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Development of a High-Throughput Assay To Measure the Neutralization Capability of Anti-Cytomegalovirus Antibodies

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Infection by human cytomegalovirus (CMV) elicits a strong humoral immune response and robust anti-CMV antibody production. Diagnosis of virus infection can be carried out by using a variety of serological assays; however, quantification of serum antibodies against CMV may not present an accurate measure of a patient's ability to control a virus infection. CMV strains that express green fluorescent protein (GFP) fusion proteins can be used as screening tools for evaluating characteristics of CMV infection in vitro. In this study, we employed a CMV virus strain, AD169, that ectopically expresses a yellow fluorescent protein (YFP) fused to the immediate-early 2 (IE2) protein product (AD169$_{IE2-YFP}$) to quantify a CMV infection in human cells. We created a high-throughput cell-based assay that requires minimal amounts of material and provides a platform for rapid analysis of the initial phase of virus infection, including virus attachment, fusion, and immediate-early viral gene expression. The AD169$_{IE2-YFP}$ cell infection system was utilized to develop a neutralization assay with a monoclonal antibody against the viral surface glycoprotein gH. The high-throughput assay was extended to measure the neutralization capacity of serum from CMV-positive subjects. These findings describe a sensitive and specific assay for the quantification of a key immunological response that plays a role in limiting CMV dissemination and transmission. Collectively, we have demonstrated that a robust high-throughput infection assay can analyze the early steps of the CMV life cycle and quantify the potency of biological reagents to attenuate a virus infection.

The coevolution of human herpesviruses with their hosts over the past millions of years has led to the development of complex strategies of immune evasion that allow persistent viral infection despite the presence of an active immune response (1). A comprehensive understanding of cytomegalovirus (CMV) infection is essential to delineate the molecular and cellular interactions necessary for priming a targeted humoral immune response and how a pathogen may coopt these processes to establish a persistent and lifelong infection (2). Furthermore, a more complete comprehension of CMV entry, replication, and immune evasion is paramount in developing strategies to diagnose and alleviate CMV disease in immunocompromised patients such as transplant recipients, AIDS patients, and neonates.

Analysis of viral protein expression during CMV infection can be useful in studying viral entry, cellular manipulation, and egress. The replication cycle of CMV is temporally controlled and regulated by different segments of the viral genome. The replicative cycle is divided into immediate-early (IE), early (E), and late (L) phases of replication. CMV IE proteins are produced first and appear within 6 h postinfection (hpi). IE proteins are potent transactivators that stimulate the transcription of E genes (3, 4). IE1 and IE2 are the best-characterized IE gene products and are essential for viral replication and controlling downstream transcription factors (5–8).

Recombinant green fluorescent protein (GFP)-expressing virus strains have contributed to our understanding of the contribution of viral genes to CMV replication and dissemination (9), including viral latency (10) and cell cycle manipulation (11). Such strains have also been used to examine the kinetics and localization of viral protein expression during CMV and herpes simplex virus 1 (HSV-1) infections (12). Viral strains that express fluorescent proteins alone or as a viral chimera are also useful for measuring virus infectivity, testing the antiviral properties of small molecules and the neutralizing capability of monoclonal anti-CMV antibodies, and elucidating early steps in viral binding and entry (13–15).

In this study, we employed a CMV strain that ectopically expresses a yellow fluorescent protein (YFP) fused to the IE2 transcript, AD169$_{IE2-YFP}$, to establish a high-throughput cell-based assay that can quantify viral entry into human cells by measuring fluorescence of infected cells. The high-throughput format of the assay offers an easy and rapid approach for evaluating viral growth kinetics and, as we demonstrate, can be employed to test the virus-neutralizing capability of monoclonal antibodies and human serum from CMV-positive patients. The infection readout of YFP fluorescence can assist in elucidating the early steps of the CMV life cycle and can be utilized for identification of anti-CMV therapeutics.

MATERIALS AND METHODS

Cells and antibodies. MRC5 lung fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal bovine serum, 1 mM HEPES, 100 U/ml penicillin, and 100 g/ml streptomycin at 37°C in a humidified atmosphere (95% air–5% CO_2). The monoclonal anti-gH antibody (clone 14-4b) was purified from hybridoma culture supernatant (16). Monoclonal antibody HC-10 (anti-HC) (recognizes free major histocompatibility complex [MHC] class I heavy chains) (17) was purified from hybridoma culture supernatant. For HCMV IE1 and IE2 detection, a primary mouse monoclonal antibody against a shared epitope present in IE1 and IE2 was used (MAB810; Millipore). Actin was detected by using a primary goat polyclonal IgG against human actin (sc-2005; Santa Cruz Biotechnology, Inc.).

Cloning of recombinant viruses. CMV $AD169_{IE2-YFP}$ was constructed in the CMV AD169 background (18) by inserting enhanced YFP (Clontech) into the 3′ end of IE2 exon 5 in the parent AD169 bacterial artificial chromosome (BAC), as previously described (19–21). The following IE2-targeting primers were used (sequences in uppercase type are the homology arms to the IE2 sequence): 5′-CTGAGCCTGGCCATCGA GGCAGCCATCCAGGACCTGAGGAACAAGTCTCAGgccggaagaagatg gaaaaag-3′ (forward) and 5′-ACGGGGAATCACTATGTACAAGAGTCCA TGTCTCTCTTTCCAGTTTTTTCACcgtcgtggaatgccttcg-3′ (reverse). The CMV_{GFP} control virus (22) encodes a simian virus 40 (SV40) promoter-enhanced GFP cassette. The CMV Δcrs IE2-YFP strain was constructed by BAC "recombineering" (23) of the CMV IE2-YFP strain, as previously described (24). The integrity of all BAC recombinant viruses was verified by (i) restriction digestion using HindIII and EcoRI to verify banding patterns (New England BioLabs), (ii) direct sequencing of the recombineered locus and 1,000 bp in each direction, (iii) replication kinetics of the resulting virus, and (iv) YFP or GFP expression. To propagate and purify virus, BAC DNA was electroporated (19) into MRC5 cells (American Type Culture Collection) by using a GenePulser Xcell electroporation system (Bio-Rad). Upon infection reaching a 100% viral cytopathic effect or 100% GFP, the culture supernatant was collected and filtered with a 0.2-μm filter (Corning, Inc.). Titers of virus stocks were determined by the 50% tissue culture infectious dose ($TCID_{50}$) and converted to PFU/ml (25).

Replication kinetics. MRC5 cells were seeded into cell culture plates and maintained for 1 to 2 days until cells reached confluence at $\sim 5 \times 10^4$ cells per well in 24-well tissue culture plates. The monolayers were then infected with recombinant CMV IE2-YFP or CMV_{GFP} as a control. Cells were infected at a multiplicity of infection (MOI) of 1. Inoculum was prepared by using virus stock diluted in culture medium and adsorbed onto cells in a volume of 200 μl for 1 h at 37°C in a humidified CO_2 incubator. The inoculum was then removed and replaced with 1 ml of fresh medium. The amount of infectious virus used to prepare the inoculum was based on plaque assay titrations (26) of virus stocks and is shown as time point zero in each figure. At each time point, three separate sample wells were collected. Infectious cell-free virus was collected by harvesting medium from infected culture wells at the indicated time points and stored at −80°C. To measure replication, samples were thawed and prepared as a 10-fold serial dilution series in culture medium and then analyzed by $TCID_{50}$. The results were then converted to PFU/ml. Error ranges were calculated by using standard deviations.

