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Flow Cytometry-Based Assay for Titrating Dengue Virus

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Plaque assays for titrating dengue virus (DENV) are time-consuming and not suitable for strains that do not plaque. Fluorescence-activated cell sorting (FACS) has been used to detect DENV-infected cells. Here we describe a FACS-based assay for titrating DENV. We determined that at 24 h postinfection, the number of infected cells detected by FACS represented the first round of infection and therefore could be used as a readout of the number of infectious particles in the inoculum. When the titers of different laboratory and clinical strains of DENV were compared using FACS, plaque, and endpoint dilution assays, for most strains the FACS titers were comparable to titers obtained by plaque or endpoint dilution assays. The FACS assay is an improvement over the plaque assay because the infection period is reduced from 5 to 7 days to 24 h and the assay can be used to titrate clinical isolates that frequently do not form clear plaques on cell monolayers. The novel FACS-based methods described here will facilitate laboratory studies of dengue.

Dengue is a mosquito-borne viral disease of global public health significance. Throughout the world there are more than 2.5 billion people at risk of dengue virus (DENV) infection. Each year there are an estimated 100 million cases of dengue viral infection worldwide, with 250,000 people developing the more severe dengue hemorrhagic fever/dengue shock syndrome, which is often fatal (5). DENV is a positive-polarity RNA virus in the family *Flaviviridae*, and the DENV complex consists of four distinct serotypes designated DENV1 through DENV4 (10).

Laboratory studies of dengue virus are difficult because the virus does not grow to high titers in cell culture and assays for titrating virus and measuring virus neutralization are time-consuming. These problems are exacerbated when working with primary clinical isolates of virus. Standard methods for titrating DENV and measuring the ability of antisera to neutralize the virus are based on plaque assays which require 5 to 7 days to complete. Some isolates, especially among primary clinical isolates, do not form clear plaques on cell monolayers. Better methods for titrating the virus and measuring the ability of antisera to neutralize dengue need to be developed. Within the past 10 years, fluorescence-activated cell sorter (FACS)-based methods have been developed to follow infection and determine titers of viruses such as human immunodeficiency virus, herpesvirus, measles virus, influenza virus, Epstein-Barr virus, and rabies virus (1, 4, 12, 13, 16). FACS has also been used to detect DENV in clinical samples and to measure the ability of the virus to infect a variety of cells (2, 3, 7, 8, 11, 17). Here we report on a FACS-based assay for titrating DENVs and for characterizing the ability of antisera to neutralize the virus.

MATERIALS AND METHODS

Cells, viruses, and antisera. *Aedes albopictus* C6/36 mosquito cells were obtained from the Centers for Disease Control and Prevention, Fort Collins. Vero 76 (African Green monkey kidney) cells were purchased from the American Type Culture Collection (Manassas, VA). C6/36 cells were maintained in Eagle's minimum essential medium (EMEM; Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin G (100 U/ml), streptomycin (100 μg/ml), L-glutamine, and nonessential amino acids at 28°C in 5% CO_2. Vero 76 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)–F-12 (Gibco BRL) containing 115 mM HEPES buffer supplemented with 10% heat-inactivated fetal bovine serum, penicillin G (100 U/ml), streptomycin (100 μg/ml), L-glutamine, and nonessential amino acids at 37°C in 5% CO_2.

The viruses used in this study included prototype DENV2 virus (New Guinea C strain), prototype DENV3 (H87 strain), and prototype DENV4 (H241) (gifts from Robert Shope, University of Texas at Galveston), one DENV1 high-passage clinical isolate (16007; gift from Robert Putnak, Walter Reed Army Institute of Research), one DENV1 clinical isolate from Sri Lanka (UNC 1002), three DENV3 clinical isolates from Sri Lanka (UNC 3001, UNC 3009, and UNC 3011), and two DENV3 clinical isolates from Nicaragua (UNC 3017 and UNC 3018; gifts from E. Harris, University of California, Berkeley). All experiments with the clinical virus isolates, unless otherwise stated, used stocks from viruses that had been passaged in cell culture fewer than four times. Viral stocks were obtained by inoculating a monolayer of C6/36 cells in a 75-cm^2 tissue culture flask with 200 μl of virus diluted in 1 ml of EMEM–2% FBS. After 1 h, 14 ml of EMEM supplemented with 2% FBS was added and the cells were cultured for 10 days at 28°C in 5% CO_2. The supernatant was removed from the cells and centrifuged for 5 min at 2,000 × *g* to pellet cellular debris. The supernatant was aliquoted and stored at −80°C. DENV1 (16007) was grown and passaged in Vero cells, whereas DENV4 (H241) was grown in C6/36 cells as described above and passaged one time in Vero cells.

