Arch Virol (2014) 159:103–116
DOI 10.1007/s00705-013-1787-3

ORIGINAL ARTICLE

Determination of antibody concentration as the main parameter in a dengue virus antibody-dependent enhancement assay using FcγR-expressing BHK cells

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Received: 9 January 2013 / Accepted: 26 April 2013 / Published online: 31 July 2013

Abstract Dengue virus (DENV) causes a life-threatening illness, with a wide range of symptoms from mild febrile illness, dengue fever (DF), to life-threatening illness, dengue hemorrhagic fever (DHF). Antibody-dependent enhancement (ADE) is considered to be a risk factor for DHF. In the present study, we determined the parameters for ADE assays using FcγR-expressing BHK cells. Monoclonal antibodies and human serum samples were used in the assays. We examined antibody concentration and virus concentration and analyzed whether antibody concentration or DENV-antibody ratio determines ADE activity. Virus growth was quantified by a conventional plaque titration method using FcγR-expressing BHK cells. The assay allowed the detection of DENV growth with inoculation doses ranging from 10^2 PFU/ml to 10^6 PFU/ml using monoclonal antibodies and undiluted or diluted serum samples. The results indicate that antibody concentration rather than DENV-antibody ratio determines the demonstration of ADE activity. Thus, antibody concentration rather than multiplicity of infection was defined as the main determinant in ADE assays using FcγR-expressing BHK cells.

Abbreviations

DENV	Dengue virus
DF	Dengue fever
DHF	Dengue hemorrhagic fever
ADE	Antibody-dependent enhancement
BHK cells	Baby hamster kidney cells
FcγR	Fc gamma receptor
PFU	Plaque-forming unit

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Introduction

Antibody-dependent enhancement (ADE) of virus infection has been reported for various viruses [1–6], and it has been suggested that ADE may contribute to disease severity in DENV infection [7–12]. In particular, the possibility that dengue-vaccine-induced ADE might potentially increase the risk of severe dengue illness has been a concern in vaccine development [13–15]. It is therefore necessary to develop assays and methodologies to investigate the infection-enhancing activity of antibodies induced by natural infection and vaccination.

Fc gamma receptor (FcγR)-expressing cell lines have proved useful in ADE studies *in vitro* [16–20]. Additionally, human peripheral blood mononuclear cells (PBMCs) have been used in the determination of ADE activity using undiluted serum samples [21]. The use of primary human monocytes has been limited because of their lesser convenience for use and limited availability compared to established cell lines [22], in addition to poor viral replication at low passages and high experimental variation [23]. Cell lines, including a human acute monocytic leukemia cell line (THP1), a human myelogenous cell line (K562), and a mouse macrophage cell line (PD3881), have also been used in studies of ADE activity using human serum samples [17, 24–28]. Although assay reproducibility has been reported previously in ADE patterns using established cell lines, the amount of infectious virus required to infect the FcγR-expressing hematopoietic cell lines varied among DENV isolates [28]. Furthermore, hematopoietic cell lines are naturally non-adherent, and thus titration of infectious virus particles requires surrogate plaque titration assays.

We have previously developed an antibody-dependent enhancement assay for DENV using BHK cells that

Table 1 Antibody-dependent enhancement activity of monoclonal antibody (mAb) 6B6C using different concentrations of DENV-2