Quantitative Western blot analysis. MRC5 cultures at $\sim 60\%$ confluence were infected at an MOI of 1 with previously described strains or recombinants of CMV. Adsorption was done at 4°C to synchronize entry of virus. After adsorption, cells were washed once in phosphate-buffered saline (PBS) (Mediatech, Inc.), and fresh medium was added at 1 ml per well. A zero-time-point sample was collected as a control for viral protein levels that could potentially be carried over from the inoculum. Cells were then placed into a 37°C incubator. Samples were collected every 2 h over 20 to 24 consecutive hours or every 1 h over 20 consecutive hours where indicated. For sample collection, cell monolayers were rinsed in PBS, and cells from each well were scraped into 50 μl of radioimmunoprecipitation

assay (RIPA) buffer and stored at −80°C. For analysis, samples were thawed, and total protein content was quantitated by using a modified Lowry assay (Bio-Rad DC protein assay kit). Equivalent amounts of total protein (10 μg) were added to appropriate volumes of 4× sample loading buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 1% beta-mercaptoethanol, 12.5 mM EDTA, and 0.02% bromophenol blue) and heated at 95°C for 5 min. Protein samples were loaded and separated on precast SDS-PAGE 10% or 7.5% bisacrylamide gels (Bio-Rad). Protein transfer and blot preparation were handled as previously described (26). For detection of bands, the blot was incubated with the respective primary and secondary antibodies, followed by incubation with chemiluminescence substrate from the Western Lightning ECL detection kit (NEN/Perkin-Elmer).

Virus preparation and infection. The $AD169_{IE2-YFP}$ virus was propagated in MRC5 cells and purified by density gradient centrifugation by spinning at 20,000 rpm at room temperature for 1.5 h over a 20% sorbitol cushion. Infectious-virus yield was assayed on human fibroblasts by $TCID_{50}$. MRC5 cells were infected with $AD169_{IE2-YFP}$ for 1 h at the respective MOIs, with agitation every 15 min at 37°C.

Human serum. Human blood was obtained from subjects from a study supported by an NIH-NIAID-funded contract (contract no. HHSN266200500028C) that studied immune changes during the course of pregnancy. All subjects gave informed consent, and the IRB included the use of samples for CMV research.

Confocal microscopy. Neonatal human dermal fibroblast (NHDF) cells were seeded overnight, infected (MOI of 5), and then fixed at 7 hpi with BD Biosciences Cytofix/Cytoperm solution (45 min at 4°C). Cells were stained by using Hoechst reagent (25 μg/ml) and washed with PBS, and data were collected for nuclear staining and YFP fluorescence by using a Molecular Devices ImageXpress Ultra (IXU) plate-scanning confocal microscope. Images were analyzed by using MetaExpress software.

Flow cytometry analysis. Flow cytometry was performed as described previously (27). In brief, MRC5 cells were plated at 250,000 MRC5 cells/well and incubated overnight at 37°C, followed by $AD169_{IE2-YFP}$ infection (MOI of 5). Cells were harvested by trypsinization, washed with PBS, and subjected to flow cytometry using a Cytomics FC 500 flow cytometer (Beckman Coulter). The data were quantified by using Flow Jo software (Tree Star, Inc.) and plotted as a normalized cell number versus the YFP fluorescence signal. The percentage of infected cells was determined by gating mock-infected cells as "negative" and all events with a fluorescent signal above mock as "positive."

Neutralization assay. $AD169_{IE2-YFP}$ was preincubated with or without the respective antibody (50-μl total inoculum volume for 2.5 h at 4°C). The inoculum was then added to MRC5 cells (5,000 to 15,000 cells/well) in a 96-well plate. After 1 h, the virus/antibody inoculum was removed and replaced with 100 μl DMEM. The plate was then read in an Acumen ^{e}X3 laser scanning fluorescence microplate cytometer to measure YFP fluorescence levels for up to 17 hpi. Serum samples were diluted with DMEM prior to virus incubation (total, 50 μl). For studies in which antibody was preincubated with cells, the anti-gH antibody was preincubated with cells for 2.5 h. Following incubation, cells were washed twice with DMEM and then exposed to virus inoculum.

Fluorometric analysis by use of an Acumen ^{e}X3 laser scanning fluorescence microplate cytometer. Cells were excited with a 488-nm laser, and shot thresholding was used to determine average background levels (shot thresholding tracks the midpoint of the background noise and sets a dividing line above this to separate data from noise). Fluorescent emission above background levels by 2 standard deviations was registered as a positive signal. To delineate between autofluorescence caused by particulate from cells or cell culture medium and emission from YFP, any fluorescent signal larger than 5 μm, smaller than 300 μm, and separated from any other emission by at least 0.5 μm in both x and y axes was considered to be an "event." One event was assumed to be equivalent to one infected cell, and thus, the number of infected cells in a given well was extrapolated from the fluorescence emission data. Background autofluorescence was

adjusted for by subtracting the mean number of events in noninfected wells from that in each infected well. Percent infection was determined by dividing the number of events in each well by the number of events in AD169$_{IE2-YFP}$-infected control wells (no antibody).

ELISA CMV diagnostic test. Patient sera were banked and stored at −80°C for use in CMV enzyme-linked immunosorbent assays (ELISAs). CMV IgG and CMV IgM ELISAs (Calbiotech) were performed according to the manufacturer's instructions. Freshly thawed sera (10 μl) were diluted 1:20 and incubated on ELISA plates precoated with the CMV pp65 protein. Plates were washed before and after the addition of enzyme conjugate. TMB (3,3′,5,5″-tetramethylbenzidine) substrate and stop solution were added before reading at 450 and 600 nm in a plate reader (Bio Tek, Winooski, VT).

Statistical analysis. Student's unpaired, two-tailed t tests and 2-way analysis of variance (ANOVA) were performed by using GraphPad Prism.

