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Research paper

Novel microneutralization assay for HCMV using automated data collection and analysis

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Abstract

In addition to being sensitive and specific, an assay for the assessment of neutralizing antibody activity from clinical trial samples must be amenable to automation for use in high-volume screening. To that effect, we developed a 96-well microplate assay for the measurement of HCMV-neutralizing activity in human sera using the HCMV-permissive human cell line HEL-299 and the laboratory strain of HCMV AD169. The degree to which neutralizing antibodies diminish HCMV infection of cells in the assay is determined by quantifying the nuclei of infected cells based on expression of the 72 kDa IE1 viral protein. Nuclear IE1 is visualized using a highly sensitive immunoperoxidase staining and the stained nuclei are counted using an automated ELISPOT analyzer. The use of Half Area 96-well microplates, with wells in which the surface area of the well bottom is half the area of a standard 96-well microplate plate, improves signal detection compared with standard microplates and economizes on the usage of indicator cells, virus, and reagents. The staining process was also streamlined by using a microplate washer and data analysis was simplified and accelerated by employing a software program that automatically plots neutralization curves and determines NT₅₀ values using 4-PL curve fitting.

The optimized assay is not only fast and convenient, but also specific, sensitive, precise and reproducible and thus has the characteristics necessary for use in measuring HCMV-neutralizing activity in the sera of vaccine trial subjects such as the recipients of Vical's HCMV pDNA vaccine candidates.

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Keywords: Microneutralization assay; Human cytomegalovirus; ELISPOT analyzer

Abbreviations: AEC, 3-amino-9-ethylcarbazole; BBS, borate buffered saline; CV, coefficient of variation; DAB, 3,3'-diaminobenzidine; EC₅₀, fifty percent effective concentration; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunospot; FBS, fetal bovine serum; gB, glycoprotein B; GPS, guinea pig serum; GPSC, complement-active guinea pig serum; HCMV, human cytomegalovirus; IEA, immediate early antigen; IE1, immediate early antigen 1; kDa, kilodalton; mAb, monoclonal antibody; NA, neutralization assay; NSC, negative serum control; NT₅₀, fifty percent neutralizing titer; PBS, phosphate buffered saline; pDNA, plasmid DNA; PSC, positive serum control; TMB, 3,3',5,5'-tetramethyl benzidine; 4-PL, four parameter logistical; %N, percent neutralization.

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1. Introduction

Humoral immunity plays a significant role in the control of HCMV infections and disease in man in that HCMV-neutralizing antibodies interfere with the receptor-mediated virus entry into susceptible cells. HCMV gB has been shown to be a major target of neutralizing antibodies induced by naturally acquired infection (Britt et al., 1990; Gonczol et al., 1991; Marshall et al., 1992). Although antigen binding ELISAs can measure antibody specific for HCMV antigens such as the gB protein, only an assay in which neutralization of viral entry into cells is measured can establish and quantify the biological activity of HCMV antigen specific antibodies. Our goal was to develop a HCMV micro-neutralization assay that is suitable for screening a large number of serum samples from vaccine clinical trials. Vical is currently testing HCMV pDNA vaccine candidates encoding the ectodomain of gB from AD169. This pDNA vaccine has been shown to induce specific antibody responses, as measured by ELISA, in animals (Selinsky et al., 2005) and humans (Wloch, in preparation).

The quantification of HCMV-neutralizing antibodies and titration of HCMV stocks often relies on tedious and long (10–14-day) plaque assays based on the cytopathic effect of the virus (Lantto et al., 2001; Andreoni et al., 2002; Nejatollahi et al., 2002; Mitchell et al., 2002). The availability of mAbs specific for the early gene products of HCMV has enabled the development of assays that can detect HCMV infection much faster (Gleaves et al., 1984; Griffiths et al., 1984; Alpert, 1985). The first overnight assay to detect HCMV infection or measure its neutralization used 24-well cluster plates with fibroblasts as indicator monolayers and a commercial mAb specific for HCMV IEA coupled with immunoperoxidase staining to detect cells infected with the HCMV lab strain AD169 or clinical CMV isolates (Chou and Scott, 1988). The first rapid HCMV neutralization assay developed for 96-well microplates (Andreoni et al., 1989) used immunofluorescent staining of infected cells for IEA and required manual counting of stained nuclei under a fluorescence microscope. Although the microplate format enables simultaneous testing of multiple samples and the assay endpoint can be reached within 24 h, the manual counting of the labeled images makes this technique slow and tedious and thus limited to small scale use.

Using IEA expression-based detection in a microplate format, we have developed a very sensitive and specific immunohistochemical staining procedure which has enabled us to quantify infected cells with

an ELISPOT analyzer. Automating the data analysis, by employing curve fitting software, has further streamlined the assay and made the determination of neutralizing activities simple, quick, and objective. We further improved the assay by selecting the optimal assay plate type, a robust indicator cell line, and the optimum assay conditions for better distinction between neutralizing and non-neutralizing sera.

The results of our extensive qualification runs show that our novel HCMV microneutralization assay has the specificity, sensitivity, linearity, precision and reproducibility that is expected from a cell-based bioassay intended for use in clinical trials.

2. Materials and methods

2.1. Cells and media

MRC-5 (CCL-171), HEL-299 (CCL-137) and WI-38 (CCL-75) human embryonic fibroblast-like cell lines were obtained from American Type Culture Collection (ATCC; Manassas, VA) and cultivated in ATCC's Minimal Essential Medium supplemented with 10% FBS (HyClone, Logan, UT).

2.2. Infectious virus

Gradient-purified infectious HCMV AD169 was purchased from Advanced Biotechnologies Incorporated (ABI; Columbia, MD). The virus stock (1.8×10^{11} virus particles/ml) was diluted 1/10 with FBS and stored in 100 μ l aliquots at -80°C until use.