The flavivirus group-reactive monoclonal antibody 4G2 (6) was purchased from the American Type Culture Collection, Rockville, MD.

FACS titration assay. C6/36 cells were seeded in six-well plates and incubated at 28°C in 5% CO_2 until cells were approximately 90 to 95% confluent (~1×10^6 cells/well). Medium was removed from cells, and 10-fold serial dilutions of virus diluted in EMEM with 2% FBS to a final volume of 200 μl were added to the cells. The cells were incubated at 28°C in 5% CO_2 for 1 h, and plates were rocked every 15 min. The medium was removed, and the cells were washed in 1× phosphate-buffered saline (PBS). Two milliliters of EMEM with 2% FBS was added to each well, and the cells were incubated for 24 h at 28°C in 5% CO_2. After 24 h the medium was removed and the cells were washed and scraped from the well and resuspended in PBS. An aliquot of cells from each well was counted using a hemocytometer. The remaining cells were centrifuged at 1,000 × *g* for 5 min and resuspended in 200 μl of Cytofix/Cytoperm solution (BD Biosciences, San Jose, CA) and incubated on ice for 20 min in the dark. All subsequent steps were performed in Cytoperm/Cytowash solution. The cells were centrifuged at 1,000 × *g* for 5 min and washed two times before resuspending in 50 μl of fluorescein isothiocyanate (FITC)-labeled 4G2 monoclonal antibody for 1 h on ice. The cells were washed two times and resuspended in the Cytoperm/Cytowash solution. The samples were analyzed on a FACScan flow cytometer using Summit software. For each sample at least 100,000 events were collected. The linear

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range of the assay under the tested parameters was determined to be between 0.20% and 25% infected cells in a well (between ~2,000 and 250,000 infected cells per well), and sample wells outside this range were not used for determining final titers. The titer of the virus was determined using the following formula: FACS infectious units/ml = [(% of infected cells) × (total number of cells in well) × (dilution factor)]/(volume of inoculum added to cells). Samples with a titer below 1×10^4 infectious particles/ml cannot be analyzed with the FACS assays, as this titer is below the level of detection.

Vero plaque assay. Vero plaque assays were performed using a modified protocol from Wu et al. (18). Briefly, Vero cells were seeded in six-well plates at a density of 2.5×10^5 cells/well and incubated overnight. Fifty microliters of DENV stock was added to 450 μl of diluent containing 1% bovine albumin in DMEM–F-12 medium supplemented with penicillin and streptomycin. The virus mixture was serially diluted using 10-fold dilutions. Two hundred microliters of each dilution of virus was added to each well of Vero cells in duplicate. The plates were incubated at 37°C in 5% CO_2 for 1 h, rocking plates every 15 min. Four milliliters of primary nutrient agar overlay (1% Seakem LE agarose, 20% Earle's balanced salt solution 10×, 7% yeast extract-lactalbumin hydrolysate, 4% FBS, 0.5% sodium bicarbonate, 1% gentamicin [50 mg/ml]) and 0.2% Fungizone (12.5 μg/ml) was added to each well, and the cells were incubated at 37°C in 5% CO_2 for 5 to 7 days. Next, 2 ml of a secondary nutrient agar overlay (primary nutrient overlay containing 1% neutral red) was added to each well and the cells were incubated overnight at 37°C in 5% CO_2 before counting plaques and calculating viral titers. The viral titers were expressed as PFU/ml, calculated as [(number of plaques per well) × (dilution)]/(inoculum volume).

Endpoint titration assay. Endpoint titration assays were performed using a modified protocol from Schoepp et al. (15). Briefly, C6/36 cells were seeded in a 96-well plate at a density of 1.3×10^5 cells/well in 225 μl and incubated overnight at 28°C in 5% CO_2. Twenty-five microliters of virus was added to the first well, and the virus was serially diluted using 10-fold dilutions. The plates were incubated at 28°C in 5% CO_2 for 5 days. To fix the plates, 50 μl of a 3:1 acetone-PBS mixture was added to each well, and the plates were incubated at −20°C for 20 min. The plates were air dried and washed with 1× PBS. The plates were blocked with 5% FBS–PBS–0.05% NaN_3 for 15 min at room temperature. Each well was resuspended in 50 μl of FITC-labeled 4G2 monoclonal antibody diluted in blocking buffer and incubated for 1 h at 4°C. The plates were analyzed using an inverted fluorescence microscope. Wells were scored for the presence or absence of infection with either a positive or a negative symbol. Replicates of 10 were performed for each virus. We calculated the 50% tissue culture infectious dose ($TCID_{50}$) of each virus using the Reed-Muench formula, based on immunofluorescence: $TCID_{50}$ (expressed as 10^x/ml) = (dilution where % of infected wells is >50%) + [(% of infected wells above 50%) − 50%]/[(% of infected wells above 50%) − (% of infected wells below 50%)] × $\log_{10}10$.

