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Antibody inhibition of human cytomegalovirus spread in epithelial cell cultures

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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 hyperimmuneglobulin 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.

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 hyperimmuneglobulin (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 hyperimmuneglobulin 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 hyperimmuneglobulin 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

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develop quantitative assays to measure antibody-mediated spread inhibition.

2. Materials and methods

2.1. Sera and CMV hyperimmuneglobulin

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 hyperimmuneglobulin (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 hyperimmuneglobulin 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 (Quiagen). Three microgram TS15 BAC DNA were co-transfected with 1 μg $UL130^{AD169}$ PCR product into ARPE-19 cells using Effectene (Quiagen) 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 U_L/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 100 pfu/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 hyperimmuneglobulin 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 (IC_{50}) 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 5000 pfu 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 IC_{50} 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 *UL131*A-repaired AD169 variant, BADrUL131-Y4 (BADr, Table 1), facilitated development of assays to quantitate epithelial entry neutralizing activities in CMV hyperimmuneglobulin, 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 U_L/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,