RESULTS

Expression kinetics and intracellular localization of IE2-YFP in AD169$_{IE2-YFP}$-infected cells. The expression of CMV gene products occurs in a tightly regulated cascade of immediate-early (IE), early (E), and late phases of replication. CMV immediate-early transcripts are produced within 6 h postinfection (hpi). IE proteins act as potent transactivators to stimulate transcription of E genes, which function primarily to replicate viral genomic DNA and alter host immune recognition (3, 4). The temporal nature of the CMV immediate-early phase of infection was exploited to study the events that permit virus entry. More specifically, a variant of the CMV AD169 strain that expresses a chimeric fusion of the IE2 protein product with enhanced yellow fluorescent protein (YFP) (AD169$_{IE2-YFP}$) (Fig. 1A) was utilized to measure a CMV infection. We examined the expression kinetics of the IE2-YFP chimeric protein in cells infected with AD169$_{IE2-YFP}$ compared to an AD169 strain expressing GFP (AD169$_{GFP}$) (Fig. 1B). Human fibroblast (MRC5) cells infected (multiplicity of infection [MOI] of 1) with AD169$_{IE2-YFP}$ and AD169$_{GFP}$ for up to 22 h were subjected to immunoblot analysis using antibodies against IE1/IE2 and actin. The IE1, wild-type IE2, and IE2-YFP fusion proteins were observed at 6 hpi, and their levels increased over 22 hpi (Fig. 1B, top). The expression kinetics of IE2-YFP were similar to those of the wild-type IE2 protein from AD169$_{GFP}$-infected cells (Fig. 1B, top). More importantly, the IE2-YFP polypeptide migrated more slowly than the IE2 protein, confirming the expression of the chimeric IE2-YFP construct. Actin levels verified equivalent protein loading (Fig. 1B, bottom). Also, the IE2-YFP construct did not alter the growth kinetics of AD169$_{IE2-YFP}$ (see Fig. S1 in the supplemental material). Collectively, these results demonstrate that IE2-YFP is expressed with immediate-early kinetics similar to those of wild-type IE2 and does not interfere with viral replication.

We next analyzed the intracellular localization of IE2-YFP signal in MRC5 cells infected with AD169$_{IE2-YFP}$ using confocal microscopy (Fig. 1C). MRC5-infected cells (MOI of 5) were fixed at 7 hpi and stained with Hoechst reagent to identify the nucleus. The YFP fluorescent signal was observed exclusively in the nucleus of cells and coincided with Hoechst staining. This exclusive nuclear localization pattern of IE2-YFP is consistent with previous reports characterizing the nuclear localization of IE2 (28–30). Together, the data demonstrate that IE2-YFP expression acts like its wild-type immediate-early counterpart.

Analysis of fluorescent signal from AD169$_{IE2-YFP}$-infected cells. To further characterize IE2-YFP expression, the kinetics of the IE2-YFP fluorescent signal from AD169$_{IE2-YFP}$-infected cells
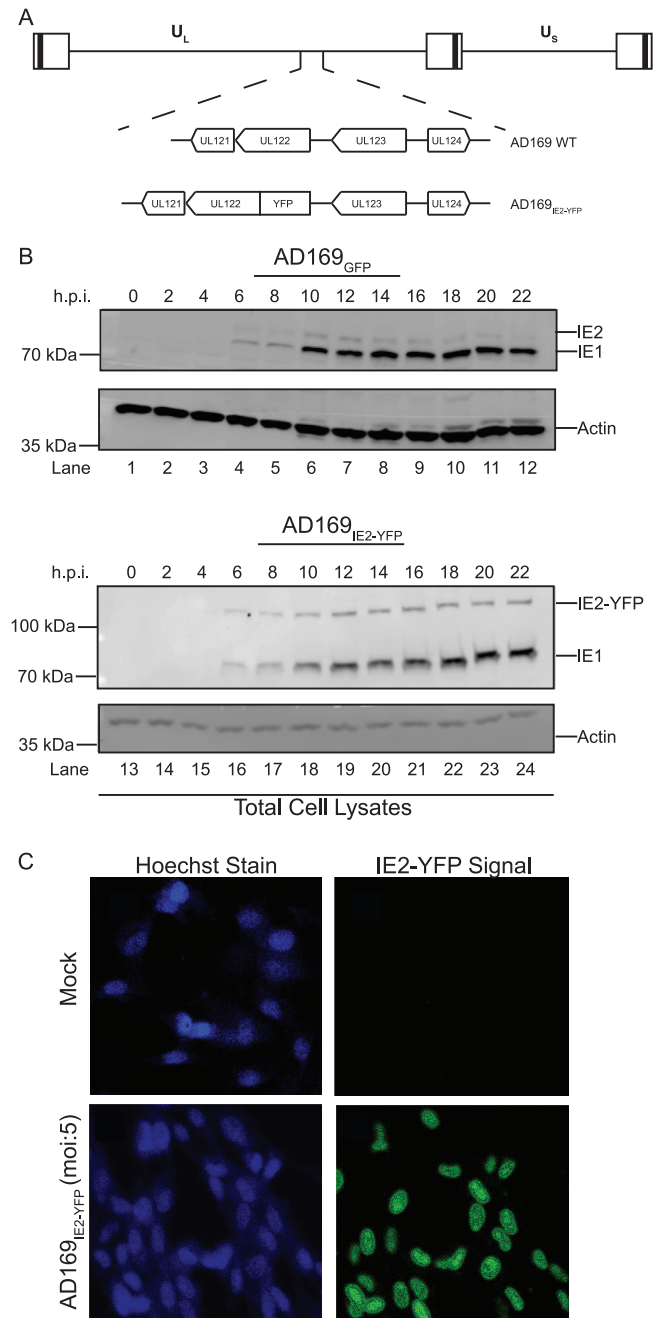
FIG 1 AD169$_{IE2-YFP}$-infected cells express IE2-YFP as an immediate-early protein. (A) Diagram of the recombinant AD169$_{IE2-YFP}$ virus. CMV unique long (UL) and unique short (US) regions of the genome, the orientation of the UL121-124 genes, and the UL122-YFP chimera are indicated. (B) AD169$_{GFP}$-infected (lanes 1 to 12) and AD169$_{IE2-YFP}$-infected (lanes 13 to 24) (MOI of 1) MRC5 cells were harvested at between 0 and 22 hpi and subjected to immunoblot analysis for IE1 and IE2 polypeptides and actin protein. The respective polypeptides and relative molecular mass markers are indicated. (C) Mock- and AD169$_{IE2-YFP}$-infected MRC5 cells (MOI of 5) were stained with Hoechst reagent and analyzed by using confocal microscopy (magnification, ×20). The Hoechst reagent and IE2-YFP signal localized exclusively to the nucleus.

