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Antibody inhibition of human cytomegalovirus spread in epithelial cell cultures

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Abstract

Anti-cytomegalovirus (CMV) antibodies reduce the incidence of CMV transmission and ameliorate the severity of CMV-associated disease. Neutralizing activity, measured as the ability of antibodies to prevent entry of cell-free virus, is an important component of natural immunity. However, in vivo CMV amplification may occur mainly via spread between adjacent cells within tissues. Thus, inhibition of cell-to-cell spread may be important when evaluating therapeutic antibodies or humoral responses to infection or immunization. In vitro CMV cell-to-cell spread is largely resistant to antibodies in fibroblast cultures but sensitive in endothelial cell cultures. In the present study antibodies in CMV hyperimmunoglobulin or seropositive human sera inhibited CMV cell-to-cell spread in epithelial cell cultures. Spread inhibition activity was quantitated with a GFP reporter assay employing GFP-tagged epithelialtropic variants of CMV strains Towne or AD169. Measurement of spread inhibition provides an additional parameter for the evaluation of candidate vaccines or immunotherapeutics and to further characterize the role of antibodies in controlling CMV transmission and disease.

Keywords

Cytomegalovirus; Antibodies; Spread inhibition; Epithelial cells

1. Introduction

Cytomegalovirus (CMV) infections cause birth defects among newborns infected in utero and morbidity and mortality in transplant and AIDS patients. Naturally acquired immunity to CMV is protective and beneficial (Yeager et al., 1981; Adler et al., 1983, 1995; Nigro et al, 2005). Cellular and humoral immunity are important for controlling CMV disease in transplant and AIDS patients. Recent trials of the glycoprotein B (gB)/MF59 vaccine, believed to act primarily through induction of neutralizing antibodies, suggest a role for antibodies both in preventing primary CMV infections (Pass et al, 2009) and in reducing CMV disease in solid organ transplant patients (Griffiths et al., 2011). In addition, use of CMV hyperimmunoglobulin (IgG isolated from the blood of CMV seropositive donors) in certain transplant settings can ameliorate post-transplant CMV disease (recently reviewed in Hsu and Safdar, 2011) and mounting evidence suggests that CMV hyperimmunoglobulin can be beneficial for prevention and treatment of congenital CMV infections (Nigro et al, 2005; la Torre et al., 2006; Nigro et al., 2008; Adler and Nigro, 2009; Maidji et al., 2010; Adler, 2012; Nigro et al, 2012a, b; Visentin et al., 2012).

Antibodies in CMV hyperimmunoglobulin or human seropositive sera potently neutralize free virus. However, the mediators, mechanisms, and neutralizing targets of CMV entry are cell type specific. Fibroblast entry requires gB and the heterodimer of glycoproteins H and L (gH/gL), whereas entry into endothelial, epithelial, and dendritic cells requires gB and a pentameric complex of gH, gL, UL128, UL130, and UL131A (Hahn et al, 2004; Gerna et al, 2005; Wang and Shenk, 2005b; Adler et al., 2006). Consequently, antibodies directed against gB impair viral entry into fibroblasts, endothelial, and epithelial cells, whereas antibodies that specifically target the pentameric complex potently and selectively block viral entry into epithelial and endothelial cells (Macagno et al, 2010; Saccoccio et al, 2011b; Fouts et al., 2012). Following natural infection the later activity is dominant as serum neutralizing titers measured with epithelial cells are significantly higher than those measured using fibroblasts (Cui et al., 2008; Gerna et al., 2008; Tang et al, 2011; Wang et al., 2011).

That CMV persists in spite of robust humoral responses suggests that in vivo CMV may evade neutralizing antibodies by spreading cell-to-cell. This may be especially relevant in transplant-associated CMV disease where pathogenesis results from viral spread within tissues of affected organs. Consistent of this, antibodies can slow but not prevent CMV spread in cultured fibroblasts (Navarro et al., 1993; Alberola et al., 1999; Sinzger et al, 2007; Jiang et al, 2008; O'Connor and Shenk, 2011; Scrivano et al, 2011). However, as viral entry is cell type-specific, mechanisms of cell-cell spread may also differ between cell types; indeed, recent evidence suggests that cell-cell spread of CMV in endothelial cell cultures is sensitive to antibody inhibition (Maidji et al., 2002; Gerna et al., 2008; Jiang et al., 2008; Scrivano et al, 2011). These observations prompted an investigation of the capacity of antibodies to impair CMV spread within epithelial cell monolayers and to develop quantitative assays to measure antibody-mediated spread inhibition.