Plaque reduction neutralization test. The plaque reduction neutralization tests were performed using modified protocols from Russell et al., Kochel et al., and Wu et al. (9, 14, 18). Vero cells were seeded in six-well plates at a density of 2.5×10^5 cells/well and incubated overnight. The test serum was heat inactivated at 56°C for 30 min. The serum was serially diluted twofold in diluent containing 1% bovine albumin in DMEM–F-12 medium supplemented with penicillin and streptomycin. DENV was diluted such that each well would be infected with 40 to 60 PFU. An equal volume of virus was added to the diluted serum and incubated at 37°C in 5% CO_2 for 1 h. The virus and antibody mixtures were added to the Vero cells for 1.5 h. Four milliliters of primary nutrient agar overlay, as described above, was added to each well and the cells were incubated at 37°C in 5% CO_2 for 6 days. Next, 2 ml of a secondary nutrient agar overlay (primary nutrient agar overlay containing 1% neutral red) was added to each well and the cells were incubated overnight at 37°C in 5% CO_2 before counting plaques and calculating viral titers. Each antibody concentration was tested in triplicate. The numbers of plaques in each well were counted and the percent reduction was determined as follows: % reduction = [(average number of control plaques − average number of plaques for each dilution)/(average number of control plaques)] × 100. The plaque reduction neutralization titers of serum equaled the reciprocal of the serum dilution in which either 50% ($PRNT_{50}$) or 80% ($PRNT_{80}$) plaque reductions were observed.

FACS neutralization test (FNT). Vero cells were seeded in six-well plates at a density of 2.5×10^5 cells/well and incubated overnight. Human serum to be tested was heat inactivated at 56°C for 30 min. The serum was serial diluted twofold in diluent containing 1% bovine albumin in DMEM–F-12 medium supplemented with penicillin and streptomycin. DENV was resuspended in diluent such that each well would receive a multiplicity of infection (MOI) of 0.1. An equal volume of virus was added to the diluted serum and incubated at 37°C in 5% CO_2 for 1 h. The virus and antibody mixture was added to the Vero cells. Each antibody dilution was assayed in triplicate. The plates were incubated at 37°C in 5% CO_2 for 1 h, rocking plates every 15 min. Two milliliters of DMEM–F-12 containing 10% FBS was added to each well, and the plates were incubated at 37°C in 5% CO_2 for 24 h. Each well was washed with 1 ml of trypsin-EDTA. Next, 0.2 ml of trypsin-EDTA was added to each well and incubated at 37°C for 5 min. One milliliter of chilled 1× PBS containing 10% FBS was added to each well to inactivate the trypsin. The cells were pipetted to breakup any clumps and centrifuged at 1,000 × *g* for 5 min. The PBS was removed, and the cells were fixed and stained for FACS as described above. Approximately 100,000 to 200,000 cells were analyzed for each sample. The percent reduction in the number of infected cells was calculated for each serum dilution. The number of infected cells in the wells infected with virus only was used to calculate the percent reduction. The titers were expressed as the reciprocal of the serum dilution that inhibited 50% (FNT_{50}) or 80% (FNT_{80}).

RESULTS

We conducted experiments to evaluate if FACS was a sensitive method for detecting dengue virus infection of C6/36 mosquito cells. Cells were infected with prototype DENV2 (New Guinea C) and DENV3 (H87) lab-adapted strains at an MOI of 0.05. Cells were harvested on days 1, 2, 3, 5, and 7 after infection and analyzed by FACS. Representative FACS scatter plots from DENV2- and DENV3-infected cells are depicted in Fig. 1. Increasing numbers of infected cells were detected as the infection progressed, indicating that the FACS assay was suitable for following the kinetics of infection.

In the FACS assay DENV-infected cells were detected using a monoclonal antibody (4G2) directed to the envelope (E) glycoprotein of the virus. It is conceivable that some positive cells detected by FACS may represent input virus binding to cells and not productive infection of cells. Experiments were performed with normal and UV-inactivated DENV2 to distinguish between infection and simple virus binding to cells. At 24 h postinfection (hpi) 5.20% of cells treated with normal virus were positive, whereas no positive cells were detected with UV-inactivated virus, indicating that simple binding of input virus does not result in a positive signal in the FACS assay (data not shown).

Titration of DENV by FACS. Ideally, a cell line for determining the titer of dengue virus by FACS should be highly permissive for infection and infected by different strains of virus with similar efficiencies. Otherwise, titers obtained for different strains may reflect differences in interactions between the viruses and cell line and may be a poor measure of the absolute number of infectious particles. We chose the C6/36 mosquito cell line because the available evidence indicates no dramatic differences in interactions between different strains or serotypes of virus with this mosquito cell line. C6/36 cells were infected with prototype DENV3 and clinical isolates of DENV3 (MOI, 0.01), and the infection was monitored daily for 7 days by FACS. The different strains of virus displayed similar growth kinetics, indicating that this cell line was suitable for titrating DENV (Fig. 2). The DENV2 prototype strain NGC displayed growth kinetics similar to the DENV3 isolates in the C6/36 cell line (data not shown).