were examined by using flow cytometry (Fig. 2A). Cells were infected (MOI of 5) and analyzed for fluorescence signals at 6, 12, and 24 hpi. YFP expression peaked by 12 hpi and remained through 24 hpi. Two distinct peaks were observed in the flow
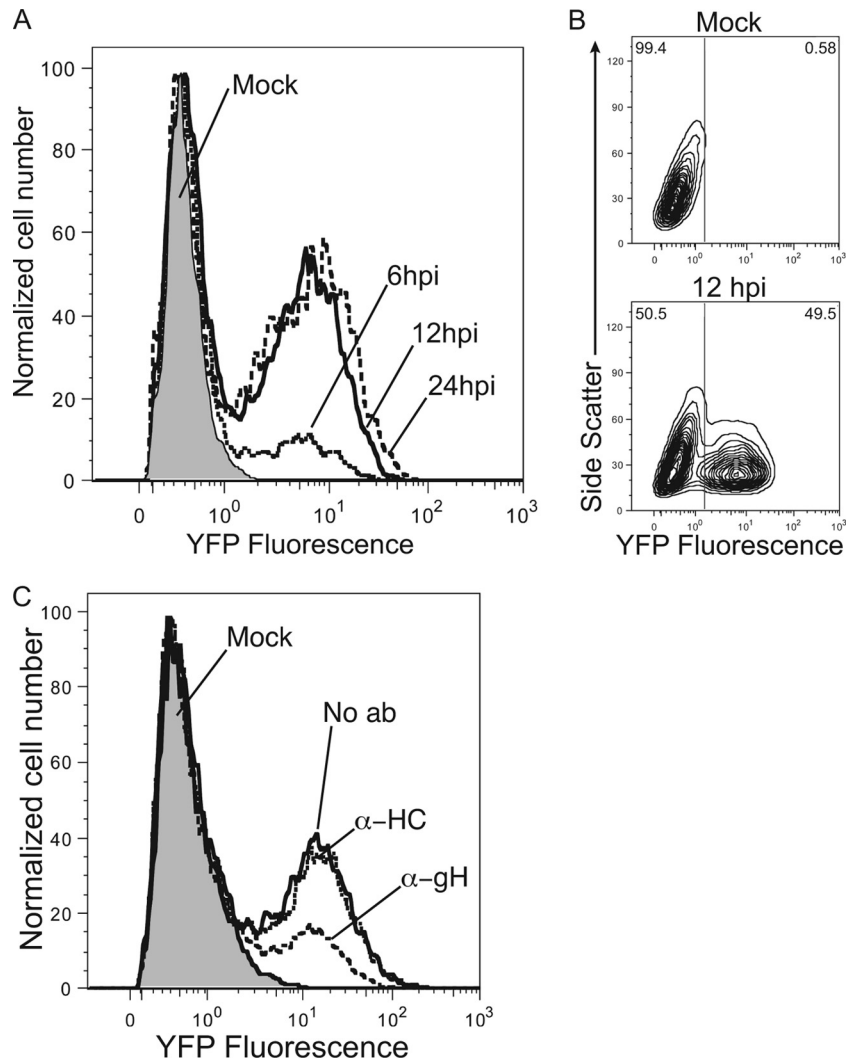
FIG 2 AD169$_{IE2-YFP}$-infected cells emit a robust fluorescent signal. (A and B) Mock- and AD169$_{IE2-YFP}$-infected MRC5 cells (MOI of 5) analyzed by flow cytometry at 6, 12, and 24 hpi for YFP fluorescent signal are represented by a histogram (A) or a contour plot (12 hpi only) (B). (C) AD169$_{IE2-YFP}$-infected cells (MOI of 3) preincubated with 4.0 μg/ml anti-gH, anti-MHC class I heavy chain (HC), or no antibody (No ab) were analyzed by flow cytometry (20 hpi) for YFP signal. Mock-infected cells are indicated by a gray peak.

cytometry plot, allowing quantification of virus-infected and non-infected cells. An increase of the fluorescence signal was observed for AD169$_{IE2-YFP}$-infected cells at between 6 hpi and 12 hpi, indicating that about 50% of the cells were IE2-YFP positive (Fig. 2B). The results demonstrate that AD169$_{IE2-YFP}$ infections can be quantified through the fluorescent signal from IE2-YFP expression.

We next determined whether AD169$_{IE2-YFP}$-infected cells could be employed in an antibody-mediated neutralization assay. Virus was preincubated with a well-characterized neutralizing antibody against CMV glycoprotein gH (anti-gH) (mAb14-4b) (16). As a control, we utilized a monoclonal antibody against the human MHC class I heavy chains (anti-HC) (17). AD169$_{IE2-YFP}$ preincubated with antibodies (4 μg/ml) was used to infect MRC5 cells, which were subsequently examined by flow cytometry. The flow cytometry results demonstrated that preincubation with anti-gH caused a significant decrease in the fluorescent signal of virus-infected cells compared to virus preincubated with anti-HC

(Fig. 2C). Quantification of the fluorescent signal from virus-infected cells revealed that preincubation of anti-gH antibody exhibited a dose-dependent neutralization and showed maximal neutralization at 8.0 μg/ml, while the anti-HC antibody did not exhibit a significant dose-dependent neutralization (data not shown). These data provide the proof of concept that AD169$_{IE2-YFP}$-infected cells can be utilized to measure the neutralizing capacity of anti-CMV antibodies.