2. Materials and methods

2.1. Sera and CMV hyperimmunoglobulin

Sera were obtained from normal healthy adults and assayed for CMV seropositivity by gB-ELISA (Jacobson et al., 2009). Consent was obtained from all subjects and protocols were approved by the Virginia Commonwealth University Committee for the Conduct of Human Research. CMV hyperimmunoglobulin (CytoGam®, CSL Behring, King of Prussia, PA) was purchased from the manufacturer. The 50 mg/ml stock was adjusted to 5 mg/ml with culture medium to approximate the concentration of IgG in human sera; dilutions of CMV hyperimmunoglobulin used in experiments represent dilutions of the 5 mg/ml stock.

2.2. Cells and viruses

Human MRC-5 fibroblasts (ATCC CCL-171), human ARPE-19 epithelial cells (ATCC CRL-2302), and viruses were propagated as described (Cui et al., 2008, 2012; Saccoccio et al., 2011b). Table 1 summarizes viruses used in this study. Virus Uxc was present in urine from a newborn with a symptomatic congenital CMV infection. Virus TS15 is a bacterial artificial chromosome (BAC) cloned virus derived from the Towne vaccine (Cui et al., 2012). BADrUL131-Y4 (BADr) is a variant of strain AD169 in which a mutation in UL131A has been repaired to express a functional UL131A protein (Wang and Shenk, 2005a). Both BADr and TS15 have been modified to express green fluorescent protein (GFP). In BADr a GFP expression cassette was inserted in the *UL21.5* region (Wang et al., 2004), whereas in TS15 a GFP expression cassette is contained within the BAC origin cassette that was inserted between *US28* and *US29* (Cui et al., 2012).

2.3. Isolation of epithelialtropic variants of TS15

ARPE-19 cultures were infected with TS15 (MOI = 1) and maintained 80 days until most of the cells were GFP+. Supernatants were passed to fresh ARPE-19 cultures and after eight days BAC TS15-rN was derived by transformation of infected cell DNA into *Escherichia coli* as described (Cui et al., 2012). Alternatively, a 719-bp fragment containing the wild type strain AD169 *UL130* open reading frame (*UL130^{AD169}*) was PCR amplified from AD169 DNA using primers UL130-1 (ATGCTGCGGCTTCTGCTTCGTCA) and UL130-2 (CGCCGTCAAGAACGGCGTCAG), gel purified, and extracted using the QIAquick kit (Qiagen). Three microgram TS15 BAC DNA were co-transfected with 1 µg *UL130^{AD169}* PCR product into ARPE-19 cells using Effectene (Qiagen) as described previously (Cui et al., 2009). After 60 days DNA was extracted, transformed into *E. coli*, and one BAC clone was selected. Virus reconstituted from this BAC by transfection of ARPE-19s was passaged an additional eight times in ARPE-19s before a final BAC clone designated TS15-rR was derived. Restriction pattern analyses and targeted Sanger dideoxy sequencing similar to that described previously (Cui et al., 2012) were used to identify a *UL130^{AD169}* insertion/7.5-kb deletion in the *UL/b* region of TS15-rR and to determine the presence or absence of the TT mutation at the native *UL130* locus.

2.4. Detection of infected cells and quantitation of viral spread

Uxc-infected cells were detected by immunohistochemical staining of immediate early (IE) antigen 48 h post infection (h.p.i.) as described (Cui et al., 2008). Based on IE staining 48 h.p.i., Uxc entry into ARPE-19s was 25-fold less efficient than entry into MRC-5s (not shown). Thus, in order to obtain cultures in which ~100 cells/well were initially infected, MRC-5 cultures were infected with 100pfu/well, while ARPE-19 cultures were infected with 2500 pfu/well. For GFP-based assays white-wall clear-bottom 96-well plates containing cell monolayers were infected with 50–100 pfu/well of GFP-tagged viruses. Inocula were removed one day post infection (d.p.i.) and cultures were incubated for up to 15 days in 200 µl/well culture medium containing dilutions of CMV hyperimmunoglobulin or human sera. GFP+ cells were photographed using a Nikon Diaphoto 300 inverted fluorescence microscope. Relative fluorescent units (RLU) of GFP were measured for each well using a Biotek Synergy HT Multi-Mode Microplate Reader seven d.p.i. Fifty-percent inhibitory concentration (IC₅₀) values were determined using Prism 5 (GraphPad Software, Inc.) as the infection points of four-parameter curves fitted to plots of mean RLUs (from triplicate wells) vs. log(dilution⁻¹) as described previously (Saccoccio et al., 2011b).