2.3. Sera

Human sera, coded for anonymity, were obtained from healthy adult HCMV-seronegative and seropositive individuals including HCMV-seronegative subjects from Vical's Phase 1 clinical trials who developed serum anti-gB IgG responses as a result of vaccination with CMV DNA vaccines and from Vical in-house volunteers. Informed consent was obtained from all subjects after the nature and possible consequences of the studies had been fully explained. Pooled AB serum (AB pool) was purchased from Pel-Freez Biologicals (Rogers, AR). Sera were classified as HCMV-seropositive or HCMV-seronegative based on their reactivity in a commercial HCMV IgG ELISA (Diamedix, Miami, FL) and were further analyzed for gB-specific serum IgG levels in an indirect gB-binding ELISA developed at Vical (Wloch, unpublished data). Negative sera as well as sera with gB ELISA titers ranging from 100 to 51,000

were chosen for neutralization analysis. All sera with anti-gB IgG titers of at least 800 were from naturally HCMV-seropositive individuals whereas the sera with titers of 100–400 were from originally HCMV-seronegative vaccinees. An NSC was prepared by combining sera from three non-vaccinated HCMV-seronegative individuals. Serum from a single volunteer donor with a gB ELISA titer of 12,800 was used for PSC. All sera were stored in aliquots at $-20\text{ }^{\circ}\text{C}$ until use. Freeze-dried GPSC, purchased from Equitech-Bio, Inc. (Kerrville, TX), was stored at $-80\text{ }^{\circ}\text{C}$ and reconstituted before use.

2.4. Substrates

The precipitating peroxidase substrates HistoMark Black (cobalt enhanced DAB), StableDAB (stabilized two-component DAB), and TrueBlue (a buffered solution of TMB and H_2O_2) were purchased from KPL (Gaithersburg, MD) whereas AEC was purchased from Vector Laboratories (Burlingame, CA).

2.5. Neutralization assay

The day before initiating an assay, cells were prepared at 200,000 cells/ml in RPMI/HEPES (GIBCO/Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Hyclone, Logan, UT) and seeded at either 20,000 cells per well into standard well-size NUNC brand or at 10,000 cells per well into Corning/Costar brand Half Area flat bottom 96-well tissue culture microplates (both from Fisher Scientific, Pittsburgh, PA).

On day one, test sera were serially diluted two-fold six-to-eight times with RPMI/HEPES medium containing $1\times$ PSF (100 U/ml penicillin G, 100 $\mu\text{g/ml}$ streptomycin sulfate, 0.25 $\mu\text{g/ml}$ amphotericin B; GIBCO/Invitrogen, Carlsbad, CA) and either 10% FBS or 10% GPSC. Fetal bovine serum, GPSC, and human control and test sera were used with or without heat inactivation of endogenous complement activity, at $56\text{ }^{\circ}\text{C}$ for 30 min, as noted. Virus stock was thawed on ice, gently resuspended, diluted in RPMI/HEPES containing $1\times$ PSF and 10% FBS, and 100 μl per well was added to the serially diluted human sera for incubation at $37\text{ }^{\circ}\text{C}$ for 1–4 h. Virus was diluted to yield from several hundred to 1500 infected cells per well in the absence of neutralizing activity. The culture medium was then carefully aspirated from the assay plates, replaced with an aliquot of the pre-incubated virus/serum mixture, and the plates were incubated at $37\text{ }^{\circ}\text{C}$ overnight (14–20 h) unless noted otherwise.

On day two, the medium was aspirated from the cells well-by-well, one assay plate at a time. The cells were fixed with 150 μl per well of absolute ethanol for 20 min at room temperature, rehydrated in the same amount of PBS for at least 10 min, blocked with 5% normal goat serum (NGS) or normal donkey serum (NDS; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA,) in PBS for 30 min, and incubated with 0.1 $\mu\text{g/ml}$ of anti-IE1 monoclonal antibody MAB810 (clone 8B1.2; Chemicon, Temecula, CA) for 1 h. Following washing of the primary antibody, a 1/1000 dilution of biotin-labeled (or for direct staining, peroxidase labeled) goat- or donkey-anti mouse IgG (Jackson ImmunoResearch Labs) was added for 30 min followed by addition of 2 $\mu\text{g/ml}$ HRP-Avidin D (Vector Labs) or HRP-Streptavidin (KPL) (this step was omitted in direct staining) for 30 min. Following washing, a peroxidase substrate was added (TrueBlue; AEC, StableDAB or HistoBlack were used for comparison) for 8–15 min. Between each staining step, the assay plates were washed 3 times with wash buffer [PBS or borate buffered saline (BBS) containing 0.01% TWEEN-20] using a microplate washer (Ultrawash Plus, Dynex Technologies, Chantilly, VA). All antibodies and the HRP conjugates were diluted in 1/20 strength Casein Blocker in PBS (Pierce, Rockford, IL). Fixation, staining, and washing were carried out at room temperature, whereas the substrate was pre-warmed to $37\text{ }^{\circ}\text{C}$ for optimal performance. Special care was taken to prevent the cell monolayer from drying out both before and after alcohol fixation. Stained plates were rinsed with distilled water followed by absolute ethanol, and then air dried and stored in the dark until scanning.

2.6. Data capture and analyses

Stained plates were scanned and counted with an ImmunoSpot Analyzer from C.T.L. (Cellular Technologies Ltd, Cleveland, OH) using the red color system. Replicate data points were averaged and %N relative to cells infected with HCMV in the presence of NSC was calculated according to the following formula:

$$\%N = (1 - \text{Mean Infected Cells}_{\text{Test}} / \text{Mean Infected Cells}_{\text{NegControl}}) \times 100.$$

Neutralization curves were automatically plotted in Prism 4 graphing software (GraphPad Software, San Diego, CA) using 4-PL curve fitting constrained to 0% at the bottom and to 100% at the top and the NT_{50} value was determined from each curve. The fifty percent neutralizing titer or NT_{50} (EC_{50} in PRISM 4) is defined

by the midpoint or inflection point of the sample-specific neutralization curve. The program also reports the goodness of fit (R^2 value) for the curve and the 95% confidence interval for EC_{50} . For samples assayed after predilution, NT_{50} equals EC_{50} multiplied by the predilution factor. The global fitting feature in the PRISM software was used to statistically compare neutralization curves and corresponding EC_{50} (NT_{50}) values for two or more data sets.

