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Comparison of Plaque- and Flow Cytometry-Based Methods for Measuring Dengue Virus Neutralization▽

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As dengue vaccines enter clinical trials, there is a need for rapid and quantitative assays to measure neutralization. We have developed flow-based neutralization assays which generated results similar to those generated by the established, plaque reduction neutralization test. The flow assays are an improvement, as they use human cells and allow for high-throughput screening.

Dengue viruses (DENVs) are members of the family *Flaviviridae* and consist of four serotypes, designated DENV serotype 1 (DENV1) through DENV4 (6, 11). Although there is extensive cross-reactivity among DENVs in serological tests, there is only limited cross-protective immunity in humans, and a person can have multiple infections with different serotypes during his or her life (19). People exposed to secondary DENV infections face a greater risk of severe disease, indicating that preexisting cross-reactive immunity can exacerbate disease (5, 8).

Given the evidence that immunity to DENV can lead to more severe disease, an effective dengue vaccine must provide balanced, long-term protection against all four serotypes. To evaluate the safety and efficacy of vaccines, we need precise and easy-to-use assays that measure DENV neutralization. The current "gold standard" for measuring neutralization is the plaque reduction neutralization test (PRNT) (17). PRNT is time-consuming and uses nonhuman cell lines. Some strains of DENV, especially clinical isolates, do not form plaques and cannot be used in PRNT. PRNT is also not suitable for high-throughput screening of large collections of serum specimens.

Flow cytometry has been used to detect DENV in clinical samples and to measure the ability of the virus to infect a variety of cells (1, 13). More recently, a flow cytometry-based DENV titration assay was introduced (9). Flow cytometry-based assays have also been used to measure antibody-mediated neutralization and enhancement of DENV (4, 10, 14). In this study we report on the development of 96-well-format, flow cytometry-based assays for the measurement of DENV neutralization and a comparison of the flow cytometry-based assays to the classical PRNT.

A 96-well-format, flow cytometry-based DENV neutralization assay was developed by using Vero (clone 81) cells or U937 cells expressing dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), a known attachment factor for DENV (15, 18). The DC-SIGN-transfected U937 cells were kindly provided by Thomas Morrison and Mark Heise (University of North Carolina School of Medicine). The DENVs used in this study were the reference strains, designated DENV1 WestPac-74, DENV2 S-16803, DENV3 CH-53489, and DENV4 TVP-360 (provided by Robert Putnak from the Walter Reed Army Institute of Research, Silver Spring, MD). A reference panel of human monovalent sera with neutralizing antibodies to DENV1 through DENV4 and a polyvalent serum with neutralizing antibodies to all four DENV serotypes was assembled by the World Health Organization (WHO) and was provided to us by Morag Ferguson of the National Institute for Biological Standards and Control in the United Kingdom.

The flow cytometry-based neutralization assays were performed in triplicate in 96-well cell culture plates with flat-bottom wells. Each well contained 5×10^4 Vero cells or DC-SIGN-expressing U937 cells. The amount of virus used in the assay infected between 7 and 15% of the cells. This amount of virus was used because a direct positive correlation was observed between the amount of virus added and the number of infected cells when between 1 and 30% of the cells were infected (data not shown). With greater than 30% infection, the number of infected cells did not increase in proportion to the amount of virus added, most likely because the cells become limiting in the infection assay or because not all cells are equally susceptible to infection. Human immune sera were serially diluted in fourfold steps (1:20 to 1:20,480), and the virus was preincubated with the sera in a final volume of 100 μl for 1 h at 37°C. The cells were washed, and 100 μl of the virus and serum mixture was added to the cells for 1 h at 37°C. Next, the wells were filled with cell culture medium to a total volume of 250 μl, and the plates were incubated for 24 to 48 h at 37°C in 5% CO_2. The cells were prepared for flow cytometry analysis by washing them in phosphate-buffered saline, treating them with trypsin to remove adherent cells (Vero cells only), and transferring them to 96-well plates with round-bottom wells. The cells were fixed and permeabilized by using a Cytofix/Cytoperm kit (BD-PharMingen, San Diego, CA) and stained with fluorescein isothiocyanate-conjugated monoclonal antibody 4G2, a monoclonal antibody that recognizes the flavivirus E protein, as described previously (9). The cells were analyzed with a FACScan flow cytometer (Becton Dickinson, San Diego, CA). The serum dilution that neutralized 50% of the viruses was calculated by nonlinear, dose-response regression analysis with Prism 4.0 software (GraphPad Software, Inc., San Diego, CA).

