FLOW CYTOMETRIC ANALYSIS OF DENGUE VIRUS-INFECTED CELLS IN PERIPHERAL BLOOD

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Abstract. With the development of permeabilization techniques in flow cytometry and the availability of various monoclonal antibodies (MAbs) that specifically bind with cell surface and intracellular antigens, it is now possible to use flow cytometric assay to identify dengue virus (DEN) infected cells in peripheral blood. Blood samples were analyzed using phycoerythrin (PE) labeled anti-CD3, anti-CD14, anti-CD16, and anti-CD19 antibodies and Alexa Fluor® 488 labeled anti-flavivirus monoclonal antibody (MAb) 6B6C-1. The predominant DEN-infected cells were $CD19^+$ in this study. There was dim partial to moderately bright partial expression of CD19 positive cells in the blood samples tested. Virus isolation and serotype-specific RT-PCR revealed the cells were infected with dengue serotype 3 (DEN3). Our results suggest B cells may play an important role in DEN1 and DEN3 replication, and dissemination *in vivo*.

INTRODUCTION

The four different dengue (DEN) serotypes (DEN1, DEN2, DEN3, DEN4) are major causative agents of morbidity and mortality in children in tropical regions of the world, including the Philippines. Dengue fever (DF) is a self-limited febrile disease with moderate manifestations. In some cases, patients develop life threatening complications, such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Dengue viruses replicate well in the cell clone C6/36 from *Aedes albopictus* (Igarashi, 1978; Gubler, 1998). Vero and LLC-MK2, both originating from monkey kidneys, are also permissive cell lines. *In vitro*, dengue viruses have been shown to infect a variety of human cells, including a myelomonocytic cell line (K562), a B cell line (Raji) and a T cell line (HSB-2) (Kurane *et al*, 1990). However, the identification of *in vivo* cells infected with dengue virus and the pathogenesis of this infection remain poorly understood. DEN antigen has been detected in peripheral $CD19^+$ cells (B cells) in DEN2 infected patients (Kinoshita *et al*, 2009). The present study aimed to analyze the major replication site(s) of other serotypes of DEN in the blood of patients with DF, DHF, and DSS using two-color flow cytometric

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analysis. A flavivirus group-specific MAb 6B6C-1 was used to detect and quantitate intracellular flavivirus antigens in infected cells. The double stained cells were analyzed using the FACSCalibur.

MATERIALS AND METHODS

Patients

Blood samples were collected from clinically suspected dengue cases within a five-day period from the onset of fever from August 2006 to May 2009 at the National Children's Hospital (NCH), San Lazaro Hospital (SLH) and University of the Philippines (UP) Health Service. A total 60 males and 56 females, ages 1 to 41 years old were enrolled in the study. Informed consent was obtained from all study participants. Plasma samples were analyzed by an in-house IgM-Capture ELISA, RT-PCR and virus isolation.

Flow cytometric analysis

Peripheral blood mononuclear cells (PBMCs) were fixed and permeabilized into subsets, each defined by a MAb to a cluster designation (CD) marker. The subsets and CDs were thymocytes and mature T lymphocytes (CD3); monocytes and macrophages (CD14); NK cells, granulocytes and macrophages (CD16); and Pan B cells (CD19). Briefly, 100 µl of EDTA whole blood cells were stained with PE-labeled MAb against the cell surface marker CD3 (eBioscience, San Diego, CA), CD14 (BioLegend, San Diego, CA), CD16 (BD Pharmingen™, New Jersey), and CD19 (eBioscience, San Diego, CA), washed twice with phosphate buffered saline (PBS) and then fixed with IC Fix Buffer™ (Biosource, Camarillo, CA) for 10 minutes on ice. The cells were washed twice with PBS and permeabilized with IC Perm™ Buffer (Biosource, Camarillo, CA) for 1 minute at room temperature. The permeabilized cells were then washed and stained with Alexa Fluor® 488 labeled anti-flavivirus 6B6C-1 for 30 minutes on ice. Alexa Fluor® 488 conjugated mouse IgG2a (eBioscience, San Diego, CA) was used as the isotype control for Alexa Fluor® 488 conjugated 6B6C-1. After incubation, the cells were washed twice with IC Perm™ Buffer (Biosource, Camarillo, CA) and then suspended in PBS. Fluorescence activated cell sorter (FACS) data acquisition of the double-stained cells was performed using the FACSCalibur (Beckton Dickinson Immunocytometry Systems). Ten thousand (10,000) cells were acquired and analyzed using CellQuest (Beckton Dickinson Biosciences) software.

Virus isolation

Plasma samples were inoculated into a monolayer culture of the C6/36 cell line and incubated in Eagle's minimum essential medium (EMEM) as previously described (Igarashi *et al*, 1978).