Virus dose/antibody dilution	FcγR-negative BHK cells		FcγR-expressing BHK cells	
	Virus titer (log10 PFU/ml)	Fold enhancement	Virus titer (log10 PFU/ml)	Fold enhancement
mAb 6B6C				
(a) 6 (log10) PFU/ml				
No dilution	4.12	0.01	4.51	0.04
10^1	4.90	0.08	5.56	0.43
10^2	4.90	0.08	6.03	1.29
10^3	5.60	0.38	6.64	5.24
10^4	5.78	0.58	6.88	9.05
No antibody	6.02	1.00	5.92	1.00
(b) 5 (log10) PFU/ml				
No dilution	3.86	0.09	4.72	0.43
10^1	4.08	0.14	5.17	1.23
10^2	4.45	0.33	5.51	2.67
10^3	4.72	0.62	5.68	4.00
10^4	4.92	1.00	5.92	7.00
No antibody	4.92	1.00	5.08	1.00
(c) 4 (log10) PFU/ml				
No dilution	NT[a]	-[b]	3.56	0.20
10^1	3.08	0.07	4.56	1.96
10^2	3.56	0.21	4.72	2.83
10^3	4.08	0.71	5.05	6.09
10^4	4.16	0.85	5.00	5.43
No antibody	4.23	1.00	4.26	1.00
(d) 3 (log10) PFU/ml				
No dilution	1.90	0.06	2.30	0.11
10^1	2.51	0.24	3.60	2.13
10^2	2.83	0.50	4.00	5.32
10^3	3.15	1.03	4.18	8.09
10^4	3.16	1.06	3.96	4.89
No antibody	3.13	1.00	3.27	1.00
(e) 2 (log10) PFU/ml				
No dilution	NT	-	0.60	0.13
10^1	NT	-	1.20	0.50
10^2	0.61	0.34	1.38	0.75
10^3	0.91	0.67	2.00	3.13
10^4	1.08	1.00	1.94	2.75
No antibody	1.08	1.00	1.51	1.00

[a] NT indicates that virus was below the detection limit

[b] - indicates that fold enhancement values could not be determined

stably expresses human FcγR [29]. ADE activity was constantly detected in DENV-antibody-positive human serum samples by using the FcγR-expressing cells. In this paper, we report the parameters that influence ADE activity levels using monoclonal antibodies and serum samples from dengue patients in the early and late phases of the disease.

Materials and methods

Virus

Dengue virus (DENV) strain DENV-2 (D2/Hu/OPD030-NIID/2005, GenBank accession no. AB219135) was used in all infection assays. The DENV-2 strain was isolated

Table 2 Antibody-dependent enhancement activity of monoclonal antibody (mAb) 4G2 using different concentrations of DENV-2

Virus dose/antibody dilution	FcγR-negative BHK cells		FcγR-expressing BHK cells	
	Virus titer (log10 PFU/ml)	Fold enhancement	Virus titer (log10 PFU/ml)	Fold enhancement
mAb 4G2				
(a) 6 (log10) PFU/ml				
No dilution	4.30	0.04	4.60	0.06
10^1	4.60	0.08	5.45	0.39
10^2	5.08	0.23	5.75	0.78
10^3	5.51	0.62	6.90	11.11
10^4	5.88	1.46	6.20	2.22
No antibody	5.72	1.00	5.86	1.00
(b) 5 (log10) PFU/ml				
No dilution	4.48	0.36	4.08	0.13
10^1	4.60	0.48	4.08	0.13
10^2	4.08	0.14	4.83	0.74
10^3	4.68	0.57	5.81	7.07
10^4	4.72	0.62	5.38	2.61
No antibody	4.92	1.00	4.96	1.00
(c) 4 (log10) PFU/ml				
No dilution	3.74	0.20	4.19	0.58
10^1	4.17	0.54	5.35	8.36
10^2	4.24	0.62	5.46	10.75
10^3	4.35	0.81	5.23	6.27
10^4	4.35	0.81	4.81	2.39
No antibody	4.44	1.00	4.41	1.00
(d) 3 (log10) PFU/ml				
No dilution	2.08	0.07	3.08	0.68
10^1	2.38	0.14	3.60	2.27
10^2	2.92	0.50	4.02	5.91
10^3	3.20	0.95	4.11	7.27
10^4	3.20	0.95	3.54	1.99
No antibody	3.23	1.00	3.25	1.00
(e) 2 (log10) PFU/ml				
No dilution	NT[a]	-[b]	NT	-
10^1	0.61	0.20	1.08	0.43
10^2	0.91	0.40	1.92	3.00
10^3	0.91	0.40	1.78	2.14
10^4	1.30	1.00	1.68	1.71
No antibody	1.30	1.00	1.45	1.00

[a] NT indicates that virus was below the detection limit

[b] - indicates that fold enhancement values could not be determined

from a patient in East Timor and belonged to the genotype 2 group [30].