The optimized final assay protocol used HEL-299 cells, Half Area 96-well microplates, and the AD169 strain of HCMV at an input that yielded approximately 1000 infected cells per well. Test and control human sera as well as the FBS used in the assay medium were heat-inactivated and the assay medium was supplemented with 5% non-inactivated GPSC to provide exogenous complement. Virus and sera were preincubated for 4 h and the viral inoculum left on the cells for the entire 14–20-hour incubation. On each plate, NSC was included as reference for infectivity and PSC as control for assay quality. After a 14–20-hour incubation, the wells were blocked with NDS and the infected cells were stained for nuclear IE1 using MAB810, followed by the addition of biotinylated donkey-anti-mouse IgG as secondary antibody, and then streptavidin-HRP. Infected cells were visualized with TrueBlue substrate.

3. Results

3.1. Substrate selection and staining optimization

Using biotin–avidin amplification and peroxidase as signal generator, we compared four histochemical peroxidase substrates for visualizing AD169-infected MRC-5 cells immunostained with mAb to IE1. Although AEC, StableDAB and HistoBlack all produced stained nuclear images easily detected and quantified by the ImmunoSpot Analyzer, the use of TrueBlue produced images that were superior in size and contrast, and resulted in the highest accuracy and sensitivity (Fig. 1). Therefore, the TrueBlue substrate was used in all further experiments.

The primary antibody, MAB810 (anti-IE1) was tested at concentrations ranging from 2 $\mu\text{g/ml}$ to 0.0625 $\mu\text{g/ml}$ and the lowest two, 0.125 $\mu\text{g/ml}$ and 0.0625 $\mu\text{g/ml}$, gave the highest sensitivity and lowest background staining (data not shown). A 0.1 $\mu\text{g/ml}$ working concentration of the primary antibody was chosen for all further studies. The secondary biotinylated antibody was tested at dilutions ranging from 1/1000 to 1/8000, and the highest assay sensitivity and lowest background was achieved using 1/1000 dilution

(data not shown). HRP-avidin and HRP-streptavidin, both used at 2 $\mu\text{g/ml}$, gave indistinguishable results (data not shown).

In side-by-side testing, we compared a direct staining method with the optimized biotin–streptavidin-amplified staining procedure. Direct staining of the infected nuclei resulted in a smaller average spot size than biotin–streptavidin-amplified staining ($1.58 \pm 0.16 \text{ mm}^2$ for direct staining versus $2.95 \pm 0.34 \text{ mm}^2$ for amplified staining), yielded a smaller and less precise nuclear count (490 ± 142 , $CV = 28.9\%$ for direct staining versus 826 ± 96 , $CV = 11.6\%$ for amplified staining) and required a much longer substrate reaction time (50 min versus 12 min for direct and amplified staining, respectively). Based on these findings, the biotin–streptavidin-amplified staining procedure was chosen for all further experiments.

The optimized staining procedure routinely yielded low background, with ImmunoSpot Analyzer counts in the virus-free wells being <3% of those in the wells with infected cells (data not shown). The subtraction of background counts for data analysis was therefore deemed unnecessary.

3.2. Optimizing serum–virus preincubation and infection

The microneutralization assay described by Andreoni et al. in 1989 calls for a 1-hour preincubation of virus and test sera, followed by a 4-hour incubation of the virus–serum inoculum with the indicator cell monolayer, after which the cells are incubated for an additional 16 h in virus-free medium. In a modified version of this assay, virus and sera are preincubated for 4 h and the virus–serum inoculum is incubated with the indicator cells for 16 h (Schoppel et al., 1996; Klein et al., 1999). In a direct comparison of these two methods, a 4-hour virus/serum preincubation time in combination with a 14–18-hour incubation without removal of the virus resulted in a far better distinction between neutralizing and non-neutralizing sera than a 1-hour virus/serum preincubation time in combination with a 4-hour incubation of cells with the virus/serum inoculum before removal (Fig. 2). Our results also confirm the observation made by Klein et al. (1999) that human sera diluted less than 1/50 can have a non-specific toxic effect on infectivity, mimicking neutralization (Fig. 2). For all further experiments, a 4-hour virus/serum preincubation time followed by a 14–20-hour incubation was used. To avoid non-specific inhibition of infectivity and thus ensure high assay specificity, 1/50 was the lowest dilution used for assaying human sera.

