrogen 11995; FBS: Hyclone SH30396-03; Penicillin–Streptomycin: Invitrogen, 15140163). ARPE-19 cells were cultured in DMEM/F12 (1:1, Invitrogen, 11320033) supplemented with 10% fetal bovine serum and 1% Penicillin–Streptomycin. All assays are performed using culture media supplemented with 5% FBS and 1% Penicillin–Streptomycin (assay media) to allow an increased infectivity of the virus.

CMV strain AD169 was obtained from ATCC (VR-538). VR1814 was kindly provided by Giuseppe Gerna (Servizio di Virologia, Fondazione IRCCS Policlinico San Matteo, Italy) (Revello et al., 2001). The viruses were propagated on MRC-5 cells for amplification until a complete cytopathic effect was observed. The culture harvest was clarified by centrifugation at 1000g, and titered by immunofocus assay. Briefly, virus was serially diluted 1/10 and used to infect 96-well plates of MRC-5 (3×10^4 cells/well) or ARPE-19 cells (3×10^4 cells/well) for 16–18 h at 37 °C and 5% CO_2. Infected cells were detected with anti-CMV staining and counted as described below in the neutralization assay protocol.

Antibodies

All monoclonal antibody sequences were obtained from Seq IDs in patent applications (see Supplemental Data Table S1 for more details). The DNA was synthesized by a contract research organization. The CMV antibody genes were removed from the commercial vectors by enzymatic digestion; purified, and cloned into IgG expression plasmids. The IgG clones were transfected into HEK293 cells with heavy and light chain DNA clones, using 293fectin (Life Technologies). The cells were harvested 6 days post-transfection for antibody purification. MabSelectSure (GE Healthcare) columns were equilibrated with Dulbecco's PBS. The clarified culture supernatant was loaded onto the equilibrated columns, washed 3X with wash buffer (DPBS/DPBS with 1 M NaCl/DPBS), then eluted with 30 mM sodium acetate pH 3.5. All the eluted fractions were pooled together and the pH was adjusted to ~5 in 60 mM sodium acetate buffer.

Cytogam® was obtained as a Cytomegalovirus Immune Globulin Intravenous solution from CSL Behring (50 mg Ig/ml).

Compounds

Ganciclovir was purchased from Sigma-Aldrich (G5236) and CFI02 was kindly provided by Dr. Lee Fader, Boehringer Ingelheim (Canada) Ltd.

Immunofocus neutralization assay

In a 384-deep well plate, antibodies were serially diluted 2.5 fold in columns 2 to 11 and 14 to 23 in the appropriate assay media. Assay media containing no antibody was added in column 1, 12, 13 and 24. 20 μl of the dilutions was transferred in duplicate to a black 384-well plate with a clear bottom (Greiner, GR781091). CMV was diluted to yield a concentration of 5×10^4 IU/ml and 20 μl of the virus inoculum was added to columns 12 and 24 (0% inhibition) as well as to the wells containing the diluted antibodies. Assay media without virus was added in column 1 and 13 (100% inhibition). This mixture was incubated for 2 h at 37 °C, 5% CO_2. 2.25×10^4 cells/well prepared in 40 ul of assay media were added to the antibody/virus mixture to yield a final 0.04 IU/cell. The plates were briefly centrifuged for 10 s at 233g and incubated for 16 to 18 h at 37 °C, 5% CO_2. The following day, the plates were washed once with PBS and fixed with an acetone/methanol solution (1:1). After a 10 min incubation at room temperature, the fixing solution was discarded and the plates were left to dry for 10 min. The plates were then stained for 90 min with 1 μg/ml of anti immediate-early protein 1 (IE1) Alexa Fluor 488-conjugated antibody (Millipore, MAB810X) in PBS containing 2% normal goat serum (Medicorp, 005-000-121). To control for variations in cell number across wells cells were stained with 2.5 μg/ml of Hoechst 33342 nuclear dye (Invitrogen, H3570). The plates were washed 3 times with PBS and the plates were covered with a Top Seal film (PerkinElmer, 6005185) and fluorescent images were acquired using the Operetta (PerkinElmer) high content screening system as described below.

Plaque reduction assay

Method A

The method is an adaptation of the plaque reduction assay described previously (Landry et al., 2000). Briefly, 1.4×10^5 cells/ well were seeded in a Costar clear 24 well plate (Corning Costar, 3526) in 500 μl of cell culture media and incubated for 18 to 20 h at 37 °C, 5% CO_2. Cells were then infected at an MOI of approximately 0.0008 IU/cell in a total of 250 μl for 3 h at 37 °C, 5% CO_2 on a rocking plate. The inoculum was aspirated and cells were overlaid with a mixture of 600 μl of 0.8% agarose maintained at 45 °C in a water bath and 600 μl of room temperature 2x DMEM +10% FBS containing the appropriate antibody or compound dilution. The plates were then incubated for 6 to 8 days at 37 °C with 5% CO_2 (AD169 on MRC-5: 6 to 7 days; VR1814 on MRC-5 or ARPE-19, 7 to 8 days). After the incubation, the agarose overlays were removed and the cell layers were washed once with PBS before being fixed with acetone/methanol (1:1) for 10 min. The fixing solution was then removed and the plates were left to dry for 2–3 min. The cells were then stained with 1 μg/ml of IE1-Alexa Fluor 488-conjugated antibody in PBS with 2% normal goat serum for 1 h. Following anti-IE1 staining, cells were washed 3 times with PBS and the and the plates were covered with a Top Seal film and fluorescent images were acquired using the Operetta high content screening system as described below.

Method B

The cell seeding and infection was carried out as in method A. However, an agarose overlay was not used to limit extracellular virus propagation. Instead, following the aspiration of the inoculum, fresh assay media was added and antibodies were replenished every 24 or 48 h.

Method C

Two aspects were modified from method A. The infection was carried out by centrifugation at 600g for 60 min at room temperature. The plates were then further incubated for 30 min at room temperature in the presence or absence of antibody before replacing inoculum with the agarose overlay and antibody preparation.

Immunofluorescence data capture

Fluorescent images were acquired for the stained plates using the Operetta high content screening system and images were analyzed using the Harmony software (PerkinElmer). The Harmony software was set to recognize a plaque based on the number of infected nuclei present and a plaque was defined as > 5 adjacent infected nuclei. The IFA neutralization and PRA results were expressed as percent inhibition relative to the untreated uninfected wells (100% inhibition) and untreated infected wells (0% inhibition). The EC_{50} curves were calculated with Assay Explorer (Accelrys) using the best curve fit.

Acknowledgments

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.06.002.

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