We reasoned that the number of cells infected early in infection, before cell-to-cell spread of virus, would be a direct measure of the number of infectious virus particles in an inoculum and that this number could be used to estimate viral titer in a sample. Experiments were conducted to identify a time point which was late enough for the input virus to have infected cells and produced sufficient protein for detection by

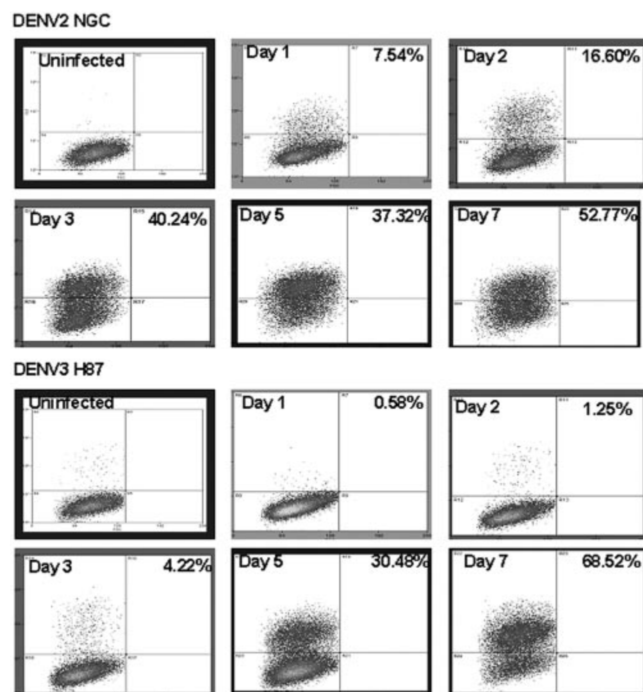

FIG. 1. Time course of DENV2 and DENV3 infection of C6/36 insect cells. C6/36 cells grown in six-well plates were infected with DENV2 strain NGC and DENV3 strain H87 at an MOI of 0.05. One well was mock infected to serve as a negative control. Cells were harvested from duplicate wells on days 1, 2, 3, 5, and 7 and processed for FACS. The cells were fixed and permeabilized using the Cytofix/Cytoperm kit from BD Biosciences and stained for FACS using monoclonal antibody 4G2, which cross-reacts with all four DENV serotypes. Cells were gated according to their size and granularity (*x* axis, side-scattered cells; *y* axis, forward-scattered cells) to identify intact, single cells. The gated cells were displayed on dot plots in which the *x* axis is the forward scatter and *y* axis is fluorescence (FITC) intensity. The dot plot was divided into four quadrants based on including 99.9% of the uninfected control cells in the lower left quadrant. The percentage of DENV-infected cells was determined by counting the number of cells in the upper left quadrant and dividing this number by the total number of cells in the dot plot.

FACS, while being too early for release and cell-to-cell spread of new virus. C6/36 insect cells were infected with DENV2 (strain NGC) at a high MOI, and the cells and supernatants were collected at 9, 18, 24, 26, 30, and 46 hpi. The number of infected cells increased from 9 to 24 hpi, and this first round of infection plateaued between 24 and 30 hpi (Fig. 3). A second round of infected cells was observed between 30 and 46 hpi. New virus was first detected in medium at 24 hpi, and the number of virus particles in the medium continued to increase over time (Fig. 3). These results demonstrate that progeny virions were first released into the medium at 24 h and that these particles initiated a second round of cellular infection that peaked at 46 hpi. We decided to harvest cells at 24 hpi for the FACS titration assay, because the first round of infection peaks at this time point and also because 24 hpi is too early to detect cell-to-cell spread of virus. Previous studies have also indicated that there is minimal cell-to-cell spread of DENV in C6/36 cells at 24 hpi (8). Next, we determined if the FACS assay was able to detect quantitative differences in the number