Quantification of CMV neutralization using an anti-CMV monoclonal antibody in a high-throughput assay. We next established conditions to quantify AD169$_{IE2-YFP}$-infected cells using a laser scanning fluorescence microplate cytometer in a high-throughput format. The YFP fluorescence was measured from AD169$_{IE2-YFP}$-infected (MOI of 2 or 5) MRC5 cells (5,000 cells/well) (Fig. 3). Numerous cells emitting a fluorescent signal were visualized in virus-infected wells (Fig. 3A). Analysis of the fluorescent intensity of infected cells revealed a Gaussian distribution of fluorescent signals that coalesced within an infected cell, demon-
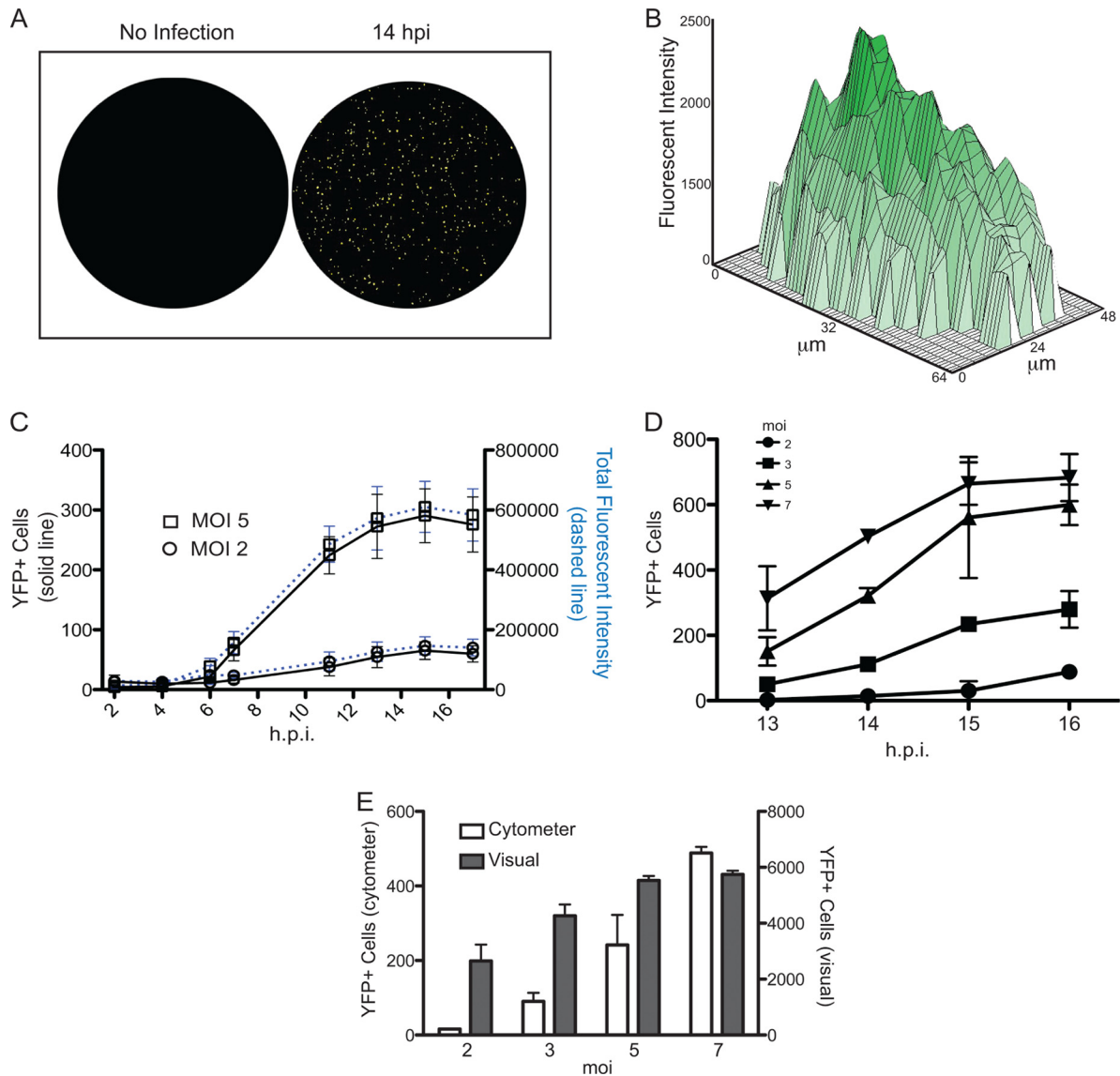
FIG 3 Characterization of AD169$_{IE2-YFP}$-infected cells. (A and B) Mock- or AD169$_{IE2-YFP}$-infected MRC5 cells (MOI of 5) analyzed with an Acumen ^{e}X3 laser scanning fluorescence microplate cytometer demonstrated points of intense fluorescence signal (A) distributed throughout the infected-cell nucleus (B). (C) The fluorescence signal from AD169$_{IE2-YFP}$-infected cells (MOI of 2 or 5) up to 17 hpi was measured by using a microplate cytometer. The number of YFP-positive cells per well (left axis) and the total fluorescence intensity in each well (right axis) were plotted over the course of infection. (D) Furthermore, the number of YFP-positive cells per well from AD169$_{IE2-YFP}$-infected cells at between 13 and 16 hpi at an MOI of 2, 3, 5, or 7 was measured by using a microplate cytometer. The 2-way ANOVA test showed a significant effect at all MOIs and time points tested ($P < 0.05$). (E) The number of YFP-positive cells from AD169$_{IE2-YFP}$-infected cells (MOIs of 2, 3, 5, and 7) at 14 hpi was measured by using a fluorescence cytometer (left axis) or by visual counting using a wide-field fluorescence microscope (right axis). Error bars represent standard errors of means.

strating an intense fluorescent signal throughout the nucleus (Fig. 3B). This punctate localization corresponded well with the nuclear localization observed in confocal microscopy images of AD169$_{IE2-YFP}$-infected cells (Fig. 1C). The quantification of the fluorescent signal was extrapolated to determine the number of infected cells/well. YFP signal increased above background levels by 6 hpi and continued to increase until 14 hpi. To confirm that measuring the number of virus-infected cells by fluorescence events was valid, the total fluorescence intensity per well was determined (Fig. 3C, right axis). Remarkably, a dramatic increase in the number of YFP-positive cells and total fluorescence intensity was quite consistent and dependent on the MOI. We then conducted infections in wells seeded at 15,000 cells per well at various MOIs (2 to 7) (Fig. 3D). The number of YFP-positive cells corresponded well with MOIs. To validate the number of fluorescent cells detected by the cytometer, we compared the number of YFP-positive cells visible by wide-field microscopy to the cytometer value (Fig. 3E). As with the cytometer readout, the number of cells with visible YFP expression corresponded well with the MOI. Thus, quantification of the fluorescent signal from AD169$_{IE2-YFP}$-infected cells provides a robust readout to measure CMV infection that is an accurate proxy for visual assessment of infection.

Furthermore, we utilized the newly developed high-throughput AD169$_{IE2-YFP}$ infection assay to measure the ability of anti-
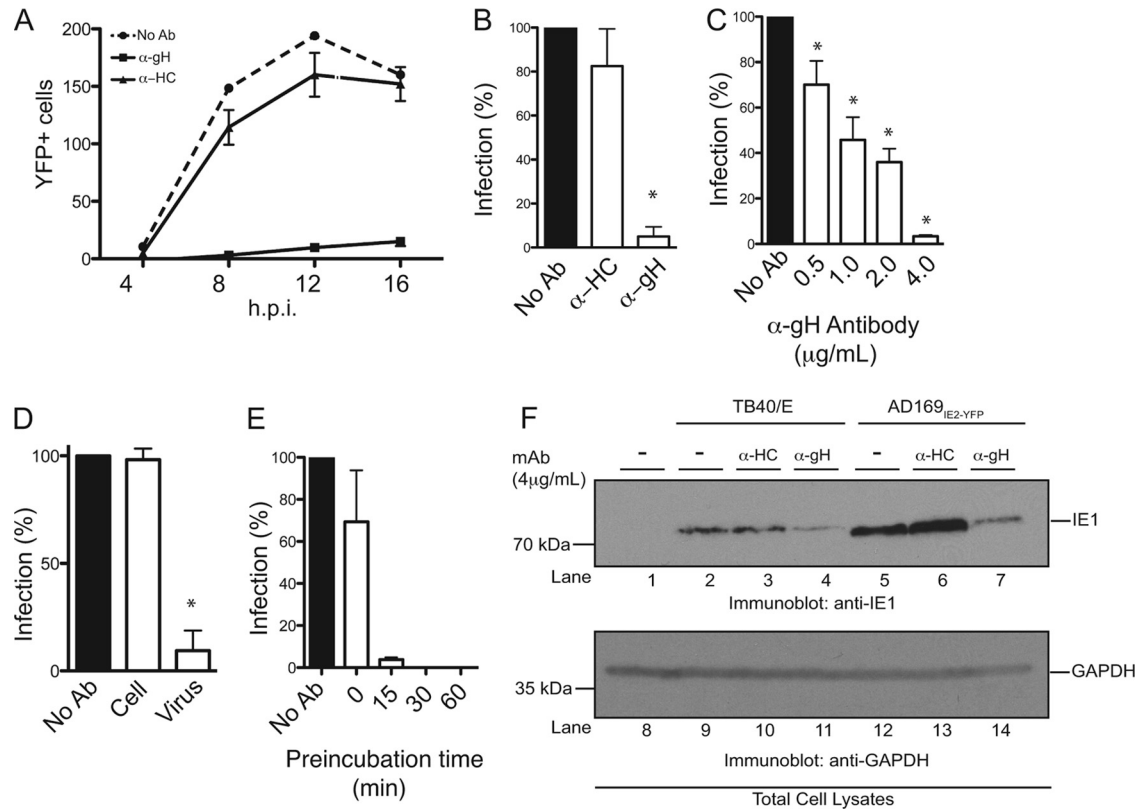
FIG 4 Establishment of a high-throughput HCMV neutralization assay. (A) MRC5 cells infected with AD169$_{IE2-YFP}$ (MOI of 5) preincubated with anti-gH, anti-MHC class I heavy chain (HC), or medium were analyzed at 4, 8, 12, and 16 hpi for the number of YFP-positive cells per well by using a microplate cytometer. (B to E) The percentage of infected cells (12 hpi) was calculated by using the number of YFP-positive cells from AD169$_{IE2-YFP}$-infected cells as 100%. Student's t test confirmed statistical significance (*, $P < 0.05$), and error bars represent standard errors of means in all experiments (B to E). (C) AD169$_{IE2-YFP}$-infected cells preincubated with various concentrations (0.5 to 4.0 μg/ml) of anti-gH or anti-HC antibody or medium were analyzed (12 hpi) to determine the optimal neutralization concentration of anti-gH antibody. (D) MRC5 cells infected with AD169$_{IE2-YFP}$ (MOI of 5) and pretreated with anti-gH antibody or cells pretreated with anti-gH prior to infection were analyzed (12 hpi) to validate the specificity of the anti-gH antibody. (E) MRC5 cells infected with AD169$_{IE2-YFP}$ (MOI of 5) and pretreated with anti-gH antibody for 0, 15, 30, and 60 min were analyzed (12 hpi) to measure the minimal time required for neutralization. (F) MRC5 cells infected with TB40/E or AD169$_{IE2-YFP}$ (MOI of 2) preincubated with anti-gH or anti-HC antibody (4 μg/ml) were subjected to immunoblot analysis (12 hpi) for IE1 (lanes 1 to 7) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (lanes 8 to 14) proteins. The respective polypeptides and relative molecular mass markers are indicated.