RESULTS

The blood samples were tested by RT-PCR, IgM-Capture ELISA, virus isolation and FACS. The detection rates of these assays were 37/115 (32%), 28/116 (24%), 31/103 (30%), and 10/116 (9%), respectively (Table 1).

PBMCs from 37 RT-PCR-positive patients were analyzed using a two-color staining flow cytometry assay. DEN antigen was detected in the PBMCs of 10 patients (27.8%). Figs 1 and 2 show representative dot plots of DEN-infected cells. The cell population identified as positive for DEN-infected cells were B cells (CD19). In this study, the positive rates for dengue virus infected cells by flow cytometry ranged from 20% to 81% for B (CD19) cells in the PBMCs from the patients. There was dim partial to moderately bright partial

Table 1
Results of dengue diagnostic tests.

Patient no.	Age	Sex	Sample date	Platelet (mm^3)	RT-PCR	ELISA	FACS	Culture	Type
1	2	F	4 days	297	+	-	CD19	NT	D1
2	8	M	4 days	ND	+	-	-	+	D3
3	6	M	5 days	144	+	-	-	+	D3
4	5	F	4 days	187	-	+	-	-	-
5	6	M	3 days	ND	-	+	-	-	-
6	13	M	ND	150	+	-	-	+	D3
7	ND	F	3 days	280	-	+	-	-	-
8	7	M	3 days	200	+	-	-	-	D3
9	13	M	4 days	125	+	-	-	+	D3
10	6	M	3 days	140	+	-	-	+	D3
11	17	F	3 days	180	-	-	-	+	-
12	9	M	4 days	103	+	-	-	-	D3
13	15	F	ND	116	-	+	-	-	-
14	10	F	3 days	230	+	-	-	+	D1
15	12	F	4 days	170	-	+	-	-	-
16	4	M	3 days	110	-	+	-	-	-
17	1	M	5 days	100	-	+	-	-	-
18	10	F	4 days	200	-	+	-	-	-
19	4	M	3 days	100	-	+	-	-	-
20	5	F	5 days	100	-	+	-	NT	-
21	25	F	8 days	265	-	+	-	NT	-
22	19	M	4 days	19	-	+	-	-	-
23	32	M	5 days	55	-	+	-	-	-
24	25	M	5 days	83	+	+	CD19	-	-
25	19	F	3 days	70	-	+	-	-	-
26	18	M	5 days	29	-	+	-	-	-
27	14	F	4 days	90	+	-	CD19	NT	D3
28	15	M	4 days	45	+	-	-	NT	D3
29	23	M	4 days	24	+	-	-	NT	D3
30	32	F	3 days	19	+	-	-	NT	D3
31	14	M	3 days	65	+	-	-	NT	D3
32	10	M	3 days	96	-	+	-	-	-
33	10	M	3 days	131	-	+	-	NT	-
34	18	F	4 days	50	+	-	-	+	D2
35	21	F	ND	88	+	-	-	+	D3
36	18	F	3 days	82	-	+	-	-	-
37	ND	ND	ND	ND	-	+	-	-	-
38	28	F	ND	106	-	-	-	+	-
39	23	F	ND	65	-	-	-	+	-
40	16	F	ND	97	-	-	-	+	-
41	15	M	ND	85	+	-	CD19	+	D3
42	17	F	ND	73	+	-	CD19	+	D3
43	11	F	ND	138	+	-	-	+	D3
44	26	F	ND	111	-	+	-	+	-

Table 1 (Continued).

Patient no.	Age	Sex	Sample date	Platelet (mm^3)	RT-PCR	ELISA	FACS	Culture	Type
45	7	M	ND	158	-	+	-	-	-
46	17	F	3 days	61	-	+	-	-	-
47	21	M	ND	105	-	+	-	-	-
48	21	M	ND	115	-	+	-	-	-
49	11	F	ND	168	+	-	CD19	+	D3
50	14	M	ND	139	+	-	CD19	+	D3
51	33	F	ND	3	+	-	-	-	D3
52	20	F	ND	134	-	+	-	NT	-
53	16	M	ND	113	+	-	CD19	NT	D3
54	41	M	ND	108	+	-	CD19	NT	D3
55	18	F	5 days	64	+	-	-	+	D3
56	13	F	4 days	139	+	-	-	+	D3
57	18	M	ND	122	+	-	CD19	+	D3
58	18	M	ND	56	+	-	CD19	+	D3
59	15	M	4 days	168	+	-	-	+	D3
60	15	M	ND	82	+	-	-	-	-
61	13	F	ND	131	+	-	-	+	D3
62	13	F	ND	62	+	-	-	-	-
63	15	F	ND	83	+	-	-	+	D3
64	8	M	ND	150	-	+	-	-	-
65	18	M	ND	79	-	+	-	-	-
66	ND	M	ND	ND	+	-	-	+	D2
67	38	F	ND	96	-	-	-	+	-
68	14	F	ND	118	-	-	-	+	-
69	19	M	2 days	81	+	-	-	+	D3
70	28	M	4 days	51	+	-	-	+	D2
71	36	F	3 days	112	+	-	-	+	-