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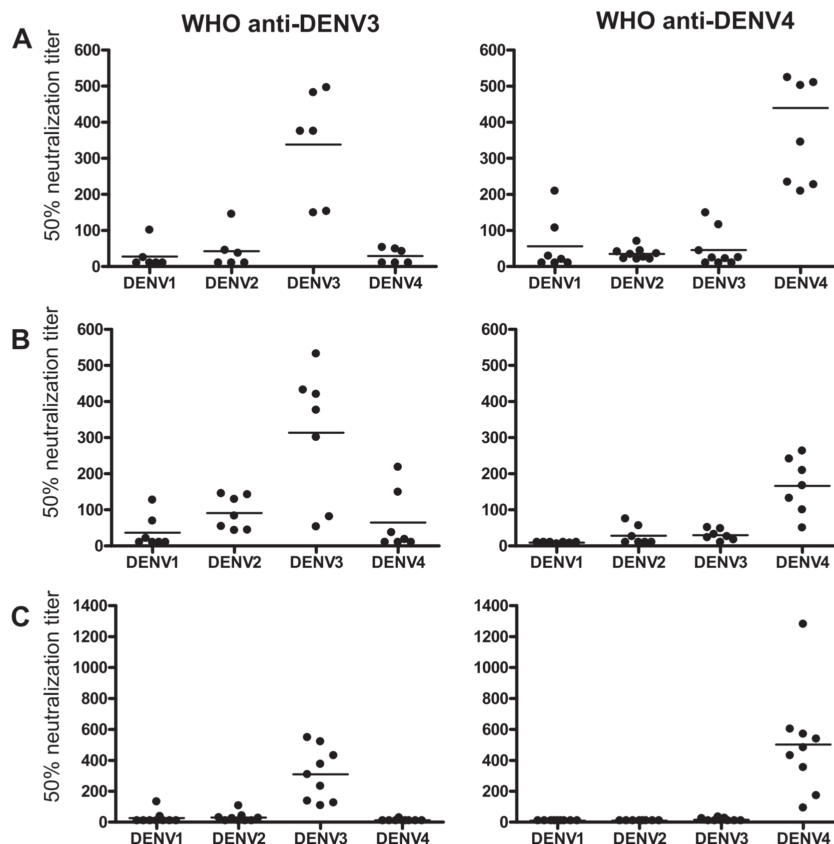

FIG. 1. Comparison of flow cytometry and plaque-based DENV neutralization assays. WHO reference dengue immune sera with monospecific immunity to DENV3 or DENV4 were analyzed by the flow cytometry-based neutralization assay with Vero cells (A) and U937 cells expressing DC-SIGN (B) and by PRNT (C). Each serum sample was tested in duplicate or triplicate against the four serotypes in three independent experiments. Dark circles, individual 50% neutralization titer measurements; horizontal lines, the mean titer for each group.

The Vero cell-based flow cytometry neutralization assay was evaluated by using WHO reference DENV immune sera specific for DENV3 (WHO anti-DENV3) and DENV4 (WHO anti-DENV4). Serial dilutions of the antisera were used in several independent neutralization experiments with all four serotypes of DENV. With the WHO anti-DENV3 immune serum sample, we obtained a mean 50% neutralization titer of 338 for DENV3 and mean 50% neutralization titers of 28, 43, and 26 for DENV1, DENV2, and DENV4, respectively (Fig. 1A). Similarly, for the WHO anti-DENV4 immune serum sample we obtained a mean 50% neutralization titer of 440 for DENV4 and mean 50% neutralization titers of 56, 35, and 46 for DENV1, DENV2, and DENV3, respectively (Fig. 1A). Thus, the flow cytometry-based neutralization assay with Vero cells gave the predicted results with these reference serum samples.

For accurate measurement of virus neutralization, the antibody must be present in excess over the amount of virus in the assay (2, 7, 16). When flow cytometry-based neutralization assays were done with all four serotypes of DENV by using twofold more virus than the amount used in the standard assay, the 50% neutralization titer remained constant, indicating that antibody was in excess when the assay was performed with our reference immune sera (data not shown).