CMV antibodies to neutralize virus infection. MRC5 cells infected with AD169$_{IE2-YFP}$ alone or preincubated with the anti-gH and anti-HC antibodies were analyzed for YFP-positive cells for up to 16 hpi (Fig. 4A). Cells infected with the AD169$_{IE2-YFP}$/anti-gH inoculum exhibited very low YFP fluorescence postinfection compared to cells infected with AD169$_{IE2-YFP}$ alone or AD169$_{IE2-YFP}$/anti-HC antibody (Fig. 4A). Quantification of the fluorescence signal at 12 hpi revealed that the AD169$_{IE2-YFP}$/anti-gH inoculum decreased the infection by >95% compared to the slight decrease in the fluorescent signal observed for AD169$_{IE2-YFP}$/anti-HC-infected cells (Fig. 4B). We verified the dose dependency of anti-gH-mediated neutralization in the high-throughput assay. As expected, the anti-gH antibody exhibited dose-dependent neutralization at concentrations of between 0.5 and 4.0 μg/ml (Fig. 4C). To validate the specificity of the high-throughput neutralization assay, cells were preincubated with anti-gH antibody, washed thoroughly, and then infected with AD169$_{IE2-YFP}$ (Fig. 4D). The anti-gH antibody reduced infection only when preincubated with virus and not cells. Finally, we investigated the effect of anti-gH preincubation times on AD169$_{IE2-YFP}$ infection (Fig. 4E). Anti-gH was incubated with a virus inoculum between 0 and 60 min prior

to infection (Fig. 4E). Strikingly, a significant reduction was observed with only a 15-min preincubation, and a complete block of infection was observed with 30- and 60-min preincubations. We further validated the fluorescence-based neutralization assay by immunoblotting. Cells were infected with two CMV strains that were incubated with anti-HC or anti-gH antibody as described above and harvested at 12 hpi (Fig. 4F). For both virus strains, expression of the CMV gene product IE1 was significantly reduced when virus inoculum was incubated with anti-gH antibody but not anti-HC antibody. Collectively, these results demonstrate that we have established a robust, high-throughput, fluorescence-based neutralization assay to quantify the neutralizing ability of anti-CMV antibodies and possibly other biological reagents.

Analysis of CMV neutralization by human serum using a high-throughput-based fluorescent assay. We examined the efficacy of our assay to measure the neutralization capability of human serum from CMV-positive donors. CMV infection elicits a robust anti-CMV antibody response by the humoral branch of the immune system. Neutralizing antibodies to CMV are important for controlling CMV disease in bone marrow transplant recipients
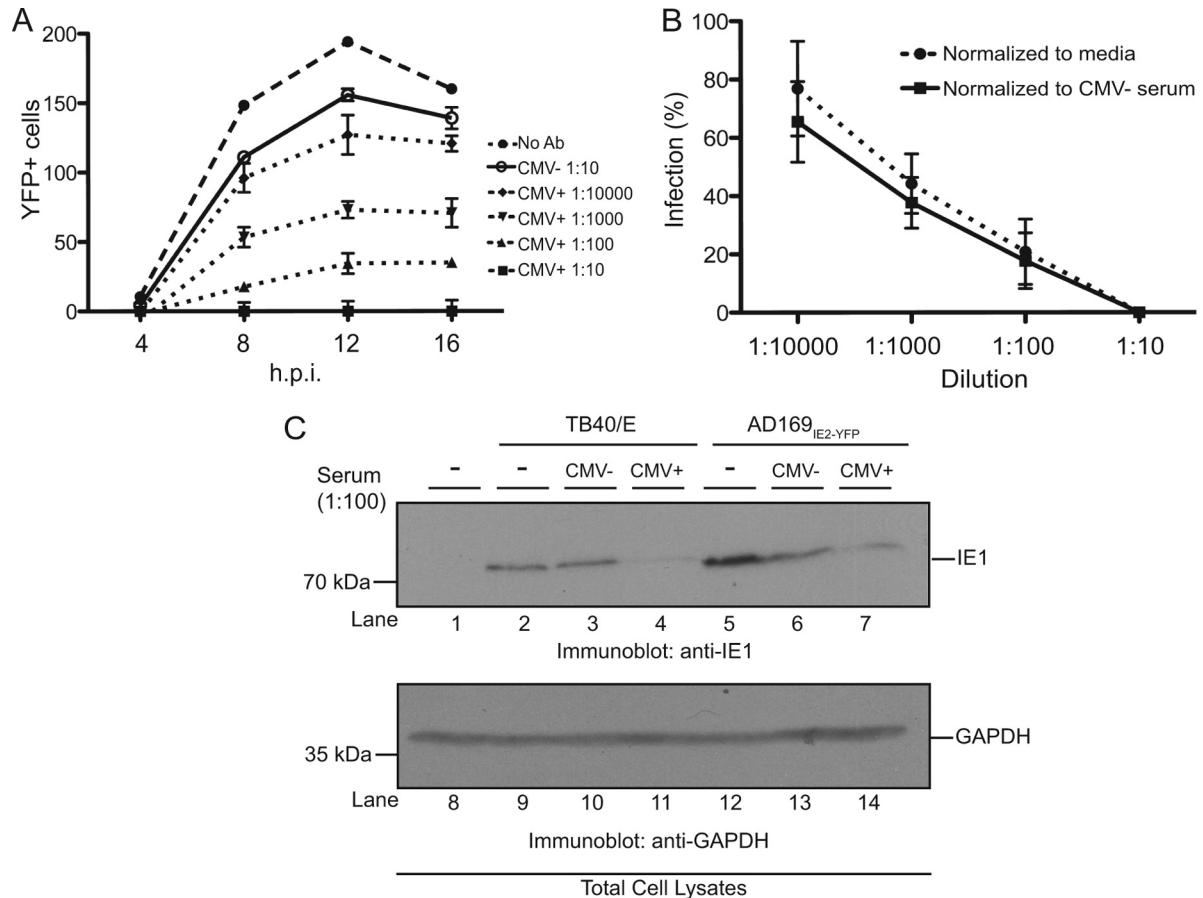
FIG 5 Serum from a CMV-positive donor neutralizes AD169$_{IE2-YFP}$ infection. (A) MRC5 cells infected with AD169$_{IE2-YFP}$ (MOI of 5) and pretreated with medium or human serum at various dilutions were analyzed with a microplate cytometer at 4, 8, 12, and 16 hpi for the number of YFP-positive cells. The 2-way ANOVA test showed significance at all dilutions compared to CMV-negative serum ($P < 0.05$). Error bars represent standard errors of means. (B) The percentage of AD169$_{IE2-YFP}$-infected cells treated with CMV-positive serum (12 hpi) was expressed as percent infection compared to cells infected with inoculum incubated with medium or inoculum incubated with CMV-negative serum. (C) MRC5 cells infected with TB40/E or AD169$_{IE2-YFP}$ (MOI of 2) preincubated with CMV-positive serum were subjected to immunoblot analysis (12 hpi) for IE1 (lanes 1 to 7) and GAPDH (lanes 8 to 14) proteins. The respective polypeptides and relative molecular mass markers are indicated.