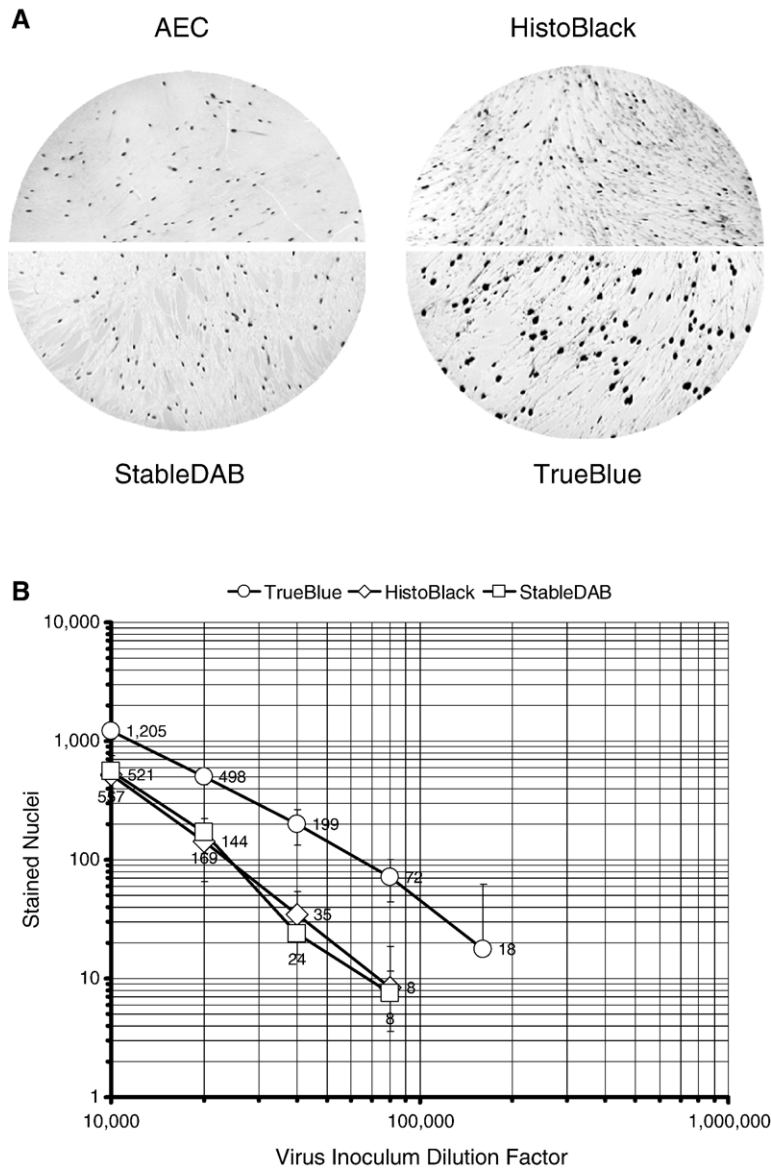


Fig. 1. HCMV-infected cells immunostained for IE1 using different commercial peroxidase substrates. A) MRC-5 cells in a standard 96-well microplate were infected with virus for 4 h, followed by a 20-hour incubation in virus-free medium. The cells were then fixed and the plate divided into four segments, which were stained for nuclear IE-1 expression using identical biotin–avidin amplified procedures except for the substrate. For each substrate, a partial close-up image of a representative well is shown at the same magnification. B) Each of six replicate wells of MRC-5 cells, seeded in a standard microplate, were infected with two-fold serially diluted AD169 (between 1/10,000 and 1/160,000) for 4 h and 20 h later stained for IE1-expression using identical procedures except for the substrate. For each dilution, the mean nuclear count from duplicate data points \pm standard deviation are plotted as a function of the virus dilution on a log–log scale.

3.3. Plate type selection

Primarily to reduce the demand for cells, we evaluated the use of Half Area well tissue culture microplates, which accommodate half as many cells and use half as much medium as standard 96-well microplates. As seen in a side-by-side comparison, the acquired nuclear images are larger and better defined

in the Half Area wells than in the standard wells because the smaller surface area of the Half Area wells requires adjustment of the camera lens to zoom in closer to the cells, resulting in a higher magnification (Fig. 3a). The standard wells contained more infected cells than the Half Area wells (mean infected cells \pm S.D. values were 861 ± 288 and 522 ± 64 , respectively), because they received twice as many plated cells and twice the

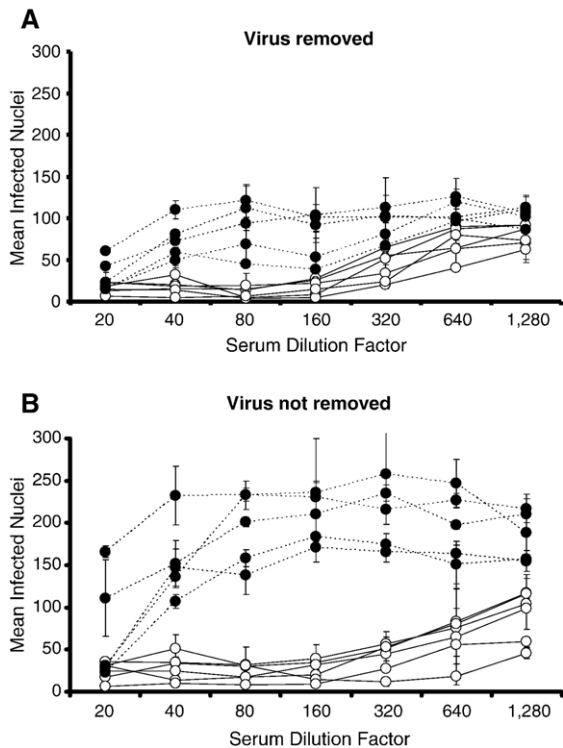


Fig. 2. Comparison of techniques for virus/serum preincubation and infection of cells. The neutralization assay was performed using standard microplates, MRC-5 indicator cells, and test sera from volunteers. Closed symbols connected by dotted lines represent five sera that tested non-reactive and open symbols connected by solid lines represent five sera that tested reactive in an HCMV gB IgG ELISA. The mean infected nuclei from duplicate wells \pm standard deviation are plotted against the serum dilution. A) Virus and sera were preincubated for 1 h and cells were exposed to virus/serum inoculum for 4 h, followed by a 17-hour further incubation in fresh culture medium. B) Virus and sera were preincubated for 4 h and cells were exposed to virus/serum inoculum for 18 h.

virus input. However, contrary to expectation, the nuclear counts for the former also showed higher variability than the Half Area wells (CV of 31% and 12%, respectively). As a consequence of this higher variability, the goodness of fit for the neutralization curves obtained on the standard plate was also inferior compared with the Half Area plate, albeit the NT_{50} values derived from the two plate types were comparable (within 85–110 %) for the same test samples (Fig. 3b and c). Therefore, Half Area microplates were used for all further experiments.

3.4. Cell monolayer optimization

A uniform and intact indicator cell monolayer enabling optimal infectivity and accuracy of automated data collection is perhaps the most critical requirement

for a microneutralization assay. Using automated data collection and analysis, we evaluated three commercially available human embryonic fibroblast-like cell lines commonly used in the diagnosis of HCMV infection as indicator cells. In side-by-side comparisons, MRC-5, HEL-299 and WI-38 cells showed similar infectivity (e.g. mean infected cells \pm S.D. of 224 ± 44 , 252 ± 40 , and 329 ± 46 , respectively) and yielded similar neutralizing curves (not shown) and NT_{50} values for several test sera (Table 1). However, HEL-299 provided us with the highest percentage of usable assay plates and had the fewest staining problems.

Having compared various seeding densities for HEL-299 cells (100,000–400,000 cells/ml), we found that plating cells at 200,000 cells/ml (10,000 cells in 50 μ l per well for the Half Area microplates or 20,000 cells in 100 μ l per well for the standard 96-well microplates), 18–24 h before assay consistently worked well (data not shown). Furthermore, HEL-299 cells worked equally well whether used at passage #2 (data not shown) or passage #18 after recovery from a frozen stock. HEL-299 cells at passage #4 and passage #18 produced mean \pm S.D. infectivity values of 380 ± 39 and 298 ± 44 , respectively, in the control wells ($n=16$, each) and yielded NT_{50} values of 379 versus 384 for one test sample and NT_{50} values of 564 versus 480 for another test sample. The neutralizing curves obtained using passage #4 versus passage #18 for generating cell monolayers were found to be not different by the comparative global fitting application of PRISM 4 (Fig. 4). HEL-299 cells passaged at least twice but not more than 18 times (the maximum passage number tested) and seeded at 200,000 cells/ml were used in all further experiments.