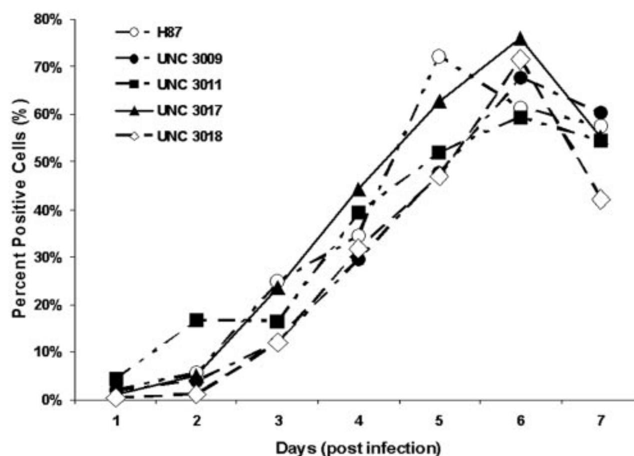

FIG. 2. Infection of C6/36 cells by different strains of DENV. The DENV3 prototype strain H87 as well as four primary DENV3 clinical isolates were used to infect C6/36 cells at an MOI of 0.01. Cells were harvested at 24-h intervals for 7 days, and infected cells were detected by FACS.

of virus particles in samples. Tenfold serial dilutions of prototype DENV2, DENV3, or clinical isolates of DENV3 were added to C6/36 cells and the number of infected cells was determined after 24 h. In initial experiments we used the prototype DENV2 (NGC) and DENV3 (H87) strains. When cells were infected with virus diluted 1:2, 1:20, and 1:200, decreasing numbers of infected cells were detected by FACS (Fig. 4A). Similar results were also obtained with clinical isolates of DENV3 (Fig. 4B). Thus, a clear correlation was observed between the concentration of virus in the inoculum and the number of infected cells detected by FACS at 24 h after infection.

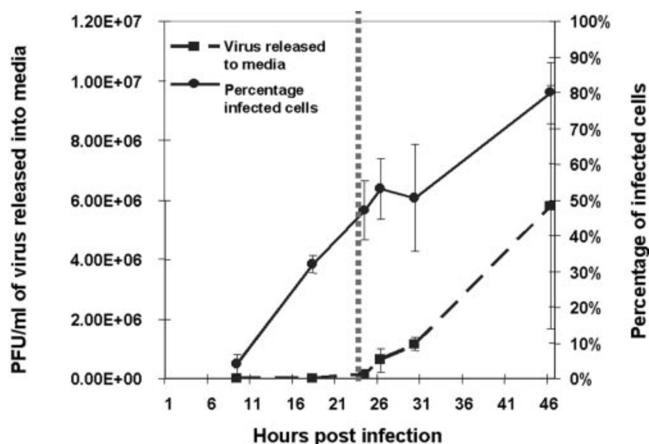

FIG. 3. Time course of cell-to-cell spread of DENV2-infected C6/36 insect cells. C6/36 cells grown in six-well plates were infected with DENV2 strain NGC at an MOI of 2. Cells and supernatants were harvested from wells at 9, 18, 24, 26, 30, and 46 h postinfection. The cells were fixed and permeabilized using the Cytofix/Cytoperm kit from BD Biosciences and stained for FACS using monoclonal antibody 4G2 to determine the percentage of infected cells; the results are expressed as a continuous line with a closed circle. The amount of virus in the supernatant at each time point was determined by plaque assay on Vero cells and is expressed as a dashed line with a closed square.