Cell lines

BHK-21, a hamster kidney cell line (Japan Health Science Research Resources Bank), was cultured in Eagle's minimum essential medium (EMEM; Sigma, St. Louis, MO, USA) supplemented with 10 % heat-inactivated FBS (Sigma) without antibiotics at 37 °C in 5 % CO_2. FcγR-expressing BHK cell lines [29] were cultured in EMEM supplemented with 10 % heat-inactivated FBS and 0.5 mg/ml neomycin (PAA Laboratories GmbH, Austria)

Table 3 Antibody-dependent enhancement activity of monoclonal antibody (mAb) 3H12 using different concentrations of DENV-2

Virus dose/antibody dilution	FcγR-negative BHK cells		FcγR-expressing BHK cells	
	Virus titer (log10 PFU/ml)	Fold enhancement	Virus titer (log10 PFU/ml)	Fold enhancement
mAb 3H12				
(a) 6 (log10)PFU/ml				
No dilution	4.30	0.02	4.60	0.07
10^1	4.90	0.10	4.90	0.14
10^2	4.60	0.05	5.56	0.64
10^3	5.56	0.43	6.68	8.57
10^4	5.92	1.00	6.83	12.14
No antibody	5.92	1.00	5.75	1.00
(b) 5 (log10) PFU/ml				
No dilution	3.90	0.15	3.60	0.05
10^1	3.90	0.15	4.45	0.37
10^2	3.90	0.15	4.45	0.37
10^3	4.45	0.54	5.72	6.84
10^4	4.72	1.00	5.90	10.53
No antibody	4.72	1.00	4.88	1.00
(c) 4 (log10) PFU/ml				
No dilution	2.90	0.06	3.51	0.23
10^1	3.30	0.14	3.72	0.37
10^2	3.92	0.60	4.51	2.29
10^3	4.11	0.91	5.23	12.00
10^4	4.24	1.23	5.09	8.86
No antibody	4.15	1.00	4.15	1.00
(d) 3 (log10) PFU/ml				
No dilution	2.08	0.09	2.51	0.19
10^1	2.75	0.44	3.00	0.58
10^2	2.92	0.66	3.96	5.35
10^3	3.13	1.06	4.24	10.00
10^4	3.21	1.28	3.98	5.58
No antibody	3.11	1.00	3.24	1.00
(e) 2 (log10) PFU/ml				
No dilution	NT[a]	-[b]	NT	-
10^1	0.61	0.09	0.90	0.22
10^2	0.61	0.09	1.88	2.11
10^3	1.51	0.73	2.41	7.11
10^4	1.60	0.91	2.18	4.22
No antibody	1.64	1.00	1.56	1.00