3.5. The effect of complement and total human serum content on neutralizing activity

The humoral immune response to HCMV comprises both complement-dependent and complement-independent neutralizing antibodies. For example, the addition of fresh guinea pig complement to human sera obtained during convalescence from HCMV infections increased their neutralizing titer 2–4 fold (Schmidt et al., 1976) and the addition of exogenous complement to various commercial IgG preparations was shown to enhance their neutralization of HCMV by 2–16 fold (Lewis et al., 1986; Eizuru et al., 1988), as measured by plaque-reduction. To establish how complement may affect serum neutralizing activity as measured in our assay, heat-inactivated test samples with a wide range of neutralizing activity were prepared by prediluting pooled

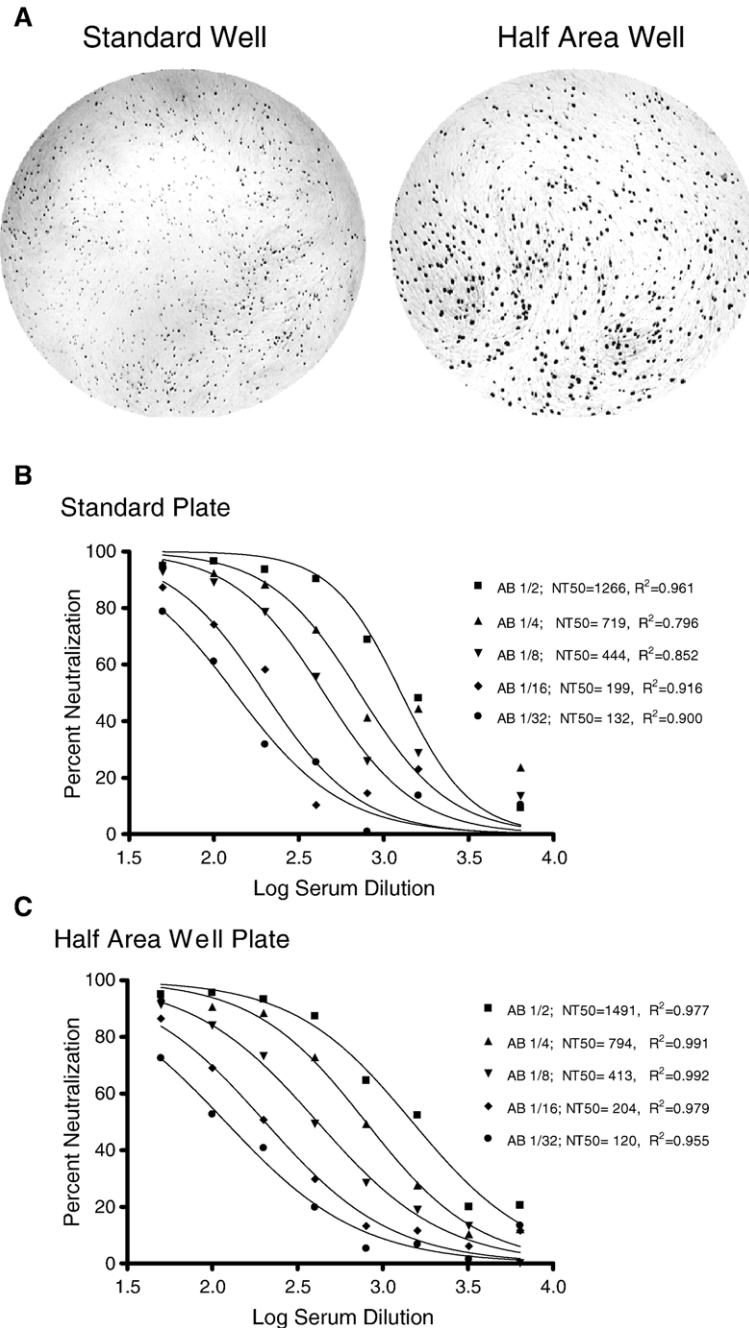


Fig. 3. HCMV neutralization using standard versus Half Area well microplates. HEL-299 cells seeded in standard well (A left panel and B) or Half Area well (A right panel and C) microplates were infected and then immunostained for IE1 using TrueBlue as substrate as described in Materials and methods section. A) For each plate type, the image of a representative well is shown. Magnification for the Half Area well is greater by necessity. B) and C) Shown are for each of five AB pool serum samples prediluted with NSC at 1/2, 1/4, 1/8, 1/16 and 1/32 the individual 4-PL neutralization curves fitted by PRISM, the corresponding R^2 values, and the derived neutralizing activities (NT_{50}).

AB serum with NSC at 1/4, 1/8, 1/16, 1/32 and 1/64. These 5 test samples were assayed for neutralizing activity with and without 5% GPSC as a source of complement, used in lieu of half the usual 10% heat-

inactivated FBS in the assay medium. The presence of 5% GPSC resulted in 3.4–5.5 times greater NT_{50} values than its absence (Fig. 5a and b) or the presence of 5% heat-inactivated GPS (data not shown).

Table 1
Comparison of three indicator cell lines in the microneutralization assay

Sample	gB IgG ELISA titer	MRC-5		HEL-299		WI-38		Global fit	
		R^2	NT ₅₀	R^2	NT ₅₀	R^2	NT ₅₀	NT ₅₀	Comparison
1063	25,600	0.932	2593	0.985	2113	0.999	2025	2207	Not different
P09	12,800	0.912	1891	0.985	1554	0.982	1928	1768	Not different
P07	3200	0.897	1419	0.985	1205	0.981	1209	1288	Not different
P05	800	0.890	374	0.957	340	0.943	463	387	Not different
P03	200	0.700	90	0.687	87	0.896	108	91	Not different

Five test sera were analyzed for neutralizing activity under identical conditions except for using MRC-5, HEL-299 or WI-38 cells as indicator monolayers. The assay was run at a starting dilution of 1/50 for all sera. NT₅₀ values for each test sample were determined using constrained 4-PL curve fitting and neutralization curves obtained using the three indicator cell strains were compared using global fitting in PRISM.