As human hematopoietic cells are the main target of DENV infection, flow cytometry-based neutralization assays were performed with a human monocytic cell line (U937 cells) stably transfected with DC-SIGN, an attachment factor for DENV (12, 15). When the percentage of U937 cells infected in the presence of DC-SIGN was divided by the percentage of control cells (U937 without DC-SIGN) that were infected, the DC-SIGN-expressing cells were found to be 10-fold or more susceptible to infection with all four serotypes of DENV (Fig. 2 and data not shown). Initially, the DC-SIGN-expressing U937 cells were evaluated by using the WHO anti-DENV3 and anti-DENV4 immune sera (Fig. 1B). With the WHO anti-DENV3 immune serum sample, we obtained a mean 50% neutralization titer of 314 for DENV3 and mean 50% neutralization titers of 37, 91, and 65 for DENV1, DENV2, and DENV4, respectively (Fig. 1B). Similarly, for the WHO anti-DENV4 immune serum sample we obtained a 50% neutralization titer of 166 for DENV4 and mean 50% neutralization titers of <20, 28, and 30 for DENV1, DENV2, and DENV3, respectively (Fig. 1B). Thus, the flow cytometry-based neutralization assays with Vero cells (Fig. 1A) and DC-SIGN-expressing U937 cells (Fig. 1B) gave comparable results, indicating that the DENV neutralization properties of these reference sera were not affected by the use of human or nonhuman cells in the assay.

PRNT is the most widely used and accepted assay for the measurement of DENV neutralization. The WHO anti-

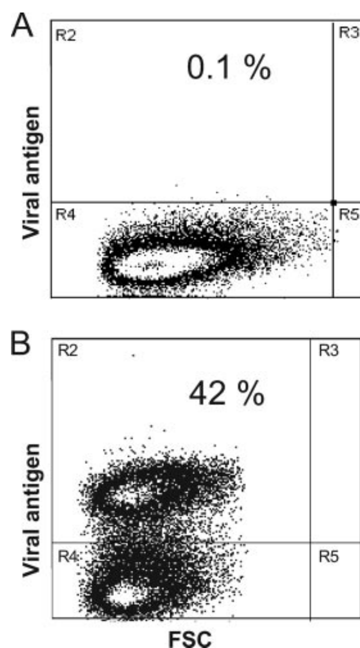

FIG. 2. U937 human monocytic cells expressing DC-SIGN are highly susceptible to DENV infection. U937 cells (A) and DC-SIGN-expressing U937 cells (B) were inoculated with DENV3 at a multiplicity of infection of 0.1. Twenty-four hours after infection, the cells were fixed, stained for viral antigen with monoclonal antibody 4G2, and analyzed by flow cytometry. The flow cytometry histograms are representative of those from three independent experiments. FSC, forward scatter.

DENV3 and anti-DENV4 immune sera were characterized by using Vero-81 cells and PRNT, as described previously (3). With the WHO anti-DENV3 immune serum sample, we obtained a mean 50% neutralization titer of 310 for DENV3 and mean 50% neutralization titers of 27, 30, and <20 for DENV1, DENV2, and DENV4, respectively (Fig. 1C). Similarly, for the WHO anti-DENV4 immune serum sample we obtained a mean 50% neutralization titer of 505 for DENV4 and mean 50% neutralization titers of <20 for DENV1, DENV2, and DENV3 (Fig. 1C). Thus, similar neutralization patterns were observed for WHO anti-DENV3 and anti-DENV4 immune sera when they were tested by PRNT and the flow cytometry-based neutralization assays (Fig. 1).

We next tested a variety of monotypic immune sera that recognized each of the four serotypes and also heterotypic immune sera from people exposed to secondary DENV infections to further compare the assays (Table 1). The sera tested consisted of monotypic WHO reference sera against each of the four serotypes (WHO anti-DENV1, anti-DENV2, anti-DENV3, and anti-DENV4 immune sera) and a heterotypic serum sample that cross-neutralized all four serotypes (WHO anti-DENV1234 immune serum). The sera also included two convalescent-phase samples from the University of North Carolina serum collection. Serum sample A was a convalescent-phase sample from a traveler who was infected with DENV2 (confirmed by virus isolation) approximately 9 years before this sample was collected. Serum sample B was from a traveler who was infected with DENV3 (confirmed by virus isolation) 3 weeks before the sample was collected. For five of the six serum samples from individuals with primary DENV infections (WHO anti-DENV1, anti-DENV2, anti-DENV3, and anti-DENV4 immune sera and serum sample A), the 50% neutralization titers for the plaque and flow cytometry-based assays were similar, in that all three assays gave the highest titers for the homologous virus (Table 1). The exception was serum sample B, which cross-neutralized all four serotypes. Serum sample B was a 3-week convalescent-phase specimen, and people exposed to primary DENV infections have neutralizing, cross-reactive antibody for several months after the ini-