The patient No. 1 was analyzed in the previous study which was conducted in 1999.
Only dengue diagnostic test positive cases are shown in Table 1.
ND, no data; NT, not tested

expression of B cells in the blood samples tested. Based on virus isolation and serotyping, the patients were found to be infected with DEN1 or DEN3.

DISCUSSION

Fc receptor-bearing cells of monocyte lineage have long been considered to be the principal target cells for DEN infection. The antibody dependent enhancement (ADE) hypothesis regarding DEN infection has yet to be demonstrated *in vivo* (Durbin *et al*, 2008).

Hypotheses for DHF/DSS have proposed a major role for T and B cells as primary target cells or effector cells for DEN infection. T and B cells produce cytokines that cause endothelial damage prior to the onset of DHF/DSS (Blackley *et al*, 2007). King *et al* (1999) demonstrated that in mononuclear cells isolated from DHF patients, the majority of virus was recovered

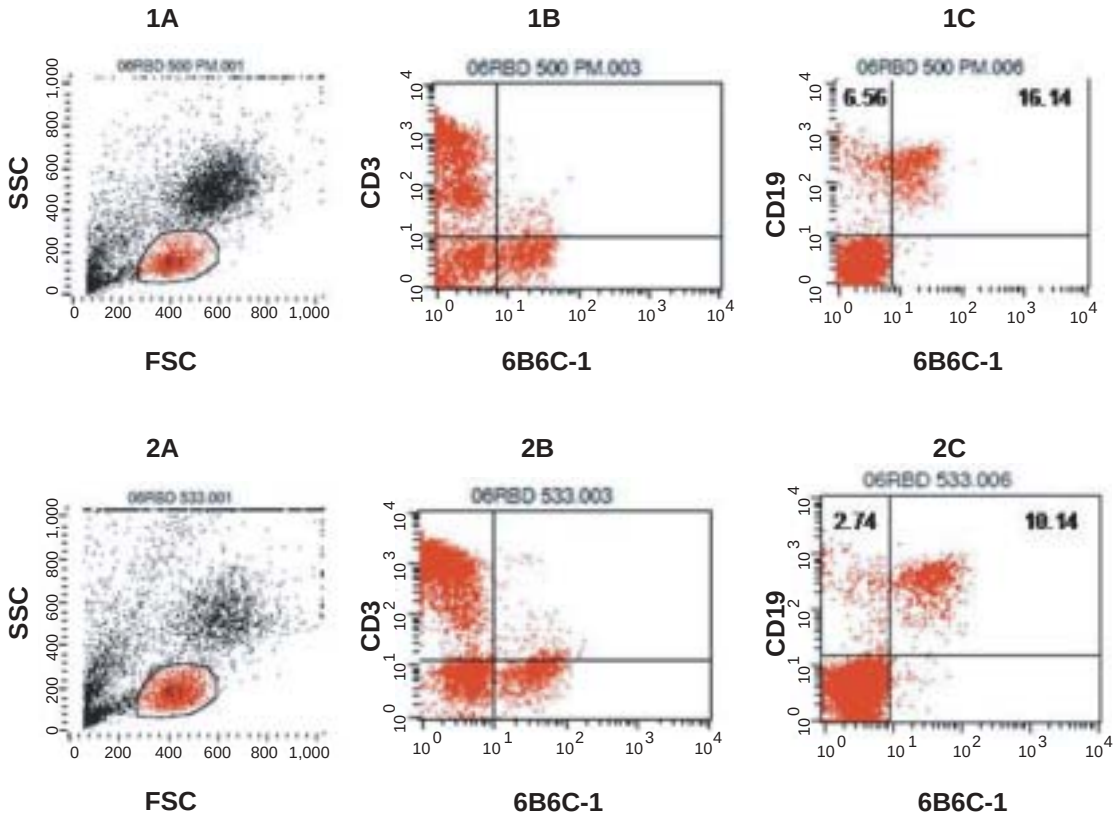

Fig 1–Dot plot encompassing the leukocyte population (1A, 2A). PBMCs stained with CD3 PE and 6B6C-1 (1B,2B). Flow cytometric analysis of B cells (1C,2C). The lower-left (LL) quadrant in 1C and 2C displays the events that were negative for both CD19 PE and Alexa Fluor® 488 labeled anti-flavivirus 6B6C-1. The upper-left (UL) quadrant in 1C and 2C contains the events that were negative for both CD19 PE and Alexa Fluor® 488 labeled anti-flavivirus 6B6C-1. The lower-right (LR) quadrant in 1C and 2C contains the events that were negative for Alexa Fluor® 488 labeled anti-flavivirus 6B6C-1. The upper-right (UR) quadrant in 1C and 2C contains the events that were positive for both CD19 PE and Alexa Fluor® 488 labeled anti-flavivirus 6B6C-1. The respective quadrant statistics for the CD19 positive isolates are shown.

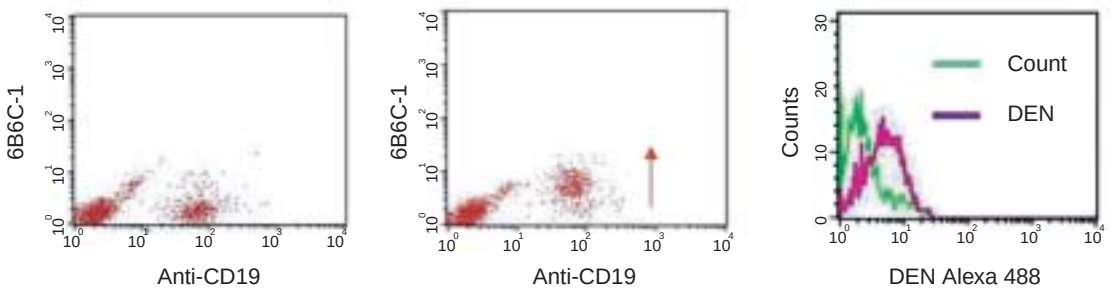

Fig 2–DEN1 infected B cells by FACS analysis.

from B cell subsets. Other studies have reported the levels of virus replication and cytokine responses observed in B cells were not much different from those in monocytes (Huang *et al*, 2000; Lin *et al*, 2002).

Based on several studies, mononuclear phagocytes, such as monocytes, macrophages, and dendritic cells, are considered to be the primary targets of DEN virus replication. Kuo *et al* (2008) showed that monocytes, but not T or B cells, are the primary target cells for DEN infection based on an *in vitro* assay using the C6/36 mosquito cell line. Durbin *et al* (2008) reported approximately 20% of cells with detectable non-structural protein 3 (NS3) were CD14 negative by flow cytometric analysis, suggesting that replication is occurring in cells other than monocytes. These cells may include NK cells, dendritic cells and B cells.