The GPSC used in our study did not itself have HCMV-neutralizing activity. In 6 independent comparisons, maximum infectivity was not substantially different in the presence of GPSC compared with its absence (values of mean infected nuclei ± standard deviation in the negative control wells with and without GPSC were 431 ± 142 versus 360 ± 63, 522 ± 64 versus 355 ± 67, 861 ± 268 versus 588 ± 174, 332 ± 95 versus 202 ± 71, 557 ± 113 versus 341 ± 119, and 757 ± 110 versus 801 ± 99, respectively). There was also no difference between GPSC and GPS in the mean number of infected nuclei (431 ± 142 versus 477 ± 127; 522 ± 64 versus 549 ± 57, respectively).

Also, to clarify whether the varying amount of human serum present in the medium during assay resulting from the serial dilution of the samples for assay has any influence on the neutralizing activities mea-

sured, we simultaneously ran a third version of the assay, in which the total human serum content of the assay medium was adjusted to a constant 2% by adding heat-inactivated NSC to every well of the assay plate (Fig. 5c). Using global fitting, the corresponding neutralization curve pairs obtained with or without added NSC, both in the presence of GPSC, were found to be statistically indistinguishable for each of the five test samples (data not shown) indicating that normalizing the concentration of human serum in the assay wells provides no advantage. Therefore, an assay medium containing 5% inactivated FBS and 5% GPSC was chosen for use in all further experiments.

3.6. Assay characteristics

To establish assay linearity, the AB pool was prediluted with NSC to provide test sera with a range of NT₅₀ values. As shown in Fig. 6a, the assay is linear for test samples with neutralizing activities in the 30–1500 range (the NT₅₀ value of 30 is extrapolated by the software program). Furthermore, neutralizing activity (NT₅₀ values) correlated closely with the levels of anti-gB serum IgG (Fig. 6b).

To establish other key performance characteristics, the assay was performed by two operators running a total of 35 assay plate pairs in thirteen separate assays using the optimized final protocol. The overall precision of 27.7%, obtained from testing the PSC ($n=39$ analyses, data not shown), is acceptable for a cell-based assay. Although the NT₅₀ range measured for PSC was fairly broad at 1032–3590, the neutralization curves for PSC consistently had good R^2 values (mean $R^2=0.986$, with an assay acceptance cutoff of mean $R^2 -2S.D. R^2=0.951$; data not shown).

Comparative evaluation of the R^2 values obtained for samples that were positive versus negative for binding in the CMV gB IgG ELISA (Wloch, unpublished data) revealed that the goodness of fit for the sample-specific

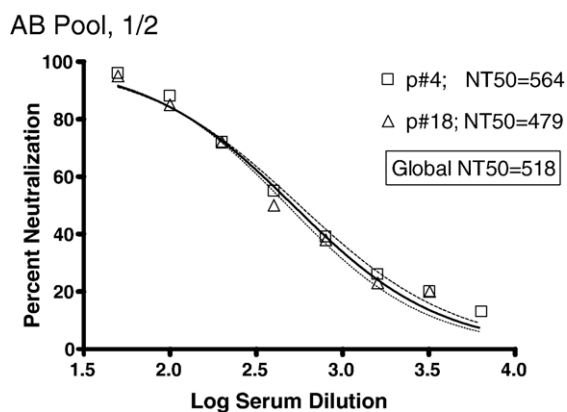


Fig. 4. The effect of cell passage number on the measured neutralizing activity. The neutralizing activities of two test sera were measured in two simultaneously run assays, using HEL-299 indicator cells at passages p#4 (squares) versus p#18 (triangles). The two neutralizing curves obtained for each of the two test samples were compared using global fitting. The individually fitted curves are represented by the dotted lines; the globally fitted (shared) curve is represented by the solid line. The corresponding NT₅₀ values are also shown.

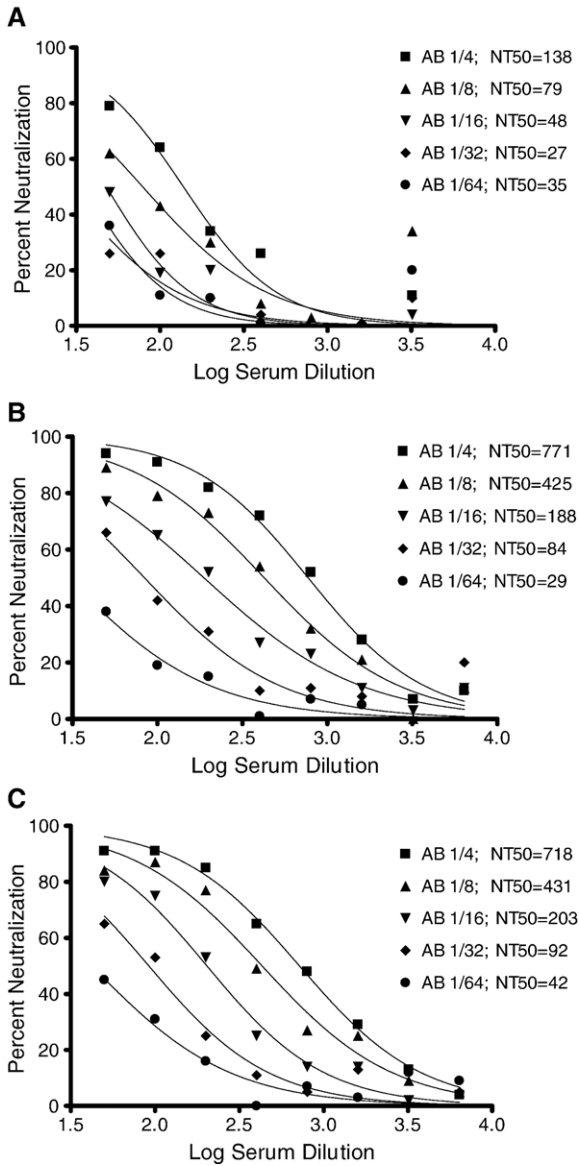


Fig. 5. The effect of complement and the total human serum content on neutralizing activity. A) Five test samples (pooled AB serum prediluted with NSC at 1/4, 1/8, 1/16, 1/32 and 1/64) were assayed for neutralizing activity in medium containing 10% FBS. FBS and the human sera were all heat-inactivated. B) The same test samples were assayed in medium containing 5% heat-inactivated FBS and 5% GPSC. C) The same test samples were assayed in medium containing 5% heat-inactivated FBS, 5% GPSC, and heat-inactivated NSC added to equalize the human serum content to 2%.