2.5. Neutralization

Human sera were evaluated for neutralizing activity against epithelial entry as described previously (Saccoccio et al., 2011b). Briefly, two-fold serial dilutions of sera in culture medium were mixed with an equal volume of culture medium containing 5000pfu of TS15-rN. After incubation for 1 h at 37°C the mixtures were added to the wells of 384-well plates containing confluent ARPE-19 monolayers. Each serum was assayed in triplicate. RLUs measured seven days post infection were used to calculate IC₅₀ values, reported as neutralizing titers, as described above.

3. Results

3.1. Construction of epithelialtropic variants of strain Towne

CMV antibody assays often use the standard reference strains Towne and AD169. Fibroblast adaptation has rendered both strains incapable of efficient entry and replication in epithelial cells (Wang and Shenk, 2005a; Saccoccio et al., 2011b; Cui et al., 2012) due to mutations that disrupt components of the pentameric complex (UL130 in Towne, UL131A in AD169 (Hahn et al., 2004)). In strain AD169 repair of the *UL131A* mutation resulted in efficient

epithelial entry and replication (Wang and Shenk, 2005a). Subsequently, a GFP-tagged *UL131A*-repaired AD169 variant, BAD Δ UL131-Y4 (BADr, Table 1), facilitated development of assays to quantitate epithelial entry neutralizing activities in CMV hyperimmunoglobulin, serum, and saliva (Cui et al., 2008; Saccoccio et al., 2011a, b). To establish similar epithelialtropic variants in the Towne genetic background, two GFP-tagged epithelialtropic variants, TS15-rR and TS15-rN, were derived from TS15 (Cui et al., 2012), a BAC-cloned virus obtained from the Towne vaccine (see Section 2). TS15-rR contains a wild type *UL130* gene from strain AD169 inserted ectopically in the *UL/b* region, while TS15-rN has a native *UL130* gene that has been restored to wild type (Table 1). Epithelial tropism of TS15-rR and TS15-rN was confirmed by infection of epithelial (ARPE-19) and fibroblast (MCR-5) cell cultures with identical amounts of each virus and monitoring the cultures for GFP expression over time. As shown in Fig. 1, BADr, TS15-rR, and TS15-rN entered and spread within both cell types with equal efficiencies. In contrast, infection of ARPE-19 cells by non-epithelialtropic parental virus TS15 was rare, and spread from infected cells was extremely limited (Fig. 1). Growth curves revealed that supernatants from TS15-infected ARPE-19 cultures peaked at 10^2 pfu/ml, while TS15-rR and TS15-rN achieved peak titers $>10^5$ pfu/ml (not shown).

3.2. Sensitivity of CMV spread to antibody inhibition

To determine the effect of antibodies on CMV cell-cell spread in epithelial cells, ARPE-19 cells in 96-well plates were infected with 100 pfu of each virus. After 24 h the virus inocula were replaced with medium containing serial dilutions of CMV hyperimmunoglobulin (adjusted to 5 mg/ml IgG, comparable to human sera) using TS15-rR, and the cultures were monitored for GFP expression for up to 12 days. As shown in Fig. 2A, CMV hyperimmunoglobulin significantly inhibited the spread of TS15-rR, and while TS15-rN and BADr were also inhibited, the effect was less pronounced. To determine if CMV hyperimmunoglobulin inhibits spread of wild type CMV, this experiment was conducted using urine containing CMV strain Uxc to infect ARPE-19 cultures directly. After 24 h the virus inocula were again replaced with medium containing serial dilutions of CMV hyperimmunoglobulin and on day 15 after infection the cultures were fixed and stained for IE antigen. CMV hyperimmunoglobulin significantly inhibited spread of Uxc in ARPE-19s (Fig. 2A). As predicted by previous reports (Navarro et al., 1993; Sinzger et al., 2007; Jiang et al., 2008, 2011; O'Connor and Shenk, 2011; Scrivano et al., 2011) CMV hyperimmunoglobulin had no apparent effect on spread of viruses in MRC-5 fibroblasts (Fig. 2B).