Based on the results of this study, RT-PCR was found to be the most sensitive assay, with a positivity rate of 32%. Virus isolation and serotyping showed the cells were infected with DEN1 or DEN3 with a detection rate of 30%. Two-color staining flow cytometric analysis detected DEN antigen in 10 dengue patients. Of the 10 FACS positive cases, the predominantly DEN infected cells were B ($CD19^+$) cells in this study. DEN3 infection was confirmed by virus isolation and serotype specific RT-PCR. This is important for viral surveillance as well as pathogenesis studies. RT-PCR provides a rapid serotype-specific diagnosis. It has similar sensitivity to virus isolation using the C6/36 cell line. However, it should not be used to replace virus isolation. IgM-Capture ELISA has the advantage of detecting dengue infections using a single sample. However, a negative finding may be false-negative because the blood sample could have been extracted before detectable IgM appeared. Consistent with the findings of King *et al* (1999), we found B cells are a major replication site for DEN3 as well as for dissemination *in vivo*. We also found a case of DEN1 infecting B cells on FACS analysis in the previous study (Fig 2). The determination of major replication site(s) of DEN in peripheral blood may lead to elucidation of dengue pathogenesis.

Flow cytometric analysis has the advantage of detecting the presence of DEN antigens and identifying a single infected cell type in PBMC's. Flow cytometry has an advantage over RT-PCR amplication in that it can quantify directly the number of infected cells by antigen detection (Sydow *et al*, 2000; Lambeth *et al*, 2005). Our study confirms the findings that flow cytometric assay is a useful tool for the identification of DEN-infected cells in PBMC's and can be used to detect both cell surface and intracellular antigens. DEN2 antigen was detected in a DHF patient at a high level of positivity (59% of peripheral CD19 cells) by flow cytometric assay (Kinoshita *et al*, 2009). In our study, 20% to 81% of B (CD19) cells in the PBMCs were confirmed to have DEN3 infection. Our results agree with the above study, in that we demonstrated B cells may have DEN infection. B cells play an important role in DEN1 and DEN3 infection.

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