neutralization curve was a decisive parameter for distinguishing neutralizing and non-neutralizing test samples in the neutralization assay. The mean R^2 value for all test samples positive for anti-gB antibody by ELISA was 0.972 and the mean-2S.D. cutoff was 0.898

($n=44$ analyses; data not shown). In sharp contrast, samples negative for anti-gB IgG had poorly fitted neutralization curves with a mean R^2 value of only 0.137 and a mean+2S.D. cutoff value of 0.510 ($n=39$ analyses; data not shown). Therefore, the R^2 value for a sample was used as a key criterion for determining whether the sample has neutralizing activity. Samples with R^2 values equal to or greater than 0.898 and an NT_{50} value of at least 50 were deemed to be “positive” and those with R^2 values below 0.510 were deemed to be “negative” for CMV-neutralizing activity. Because

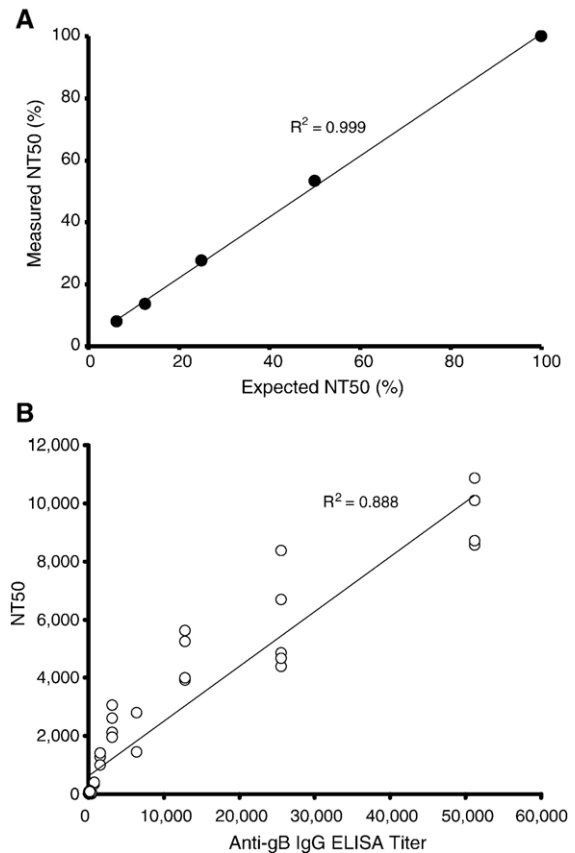


Fig. 6. Assay linearity and relationship between CMV-neutralizing activity and serum anti-gB IgG levels. Pooled AB serum was two-fold serially prediluted with NSC to obtain multiple test samples with decreasing neutralization activity, which were then assayed for neutralizing activity. The measured NT_{50} values are plotted here against the expected NT_{50} values, both expressed as a percentage of that of the most potent sample in the assay. Linear regression analysis indicated a high degree of correlation between the expected and the measured NT_{50} values for both experiments (R^2 values >0.99). B) The neutralizing activities (NT_{50} values, ranging 5–10,864) were plotted against the corresponding anti-gB IgG ELISA titers (ranging 100–51,200) from a total of 49 analyses. Linear regression analysis showed a strong positive correlation ($R^2=0.888$) between neutralizing activity (NT_{50} values) and anti-gB IgG titers.

Table 2
Summary results for HCMV-neutralizing analysis of test sera

No. tested	Anti-gB IgG ELISA titer	CMV ELISA reactivity	Neutralizing activity		
	(Vical)	(Diamedix)	No. positive	No. reactive	No. negative
9	800–50,000	Positive	9 (100%)	0 (100%)	0 (0%)
11	100–400	Negative	4 (36.4%)	4 (36.4%)	3 (27.2%)
37	Negative	Negative	0 (0%)	0 (0%)	37 (100%)

A total of 57 test sera with various anti-gB IgG levels were analyzed for neutralizing activity using the optimized neutralization assay. The sera were classified for neutralizing activity as described in Section 3.6.

NT₅₀ values below 50 are extrapolated by the software, samples with an R^2 value of at least 0.898 but with an NT₅₀ value < 50 were reported as “reactive”. Sera with an R^2 value > 0.510 but < 0.898 were re-assayed. They were considered reactive if the R^2 value in the repeat assay was at least 0.898, otherwise the samples were deemed non-reactive.

For sera with anti-gB IgG titers of 800 or greater, the assay sensitivity for detecting neutralizing activity with an NT₅₀ of at least 50 was 100% (Table 2). The results for sera with low anti-gB IgG titers of 100–400 (all were sera from HCMV DNA vaccine trials that tested negative in the Diamedix HCMV IgG ELISA but positive in Vical’s anti-gB IgG ELISA) were variable. Of the 11 sera with low anti-gB IgG titers, 8 were positive or reactive in the assay (73%) while 3 sera, 2 with anti-gB IgG titers of 100 and one with an anti-gB titer of 200, were non-reactive (negative) in the assay (27%). These results suggest that the neutralization assay is sensitive even at low levels of anti-gB antibody. The assay showed 100% specificity in that all 37 of the anti-gB IgG negative sera assayed tested were non-reactive, with only one sample prompting a re-assay because of an R^2 value > 0.510 (but < 0.898) in the first analysis.

4. Discussion

We have developed a fast, convenient, sensitive, and specific microneutralization assay for HCMV that will enable a high-throughput assessment of neutralizing antibody responses to HCMV vaccine candidates in ongoing and future clinical trials, including those testing Vical’s pDNA vaccines. As previously described by others (Andreoni et al., 1989), a 96-well microplate format was used to measure infectivity based on immunostaining for IEA. However, we replaced immu-

nofluorescent staining with a very sensitive immunohistochemical procedure, which enables automated data acquisition and analysis. This assay has the potential to measure the neutralization of other HCMV strains, including clinical isolates, with little to no procedural modification.