TABLE 1. Comparison of flow cytometry- and plaque-based neutralization assays with reference monotypic and heterotypic dengue immune sera

Immune serum and serotype	Mean 50% neutralization titer[a]		
	PRNT (Vero cells)	Flow cytometry-based neutralization assay	
		Vero cells	DC-SIGN-expressing U937 cells
WHO anti-DENV1			
DENV1	**125**	**75**	**168**
DENV2	<20	22	41
DENV3	<20	<20	<20
DENV4	<20	<20	<20
WHO anti-DENV2			
DENV1	<20	<20	<20
DENV2	**104**	**157**	**199**
DENV3	<20	<20	<20
DENV4	<20	<20	<20
WHO anti-DENV3			
DENV1	27	28	37
DENV2	30	43	91
DENV3	**310**	**338**	**314**
DENV4	<20	29	65
WHO anti-DENV4			
DENV1	<20	56	<20
DENV2	<20	35	28
DENV3	<20	46	30
DENV4	**503**	**440**	**166**
WHO anti-DENV1234			
DENV1	413	ND[b]	>1,280
DENV2	663	ND	>1,280
DENV3	518	ND	>1,280
DENV4	117	ND	>1,280
Serum sample A[c]			
DENV1	78	58	25
DENV2	**1,203**	**651**	**371**
DENV3	39	<20	35
DENV4	58	<20	44
Serum sample B[d]			
DENV1	371	>5,120	>1,280
DENV2	>1,280	996	1,145
DENV3	>1,280	>5,120	>1,280
DENV4	>1,280	3,529	209

[a] The values in each cell are the mean 50% neutralization titers determined from two to three independent experiments. With monotypic dengue immune sera, the highest titer for each assay is indicated in boldface.

[b] ND, not determined.

[c] Serum A is a convalescent-phase sample collected 9 years after a primary DENV2 infection.

[d] Serum B is a convalescent-phase sample collected 3 weeks after a primary DENV3 infection.

tial infection. The convalescent-phase serum sample from an individual exposed to a secondary DENV infection (WHO anti-DENV1234 immune serum) cross-neutralized all four serotypes when it was tested by the three different assays. Although there were differences in the mean absolute neutralization titers for each assay, this variability was no greater than the intra-assay variability typically seen for plaque and flow-based DENV neutralization assays.

It appears that DENV neutralization is primarily dependent on virus-antibody interactions and not on the cell types used in these assays or the method used to detect infected cells. Our results are in agreement with those of a similar study recently conducted by Martin and colleagues who compared a flow cytometry-based assay using Raji cells transfected with DC-SIGN to PRNT (14). Using monoclonal antibodies and DENV immune sera, those investigators observed qualitatively and quantitatively similar neutralization titers when the flow cytometry-based and plaque-based assays were compared. We recommend that the flow cytometry-based assays replace the DENV PRNT. The flow assays are rapid and suitable for the screening of large panels of sera by using the 96-well plate format described here. The flow cytometry-based assay can also be performed with human cell lines and even primary human cells. Finally, many strains of DENV, including low-passage clinical isolates, can be used in the flow cytometry-based neutralization test, as the virus is not required to form plaques.

The flow cytometry-based assays also have some limitations. The intra-assay variability was high especially when the same samples were tested on different days (Fig. 1A and B). In this respect, the flow cytometry-based assays were not very different from PRNT, which also has high variability (Fig. 1C). Experiments are currently being done to further improve the reproducibility of the flow cytometry-based neutralization assays. It is also more difficult to achieve conditions of antibody excess with the flow cytometry-based assay than with the plaque assay. In a typical PRNT setup, less than a 100 infectious units of virus are incubated with antibody per well. The flow cytometry-based assay requires 1,000 to 10,000 infectious units per well. Even with these high concentrations of virus, it was easy to achieve antibody excess with human DENV immune sera. However, with low concentrations of specific antibody such as those found in hybridoma supernatants, one might have to refine the assay to ensure antibody excess. As flow cytometry requires expensive equipment that is difficult to maintain, the flow cytometry-based neutralization assay can be performed only in selected laboratories.

In conclusion, flow cytometry-based neutralization assays offer significant advantages over PRNT for measuring antibody neutralization of DENV. As dengue vaccines are already being tested in clinical trials, there is a need for assays that can rapidly screen large numbers of serum samples for their neutralization of contemporary strains of DENV. The 96-well-format flow cytometry-based neutralization assays described here will greatly facilitate the characterization of human antibody responses following vaccination and natural infection.

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