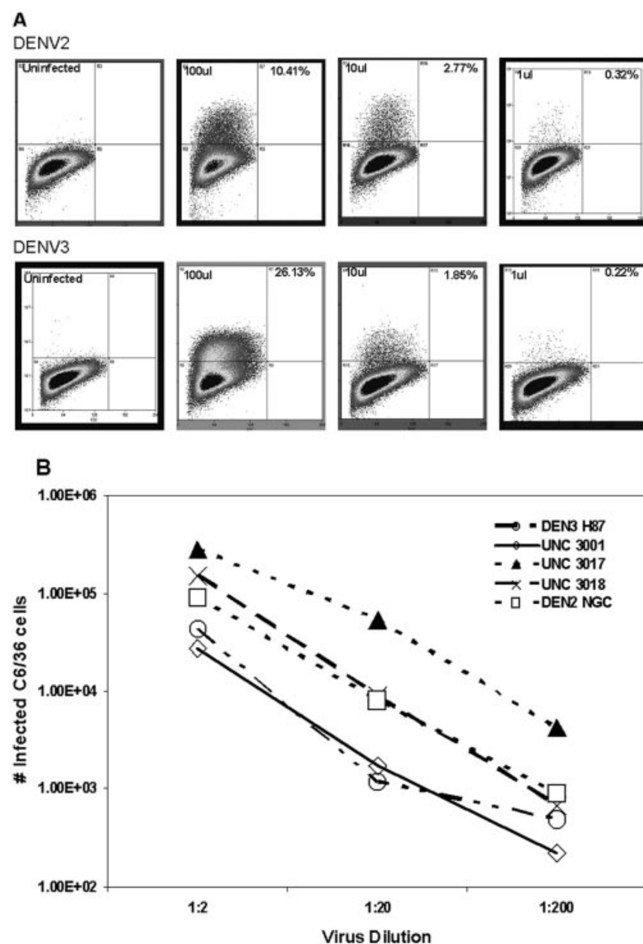

FIG. 4. Titration of dengue virus using FACS. C6/36 cells were infected with 10-fold serial dilutions of prototype strains and a clinical isolate. At 24 hpi, cells were harvested, fixed, and stained for FACS analysis. (A) Contour dot plots for DENV2- and DENV3-infected cells were obtained from FACS analysis of infected C6/36 cells at 24 h postinfection. C6/36 cells grown in six-well plates were infected for 24 h using 10-fold serial dilutions of prototype strains NGC and H87. One well served as a mock-infected control. The percentage of DENV-infected cells was determined by counting the number of cells in the upper left quadrant and dividing this number by the total number of cells in the dot plot. At 24 h postinfection, the cells in the upper left quadrant are likely a measure of the input virus, because this time point is too early for cell-to-cell spread of newly assembled virus. The dot plots shown above represent one experiment out of three and one out of six experiments for DENV2 and DENV3, respectively. (B) C6/36 cells grown in six-well plates were infected for 24 h using 10-fold serial dilutions of prototype strains NGC and H87 and clinical isolates UNC 3001, UNC 3017, and UNC 3018.

Comparison of the FACS assay to other standard dengue virus titration assays. We estimated the FACS titer of each virus stock using the following formula: titer (in infectious units/ml) = [(% of infected cells) × (total number of cells in well) × (dilution factor)]/(volume of inoculum added to cells). Plaque formation on tissue culture monolayers (Vero, LLC-MK2 [rhesus monkey kidney], and BHK [BHK 21, clone 15]) is the currently accepted method for titrating DENV. To further validate the FACS assay, we compared the titers obtained by FACS and plaque assay for different dilutions of the same preparation of virus. For these experiments, initially we used DENV2 NGC strain, which produces clear plaques on cell monolayers. The virus stock was subjected to serial fivefold dilutions, and an equal volume from each dilution was tested by plaque and FACS assays. A linear relationship was observed between the assays over 3 logs of titers ranging from 1×10^4 to 1×10^7 infectious particles or PFU/ml (Fig. 5), indicating that the two assays were similar measures of infectious virus in a sample. Furthermore, we observed that the effective range of the FACS assay was when between 0.2 and 25% of cells were infected (between ~2,000 and 25,000 infected cells per well). When the signal was below 0.2%, the assay was not sensitive and displayed high variability (data not shown). When more than 25% of the cells were infected, the assay was no longer linear, presumably because the C6/36 cells became limiting in the assay (data not shown). When titers for several strains of DENV1, DENV2, DENV3, and DENV4 were determined by FACS, plaque assay, and endpoint dilution assay, similar titers were obtained with the different assays for DENV1 (strains 16007 and UNC 1002), DENV2 (NGC), DENV3 (strains UNC 3001, UNC 3009, and UNC 3011), and DENV4 (H241) (Table 1). For DENV3 strain H87, the titers obtained with the different assays varied more than the titers of the other viruses analyzed (Table 1).

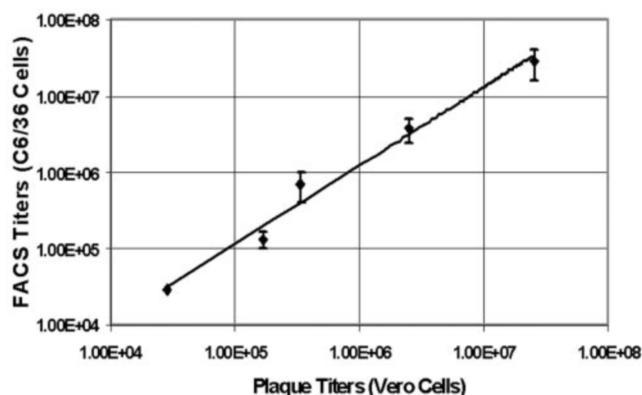

FIG. 5. Comparison of FACS titration and plaque assay methods for estimating DENV2 titers. A stock of DENV2 (1×10^7 PFU/ml) was serially diluted fivefold, and each dilution was titrated on Vero cells by plaque assay and on C6/36 cells by FACS. The lower limit of detection by FACS is 10,000 infectious units per milliliter of inoculum. The lower limit of detection by plaque assay is 50 PFU/ml.