Indirect immunoperoxidase staining using an anti-IEA mAb and the DAB substrate had been employed to detect and diagnose HCMV infection several years before the development of the immunofluorescent microneutralization assay (Swenson and Kaplan, 1985). A similar detection method had also been used in combination with the substrate AEC to titer HCMV and measure neutralizing antibody (Chou and Scott, 1988). Later, the immunofluorescent microassay was also modified for immunohistochemical staining and the substrate DAB was used to measure the neutralization of rhesus CMV by macaque serum ((Lockridge et al., 1999). Alternatively, others used the modified Andreoni assay with AEC to quantitate neutralizing antibodies in the sera of mice vaccinated with AD169 dense bodies (Pepperl et al., 2000). In all of these approaches, nuclear images were counted by an analyst using a microscope.

Recognizing the need for automated data acquisition to analyze large numbers of specimens in prospective clinical trials, Wang et al. (2004a) used a microplate spectrophotometer to measure fluorescence emitted by cells infected with EGFP-expressing recombinants of the Towne or Toledo strains of HCMV in their version of an HCMV microneutralization assay. Alternatively, they used a digital camera to capture the fluorescent images and an image processing software for data analysis. However, a neutralization assay based on intrinsic fluorescence is limited by the availability of the appropriate HCMV recombinant. Furthermore, a minimum 5-day post-infection cultivation time was needed for the cells to produce signals quantifiable by microplate spectrophotometry and edge effects made the outer 36 wells of a 96-well microplate useless for the assay (Wang et al., 2004a). Andreoni et al. (1989) also reported that the outermost columns and rows yielded uninterpretable results in their microassay and thus were used only as an evaporation barrier. This phenomenon may be specific for fluorescent detection, because in our assay we can use all 96 wells of a microplate. However, we strategically position our controls and the test samples across the plate, to minimize the potential influence of well location on our results.

Automated ELISPOT analyzers are conventionally used for scanning and counting colored immunoprecipitates on opaque white membrane-bottom microplates manufactured specifically for use in ELISPOT assays.

Cheong et al. (2003) used an ELISPOT approach to titer stocks of a recombinant virus expressing human IL-2 by plating infected cells onto anti-IL-2 Ab coated ELISPOT plates. In a novel application for a neutralization assay for the SARS coronavirus, an ELISPOT analyzer was used to count whole cells actually growing on ELISPOT plate membranes and infected with a pseudovirus, which also encoded a Lac Z gene that made cells blue upon staining with X-gal (Han et al., 2004). Our HCMV microneutralization assay appears to be the first to use an automated ELISPOT analyzer for quantitating immunostained cell nuclei. In our experience, the use of ELISPOT plates for this purpose is unnecessary and increases the assay expense. In a study in which we compared several microplate types, including MultiScreen-HA cellulose ester and MultiScreen-IP high protein binding hydrophobic Immobilon-P ELISPOT plates (Millipore, Burlington, MA), neither the HA cellulose ester ELISPOT plates nor white opaque tissue culture plates (Perkin-Elmer, Boston, MA) showed any advantage over standard clear tissue culture plates (NUNC) (data not shown). By employing an automated ELISPOT analyzer, we have made the counting of stained nuclei not only more convenient but also less prone to human error and thus more objective. Our results indicate that curve fitting of data, and with it the accuracy and sensitivity of the assay, improves with increasing viral load, which can be critical when assaying sera that have little neutralizing activity. Because the ImmunoSpot Analyzer can count a whole 96-well microplate with over a thousand stained nuclei in each well in about half an hour, our assay can easily be run at a high viral input with a high level of accuracy.

Most HCMV neutralization assays use primary human foreskin fibroblasts (HFF) as indicator monolayers, regardless of whether the endpoint is based on plaque-reduction (Gonczol et al., 1989; Adler et al., 1999; Pass et al., 1999) or immunostaining of viral proteins in the target cells (Chou and Scott, 1988; Andreoni et al., 1989; Klein et al., 1999; Lockridge et al., 1999; Pepperl et al., 2000). MRC-5 human embryonic fibroblast-like cells have long been used interchangeably with primary fibroblasts for the detection of HCMV in cell culture (Swenson and Kaplan, 1985) and MRC-5 were even found superior to HFF in shorter (24 h versus 48 h) cultures (Gleaves and Meyers, 1987). Lately, MRC-5 cells have been used in neutralization assays as well (Wang et al., 2004b). In our hands, MRC-5 cells performed very inconsistently as indicator monolayers for neutralization analysis. Although they were readily infected and expressed the IE1 protein, the quality of the monolayer varied largely

and unpredictably together with the extent of background staining, which affected the automated data acquisition detrimentally (data not shown). Prompted by a report that showed MRC-5, HEL, and WI-38 cell lines and primary HFF equally reliable (overall 100% sensitive and 97.4% specific) at detecting HCMV in urine, (as used by various clinical laboratories; Demmler et al., 2000), we compared the cell lines in our assay. Although all three cell lines could be infected equally well with AD169 and yielded comparable neutralizing activity values for several test sera, the culture and staining characteristics of HEL-299 cells were much better than those of MRC-5 and WI-38 cells. HEL-299 cells provide more uniform indicator monolayers and are harder than MRC-5 and WI-38 cells, ultimately improving the robustness of the assay.

During our studies, we switched from standard clear 96-well tissue culture microplates to a clear tissue culture plate whose wells were half the area of that of a standard plate. With cell availability being a potential bottleneck, we wanted to reduce the cell requirement and thus be able to increase the number of assay plates we could run at once. We have found that the Half Area plates are beneficial at multiple levels: they require not only fewer cells but also less reagents and virus than regular plates while yielding better data. The apparent superiority of the Half Area plates over the standard plates in our assay likely is the combined result of the indicator cell monolayer being more sheltered from or less disturbed by the necessary manipulations during assay and the nuclear images likely being counted more accurately. Half Area plates require scanning and counting at a higher magnification than standard microplates, which makes the nuclear images better defined.

Our approach to measuring viral infectivity using an ELISPOT analyzer has the potential to be useful in or adaptable to a variety of applications. Among others, it should be a great help to the developers of neutralization assays for other viruses where high-throughput screening is desirable in a clinical trial setting. It should easily be modified for the titration of various viruses, recombinant or other, and developed into a screening assay for antiviral agents.

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