3.3. Quantitative assays to measure spread inhibition in epithelial cultures

GFP-based assays for quantitating CMV neutralizing activity in CMV hyperimmunoglobulin, serum, and saliva have been described previously (Cui et al., 2008; Saccoccio et al., 2011a). To develop a GFP-based assay to measure spread inhibition activity, the experiment described above was modified to include quantitative measurement of GFP levels using a fluorescent plate reader. The timing of GFP measurement post infection (8–15 days) and the amount of virus in the inocula (50–100 pfu) were important for optimal detection of antibody inhibition. In addition, optimal conditions were specific to each virus (TS15-rR, TS15-rN, and BADr). Fig. 3 illustrates optimized quantitative assays of human sera using TS15-rR (highly sensitive) or BADr (moderately resistant) viruses. Four-parameter curve fitting of GFP values (Fig. 3B and C) was used to calculate IC_{50} concentrations for spread inhibition (shown below images in Fig. 3A). As observed for CMV hyperimmunoglobulin in Fig. 2, TS15-rR was more sensitive to inhibition by serum antibodies than BADr, and this was reflected in lower IC_{50} values for each serum when measured using BADr vs. TS15-rR. Using TS15-rR, spread inhibition titer for CMV hyperimmunoglobulin was 1:839 and lay within the range of IC_{50} values determined for the

seropositive sera (1:1566, 1:528, and 1:312). The IC_{50} values for BADr spread inhibition by the seropositive sera were proportionally lower (1:489, 1:282, and 1:173), reflecting the lower sensitivity of BADr spread to antibody inhibition. Two seronegative sera had only a slight effect on spread at very high concentrations.

The same sera were assayed for neutralizing activity using virus TS15-rN in a GFP-based neutralizing assay (Cui et al., 2008). Although a limited sample size, comparison of spread inhibition titers vs. neutralizing titers (Fig. 4) suggests that at least for convalescent sera from normal healthy adults spread inhibition titers are lower than but roughly correlate with neutralizing titers.

4. Discussion

Humoral immunity reduces the incidence of CMV transmission and ameliorates the severity of CMV-associated pathogenesis in both transplant and congenital infection settings (Nigro et al., 2005, 2008; la Torre et al., 2006; Adler and Nigro, 2009; Pass et al., 2009; Maidji et al, 2010; Griffiths et al, 2011; Hsu and Safdar, 2011; Adler, 2012; Nigro et al., 2012a, b; Visentin et al, 2012). An improved understanding of how antibodies mediate these beneficial effects may be important for development of CMV vaccines or antibody-based therapeutics. Neutralizing activity, measured as the ability of antibodies to prevent entry of cell-free virus, is regarded as an important component of humoral immunity; however, that replication of low passage CMV isolates in vitro is highly cell associated suggests that in vivo CMV amplification may occur mainly via spread between adjacent cells within tissues. Thus, antibody inhibition of CMV spread may be more clinically relevant than neutralization, and assays to quantitate spread inhibition may be important for vaccine and immunotherapeutic development.

Among enveloped viruses, mechanisms of cell-cell transfer have been described that are antibody-sensitive or antibody-resistant (Sherer et al., 2007; Sattentau, 2008; Martin et al., 2010; Mothes et al., 2010). CMV spread between fibroblasts has been reported to occur through microfusion events (Digel et al, 2006). As transferred virions presumably remain cytoplasmic, this mechanism is consistent with observations that cell-cell spread in fibroblasts is largely antibody-resistant (this study, Navarro et al., 1993; Alberola et al, 1999; Sinzger et al, 2007; Jiang et al., 2008, 2011; O'Connor and Shenk, 2011; Scrivano et al, 2011). In contrast, antibodies can inhibit CMV spread in endothelial cells and block virus transfer from endothelial cells to leukocytes (Gerna et al., 2000, 2008; Maidji et al., 2002; Jiang et al., 2008; Scrivano et al., 2011). The present study indicates that cell-cell spread within epithelial cells is also antibody-sensitive, although different viruses exhibited different degrees of sensitivity. Why different strains, such as BADr and TS15-rR, or variants of the same strain, such as TS15-rR and TS15-rN, differ in sensitivity to antibody-mediated spread inhibition is not known. For TS15-rR and TS15-rN it may relate to UL130 expression levels or kinetics since the *UL130* genes are in different genomic locations. Interestingly, these two viruses also differ somewhat in their tropism properties: TS15-rN enters ARPE-19 cells more efficiently than TS15-rR, while TS15-rR appears to spread more rapidly (data not shown). Together, these observations suggest that spread within epithelial cultures may occur by a mixture of antibody-sensitive and antibody-resistant mechanisms which are utilized in different proportions by different viruses. Importantly, evaluation of virus derived from clinical material confirmed that antibody-sensitive cell-cell spread in epithelial cells is a feature of naturally occurring CMVs.