TABLE 1. DENV titers determined by FACS, plaque assay, and and point dilution

Strain	DENV titer based on[a]:		
	FACS	Plaque assay	Endpoint dilution
DENV1 (16007)	1.5×10^7*	3.5×10^7*	Not done
DENV1 (1002)	2.9×10^6*	1.7×10^6*	Not done
DENV2 (NGC)	1.8×10^6	3.8×10^6	Not done
DENV3 (H87)	2.9×10^6	1.3×10^5	4.2×10^5
DENV3 (3001)	4.1×10^5	1.8×10^6	1.0×10^6
DENV3 (3009)	1.2×10^6	1.8×10^6	1.7×10^6
DENV3 (3011)	2.9×10^5	4.8×10^5*	1.1×10^6
DENV4 (H241)	9.7×10^6*	1.0×10^7*	Not done

[a] Most values (exceptions are marked by an asterisk) are based on at least three independent experiments.

TABLE 2. Antibody neutralization titers determined by FACS and PRNT assays[a]

Serum	FNT_{50}	FNT_{80}	$PRNT_{50}$	$PRNT_{80}$
Normal human serum	<20	<20	<20	<20
H8163	10,240	2,560	5,120	1,280
H7859	<20	<20	<20	<20

[a] Vero cells were infected with DENV2 NGC that had been incubated with serial two-fold dilutions of serum from two human patients previously infected with dengue virus. The FNT and PRNT were analyzed at 24 hpi and 6 days postinfection, respectively. The 50% and 80% neutralization titers were determined based on the percent reduction observed by each method. H8163 was from a secondary infection, and H7859 was from a primary infection, serotype unknown.

FACS-based assay to measure antibody neutralization of DENV. Interactions between antibody and DENV play roles in protective immunity and in antibody-dependent enhancement of viral infection. A current method for measuring antibody neutralization is the plaque reduction neutralization test on Vero cells (PRNT). This assay is subject to the same limitations as the plaque assay for virus titration. Experiments were conducted to determine if the FACS titration assay could be modified for measuring the ability of antisera to neutralize the virus. The DENV2 NGC strain was incubated with serial two-fold dilutions of human dengue virus immune or normal serum before adding the virus to Vero cells. At each antibody dilution the number of infectious virus particles was estimated by FACS (24 hpi) and plaque assay (6 days postinfection). The 50% and 80% neutralization titers obtained with each method were remarkably similar (Table 2).

DISCUSSION

Any assay for titrating a virus is a relative measure of infectious virus that is defined by properties of the particular virus and the methods used. The plaque assay, which has been the standard method for titrating DENV, is time-consuming and difficult to use with some primary clinical isolates. Here we describe and validate a FACS-based assay for titrating DENV that overcomes the limitations of the plaque assay. Viral titers determined by FACS were similar to titers obtained by plaque assay and endpoint dilution assay for some DENV strains but not for others. We suspect that the ability of some strains to infect the cell lines used for the different assays and/or differences in plaquing efficiency may account for the discordant titers. Another explanation for the differences in viral titers may be the time at which each experimental method was evaluated for viral infection. The FACS assay conducted at 24 h after infection measures the ability of dengue virus particles to enter a cell and produce viral antigen. The assay does not require virus assembly or cell-to-cell spread. In contrast, to obtain a positive signal in the endpoint dilution method, virus particles have to enter cells and spread from cell to cell over a period of 5 to 7 days. The FACS assay can only be used with samples with a titer greater than 1×10^4 infectious units/ml. At titers below 1×10^4/ml, the small number of infected cells in a well were below the level of detection of the FACS assay. This is a limitation of the FACS assay compared to the plaque assay, which is more sensitive. Another limitation to the FACS assay is that it requires costly equipment and trained staff and may be difficult to establish in some settings. Even with its limitations, FACS is still a powerful and effective tool for titrating virus. The FACS assay provides the ability to measure infectious viral titers as early as 24 h postinfection, whereas the plaque assay requires 5 to 7 days. Additionally, the FACS assay can be used to titrate clinical isolates that do not form clear plaques on cell monolayers.

The current method for measuring the ability of antisera to neutralize DENV is the PRNT assay. The PRNT assay is subject to the same drawbacks as the plaque titration assay. We report here that the FACS assay can also be adapted to measure the ability of antisera to neutralize the virus. When two human dengue virus immune sera were tested with the FACS and plaque neutralization assays, similar neutralization titers were obtained with both assays. Further studies are needed to validate the FACS neutralization assay. Specifically, experiments need to be done with reference sera to determine if the FACS neutralization assay can be used to identify the infecting serotype of DENV and to distinguish between primary and secondary infections. A major advantage to a FACS neutralization assay is that it could be used to measure neutralization of DENV isolates, irrespective of their ability to form plaques. Compared to existing methods for studying dengue virus, which work best with laboratory-adapted strains, we hope the FACS-based methods described here will greatly facilitate studies with primary clinical isolates of dengue virus.