(31) and solid-organ transplant recipients (32, 33) as well as for potentially reducing virus transmission in newborn infants exposed to infectious CMV (34). To analyze the presence of CMV-reactive antibodies in human serum, a CMV-positive donor and a CMV-negative donor were identified by using an ELISA-based assay that employed a virus-coated plate and colorimetric IgG detection (data not shown). Sera from these individuals were tested in our neutralization assay (Fig. 5A). Four serum dilutions of between 1:10 and 1:10,000 were tested. Serum from the CMV-positive donor exhibited effective neutralizing capability at all dilutions in a dose-dependent manner, while serum from the CMV-negative donor only slightly decreased YFP fluorescence at the highest concentration. To ensure that the observed neutralization capacity of the CMV-positive serum was not due to nonspecific interactions, the number of infected cells from the AD169$_{IE2-YFP}$/CMV-positive reaction was normalized to the numbers of AD169$_{IE2-YFP}$-infected cells incubated with medium and CMV-negative serum, respectively (Fig. 5B). In both cases, incubation with CMV-positive serum was found to significantly and specifically reduce infection in a dose-dependent manner. We validated these results by immunoblotting. Cells were infected with either

TB40/E or AD169$_{IE2-YFP}$ inoculum that was incubated with serum from a CMV-negative donor or serum from a CMV-positive donor. Infected cells were harvested at 12 hpi (Fig. 5C). In cells infected with either virus strain, the expression level of the CMV gene product IE1 was significantly reduced when virus inoculum was incubated with CMV-positive serum but not CMV-negative serum. We conclude that our neutralization assay can be employed to measure the CMV-neutralizing activity of human serum.

How does the CMV-neutralizing capability of human serum compare to anti-CMV immunoglobulin levels? Serum samples from four CMV-positive donors and one CMV-negative donor were tested for the presence of anti-CMV pp65 IgG and IgM levels by using a commercially available diagnostic kit (Fig. 6A). Sera from three donors were found to contain high titers of anti-CMV IgG that ranked above the cutoff for a "CMV-positive" designation. Donor 4 ranked just below the cutoff for the CMV-positive designation. To determine whether the various levels of IgG were due to a primary or recurrent infection, we measured the levels of anti-CMV IgM (Fig. 6A). Interestingly, donor 4 exhibited IgM levels above the cutoff for the CMV-positive designation, possibly
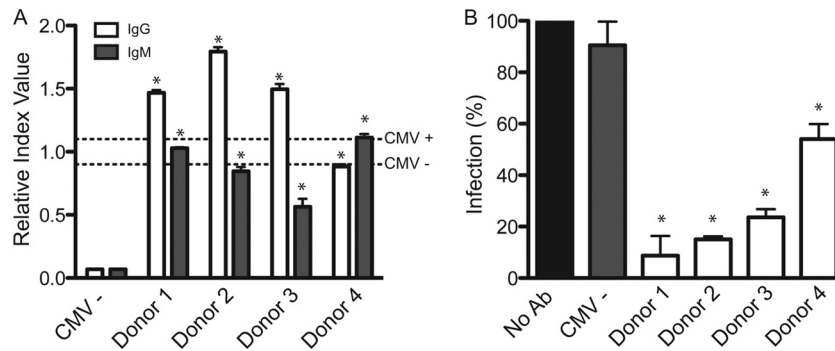
FIG 6 Analysis of the ability of sera from different donors to neutralize AD169$_{IE2-YFP}$ infection. (A) Anti-CMV IgG and IgM levels from sera of five human donors were determined by using a commercial ELISA-based anti-CMV kit. Cutoff values for CMV-positive and CMV-negative donors, as recommended by the manufacturer, are indicated with dashed lines. Student's t test showed significance of IgG and IgM levels in all samples compared to the CMV-negative control (*, $P < 0.05$). (B) AD169$_{IE2-YFP}$-infected MRC5 cells (MOI of 5) preincubated with serum (1:100 dilution) from all five donors were analyzed (14 hpi) by using a microplate cytometer to determine YFP-positive cells. The percentage of infected cells was calculated by using AD169$_{IE2-YFP}$-infected cells as 100%. Student's t test showed significance in all samples compared to the CMV-negative control (*, $P < 0.05$). Error bars represent standard errors of means.

suggesting that donor 4 recently experienced a primary or recurrent infection. Collectively, the data provide an overall "snapshot" of the anti-CMV immunoglobulins from various donors.