Because CMV epithelial entry is more sensitive than fibroblast entry to neutralizing antibodies in CMV hyperimmunoglobulin or seropositive sera (Cui et al., 2008), it could be argued that the sensitivity of epithelial spread vs. insensitivity of fibroblast spread to

antibodies is merely a manifestation of this difference in neutralizing potency. However, the differential inhibition of spread in the two cell types is maintained even when dilutions predicted to have equivalent neutralizing potency are used. For example, because the neutralizing activity of CMV hyperimmunoglobulin is 60-fold higher when measured using epithelial cells vs. fibroblasts (Cui et al., 2008), the neutralizing potency of a 1:4 dilution against fibroblast entry should be equivalent to the neutralizing potency of a 1:256 dilution against epithelial entry. However, a 1:256 dilution of CMV hyperimmunoglobulin inhibited viral spread in epithelial cells (Fig. 2A) while a 1:4 dilution failed to inhibit spread in fibroblasts (Fig. 2B). Thus, these data support a genuine mechanistic difference (one sensitive to antibodies, one resistant) for epithelial vs. fibroblast spread.

Quantitative assays of spread inhibition were feasible using GFP as a reporter and may be valuable for evaluating therapeutic antibodies or humoral responses to natural infection or experimental immunizations. While spread assays described here were conducted in 96-well format, adaptation to 384-well format, currently used for GFP-based neutralizing assays (Saccoccio et al, 2011a), should be feasible. Three hundred and eighty-four-well format assays require smaller sample volumes (particularly important for mouse studies) and are amenable to high-throughput platforms. In addition, by evaluating the effects of monospecific antibodies it should be possible to identify viral mediators of cell-cell spread, and indeed, specific epitopes. This will be important, as targets and epitopes that mediate virus neutralization may not precisely match those involved in inhibiting cell-cell spread. Defining these epitopes for both pathways could be important for development of vaccines or therapeutic antibodies.

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Fig. 1. Evaluation of epithelial-tropic strain Towne variants. Fibroblast (MRC-5) or epithelial (ARPE-19) cell monolayers in 96-well plates were infected with 100 pfu of the indicated GFP-tagged viruses and representative micrographs were taken with an inverted UV microscope 10–12 (MRC-5) or 14–17 (ARPE-19) days post infection.

Fig. 2.

CMV hyperimmunoglobulin inhibition of CMV spread. (A) ARPE-19 cell monolayers in 96-well plates were infected with 100pfu/well GFP-tagged viruses (TS15, TS15-rR, TS15-rN, BADr) or with urine containing 2500pfu/well Uxc virus (empirically determined in advance to result in approximately 100 initially infected cells per well (see Section 2.4)). After 24 h the inocula were replaced with either medium (∅) or medium containing CMV hyperimmunoglobulin (HIG) at the indicated dilutions. Viral spread was determined by staining for IE antigen (Uxc) on day 15 p.i. or by GFP fluorescence on day 12p.i. (others). (B) MRC-5 cell monolayers in 96-well plates were infected with 50 pfu/well GFP-tagged viruses or with urine containing 50 pfu/well Uxc virus. After 24 h the inocula were replaced

with either medium (Ø) or medium containing CMV hyperimmunoglobulin diluted 1:4 in medium. Viral spread was determined by staining for IE antigen (Uxc) or by GFP fluorescence on day 12 p.i.

Fig. 3.

Quantitative assays of spread inhibition. (A) ARPE-19 cells in 96-well plates were infected for 24 h with 60 pfu/well and after 24 h the inocula were replaced with medium alone ($\emptyset$) or medium containing 4-fold serial dilutions of human seropositive sera (pos-1, pos-2, pos-3) or seronegative sera (neg-1, neg-2). Representative micrographs were taken and GFP values for each well were measured 12 d.p.i. for TS15-rR and 14 d.p.i. for BADr. (B) the means of GFP values from triplicate wells were plotted vs. $\log(\text{serum dilution}^{-1})$ and fitted to four-parameter curves to calculate IC₅₀ values.

Fig. 4. Comparison of neutralizing and spread inhibition titers. Epithelial entry neutralizing titers for the human seropositive sera assayed in Fig. 3 (pos-1, pos-2, pos-3) were determined using TS15-rN and compared with the spread inhibition titers obtained in Fig. 3.

Table 1

Virus strains and variants used in this study.

Virus strain	Variant	Mutations impacting the pentameric complex	Epithelial tropism
Uxc	Urine	ND ^a	ND ^a
AD169	BADr	UL131A-repaired	+
Towne	TS15	UL130-mutated	–
	TS15-rR	UL130 ^{AD169} -ectopic insertion	+
	TS15-rN	UL130-repaired	+

^aND, not determined.