REFERENCES

1. **Bordignon, J., S. C. Pires Ferreira, G. M. Medeiros Caporale, M. L. Carrieri, I. Kotait, H. C. Lima, and C. R. Zanetti.** 2002. Flow cytometry assay for intracellular rabies virus detection. J. Virol. Methods **105:**181–186.
2. **Diamond, M. S., D. Edgil, T. G. Roberts, B. Lu, and E. Harris.** 2000. Infection of human cells by dengue virus is modulated by different cell types and viral strains. J. Virol. **74:**7814–7823.
3. **Diamond, M. S., T. G. Roberts, D. Edgil, B. Lu, J. Ernst, and E. Harris.** 2000. Modulation of dengue virus infection in human cells by alpha, beta, and gamma interferons. J. Virol. **74:**4957–4966.
4. **Dominguez, J., M. M. Lorenzo, and R. Blasco.** 1998. Green fluorescent protein expressed by a recombinant vaccinia virus permits early detection of infected cells by flow cytometry. J. Immunol. Methods **220:**115–121.
5. **Halstead, S. B.** 1988. Pathogenesis of dengue: challenges to molecular biology. Science **239:**476–481.
6. **Henchal, E. A., M. K. Gentry, J. M. McCown, and W. E. Brandt.** 1982. Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. Am. J. Trop. Med. Hyg. **31:**830–836.
7. **Ho, L. J., J. J. Wang, M. F. Shaio, C. L. Kao, D. M. Chang, S. W. Han, and J. H. Lai.** 2001. Infection of human dendritic cells by dengue virus causes cell maturation and cytokine production. J. Immunol. **166:**1499–1506.
8. **Kao, C. L., M. C. Wu, Y. H. Chiu, J. L. Lin, Y. C. Wu, Y. Y. Yueh, L. K. Chen, M. F. Shaio, and C. C. King.** 2001. Flow cytometry compared with indirect immunofluorescence for rapid detection of dengue virus type 1 after amplification in tissue culture. J. Clin. Microbiol. **39:**3672–3677.
9. **Kochel, T. J., D. M. Watts, S. B. Halstead, C. G. Hayes, A. Espinoza, V. Felices, R. Caceda, C. T. Bautista, Y. Montoya, S. Douglas, and K. L. Russell.** 2002. Effect of dengue-1 antibodies on American dengue-2 viral infection and dengue haemorrhagic fever. Lancet **360:**310–312.
10. **Lindenbach, B. R., and C. M. Rice.** 2001. Flaviviridae: the viruses and their replication, p. 991–1042. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, Pa.
11. **Marovich, M., G. Grouard-Vogel, M. Louder, M. Eller, W. Sun, S. J. Wu, R. Putvatana, G. Murphy, B. Tassaneetrithep, T. Burgess, D. Birx, C. Hayes, S. Schlesinger-Frankel, and J. Mascola.** 2001. Human dendritic cells as targets of dengue virus infection. J. Investig. Dermatol. **6**(Symp. Proc.)**:**219–224.
12. **McSharry, J. J.** 2000. Analysis of virus-infected cells by flow cytometry. Methods **21:**249–257.

13. **Roberts, T. G., D. Edgil, B. Lu, J. Ernst, E. Harris, and R. Lonsdale.** 2000. A rapid method for immunotitration of influenza viruses using flow cytometry. J. Virol. Methods **110:**67–71.
14. **Russell, P. K., A. Nisalak, P. Sukhavachana, and S. Vivona.** 1967. A plaque reduction test for dengue virus neutralizing antibodies. J. Immunol. **99:**285–290.
15. **Schoepp, R. J., and B. J. Beaty.** 1984. Titration of dengue viruses by immunofluorescence in microtiter plates. J. Clin. Microbiol. **20:**1017–1019.
16. **Stowe, R. P., M. L. Cubbage, C. F. Sams, D. L. Pierson, and A. D. Barrett.** 1998. Detection and quantification of Epstein-Barr virus EBER1 in EBV-infected cells by fluorescent in situ hybridization and flow cytometry. J. Virol. Methods **75:**83–91.
17. **Sydow, F. F., M. A. Santiago, P. C. Neves-Souza, D. I. Cerqueira, A. S. Gouvea, M. F. Lavatori, A. L. Bertho, and C. F. Kubelka.** 2000. Comparison of dengue infection in human mononuclear leukocytes with mosquito C6/36 and mammalian Vero cells using flow cytometry to detect virus antigen. Mem. Instit. Oswaldo Cruz **95:**483–489.
18. **Wu, S. J., E. M. Lee, R. Putvatana, R. N. Shurtliff, K. R. Porter, W. Suharyono, D. M. Watts, C. C. King, G. S. Murphy, C. G. Hayes, and J. W. Romano.** 2001. Detection of dengue viral RNA using a nucleic acid sequence-based amplification assay. J. Clin. Microbiol. **39:**2794–2798.