Sera from the four donors and one CMV-negative donor were preincubated with the AD169$_{IE2-YFP}$ inoculum at a 1:100 dilution, and the inoculum was then used to infect MRC5 cells. The fluorescence signal from AD169$_{IE2-YFP}$-infected cells was quantified by using nontreated AD169$_{IE2-YFP}$-infected cells as 100% (Fig. 6B). Virus-neutralizing activity was detected in the sera from all CMV-positive donors, resulting in 8.73% ± 13.5%, 15.01% ± 0.82%, 23.63% ± 2.22%, and 54.03% ± 4.12% infection for donors 1 to 4, respectively. The relative levels of CMV-reactive antibodies corresponded well with the neutralizing capability of serum from donors 1 to 3. Importantly, the neutralization assay identified donor 4 to be capable of substantially neutralizing a CMV infection despite the ELISA results that ranked donor 4 as borderline negative for anti-CMV antibodies. These results suggest that the high-throughput AD169$_{IE2-YFP}$ neutralization assay would provide a robust readout that directly measures CMV-neutralizing antibodies in human serum. In summary, the neutralization assay offers a rapid and quantitative assessment of the neutralizing capability of both monoclonal antibodies directed at CMV and human serum. The assay was optimized for use in a high-throughput format that requires minimal material and allows thorough time course analysis of viral infection, as measured by IE2-YFP signals. Our assay can provide an immunological parameter that assesses a patient's capacity to control CMV proliferation.

DISCUSSION

In this study, we employed a recombinant CMV virus, AD169$_{IE2-YFP}$, that expresses a fluorescently tagged IE2-YFP protein to visualize fluorescent expression and quantify virus infectivity. We established conditions for an antibody neutralization assay that uses YFP expression as a quantitative readout. Finally, we adapted our assay for use in a high-throughput format that uses minimal material to quantify virus infection with a rapid turnover. There are many potential applications in which the fluorescent readout of AD169$_{IE2-YFP}$ infection could be employed. One such example is high-throughput screening for identifying compounds or antibodies that attenuate or block infection as well as for diagnostic purposes in which the neutralizing capability of human serum can

be tested in a rapid and accurate assay. Indeed, a recent report demonstrated the usefulness of CMV strains that express fluorescent fusion proteins for such applications (13).

The high-throughput AD169$_{IE2-YFP}$ neutralization assay could be utilized to evaluate the neutralizing capability of human serum from patients at high risk for CMV-associated diseases. For example, determining the neutralizing ability of serum from an immunocompromised patient may indicate whether an individual has CMV-neutralizing immunoglobulins, possibly due to a bout of CMV reactivation. Indeed, neutralizing antibodies have been proposed to play critical roles in protective immunity in the case of CMV pneumonitis (35) and may thus serve as critical indicators of a reactivated CMV infection. Remarkably, the assay (Fig. 5A and 6B) requires only 0.5 μl of human serum, which could likely be obtained from a finger prick. Sera from patients who had tested positive for CMV by ELISA displayed significant neutralizing capability compared to serum from a CMV-negative donor. Relative levels of CMV neutralization corresponded to levels of CMV-reactive IgG, as measured by a commercial diagnostic ELISA kit. One donor (donor 4) displayed approximately 60% infection (or 40% neutralizing capability), while the ELISA-based diagnostic kit ranked the same donor as just below the cutoff for CMV positivity. Thus, our assay is a sensitive evaluation of the ability of an individual's serum to neutralize a CMV infection and a method to survey the overall health of the patient's humoral immunity. A potential extension of our assay would be to test the neutralizing capability of antibodies found in saliva and urine. For example, a recent report demonstrated the presence of CMV-neutralizing antibodies in saliva and indicated an important distinction in neutralizing capability between fibroblast and epithelial cells (36).

One limitation of the assay developed here is that it employs a laboratory CMV strain that lacks the UL128-131 region, known to be essential for pH-dependent membrane fusion in endothelial and epithelial cells (37–40). Our assay could thereby "miss" the detection of neutralizing antibodies against this glycoprotein complex. Indeed, anti-UL130 and -UL131 antibodies can block CMV entry into mucosal epithelial cells (41). However, CMV infects fibroblasts, such as those utilized in this assay, by fusion at the cell surface in a gH/gL/UL128-131-independent fashion (42). Additionally, serum-mediated neutralization measures a polyclonal

antibody response that is unlikely to be directed against only one glycoprotein complex (43, 44). Furthermore, neutralizing antibodies against the gB glycoprotein, present in AD169, are very common in CMV-seropositive individuals. One study found neutralizing antibodies to gB in 86% of 600 CMV-positive individuals (45). As several CMV vaccine strategies utilize the gB protein as an immunogen, our assay could be ideal for assessing vaccine efficacy (46). We show here (Fig. 5C) that serum from a CMV-positive individual is capable of neutralizing both a clinical (TB40/E) and a laboratory (AD169$_{IE2\text{-}YFP}$) CMV strain. A clinical CMV strain that employs a similar YFP chimera or a reporter cell line capable of infection by various CMV strains (47) may be ideal for quantifying infection under a variety of conditions.

The transmission of CMV from mother to child is a result of a primary or recurrent maternal CMV infection and carries a risk of transmission of between 24 and 75% (48–50). Anti-CMV IgG avidity tests are a reliable determinant of primary versus secondary CMV infection (51–54). Combining the results from avidity and neutralization tests could strengthen the diagnostic value of these assays alone and identify patients with a higher risk of CMV transmission, potentially secondary to poor virus-neutralizing activity. A longitudinal study that could correlate virus-neutralizing antibodies with the postulated gestational age at which intrauterine transmission occurred could identify parameters that result in a high risk of transmission (55). Naturally, these studies could extend beyond maternal and congenital CMV infections to transplant recipients and patients who suffer from chronic immune-regulatory disorders such as vascular and autoimmune diseases as well as chronic immunosuppression (56).

CMV can produce noninfectious particles termed dense bodies (DB) and noninfectious enveloped particles (NIEPs) (57–60). Importantly, CMV-positive human sera react to these noninfectious particles (61), thereby demonstrating their antigenic properties. In fact, most commercially available test kits for detection of anti-CMV antibodies contain large amounts of DB in the antigen preparation (62). Previous studies indicated that the neutralization capacity of human sera is not influenced by the presence of noninfectious envelope glycoproteins, even when added at a 100-fold excess (63). Thus, only virus-neutralizing antibodies and not ELISA-reactive antibodies should be associated with protection. Our assay offers the unique advantage of measuring the protective quality of a patient's antibody response. Furthermore, the high-throughput format will allow investigators to assay large numbers of patient samples to quantify the protective response with sufficient numbers of patients to allow statistically meaningful results.

In conclusion, we developed a high-throughput assay utilizing a recombinant CMV that expresses a fluorogenic viral protein to measure the neutralizing capability of antibodies directed against the CMV virion and human serum. The assay significantly reduces the amount of material needed for analysis and measures data from multiple time points during viral replication to demonstrate IE2 expression kinetics during a lytic CMV infection. Furthermore, our high-throughput, fluorescence-based assay will be useful in the future for characterizing the neutralizing capability of human patient cohorts as well as investigating the biology of the humoral immune response toward a CMV infection in various disease states.

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