[a] NT indicates that virus was below the detection limit

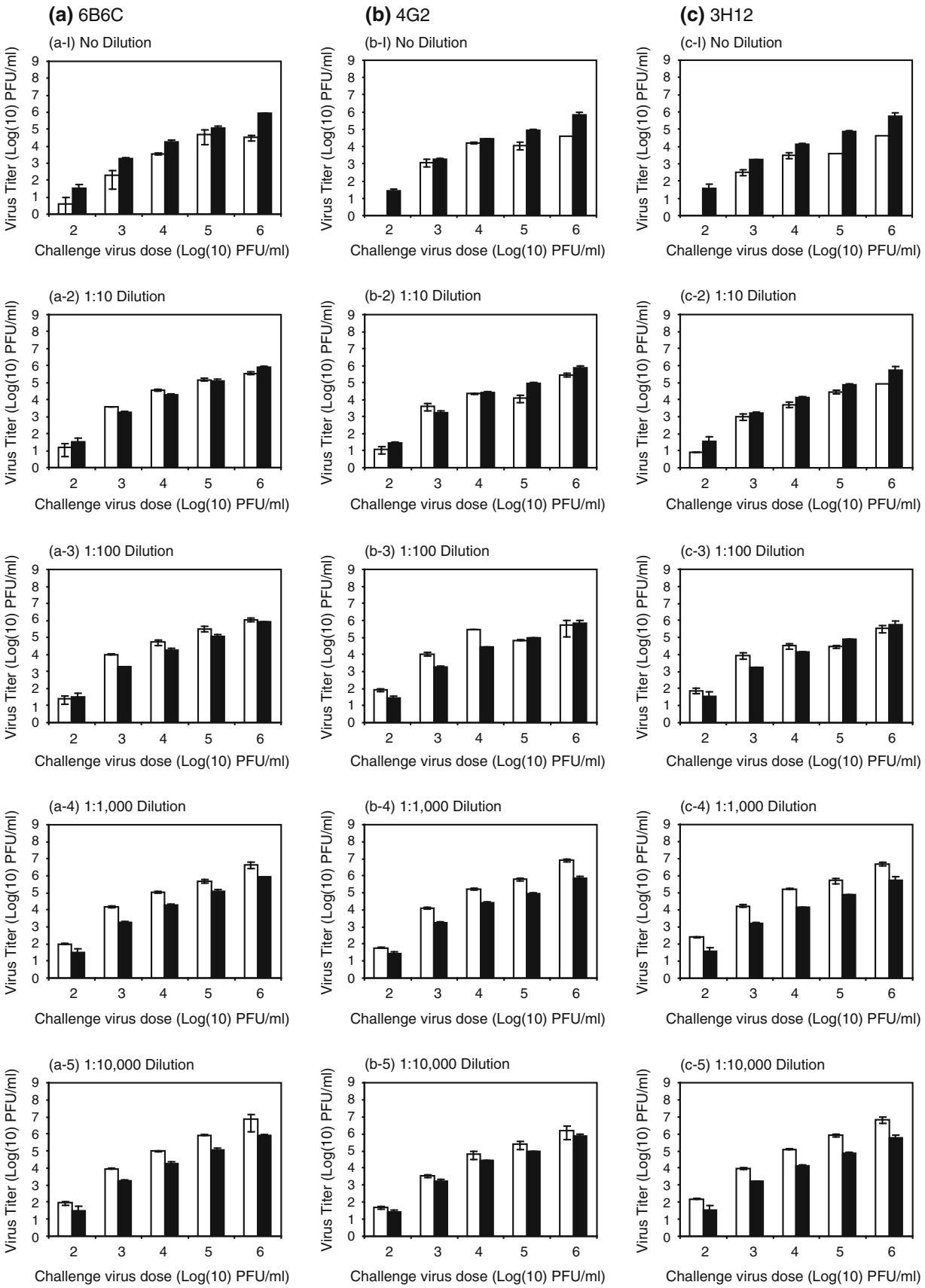
[b] - indicates that fold enhancement values could not be determined

Monoclonal antibodies and human serum samples

Flavivirus cross-reactive mouse monoclonal IgG antibodies (mAb) used in this study were 6B6C (5.9 mg/ml), 4G2 (2.6 mg/ml, ATCC MAb HB-112 D1-4G2-4-15), and 3H12 (1.6 mg/ml) [31–33].

Four serum samples were obtained from two patients (two samples each) with secondary DENV-2 infection.

Fig. 1 Enhancement of DENV-2 infection by monoclonal antibodies. ▶ **a** mAb 6B6C, **b** mAb 4G2 and, **c** mAb 3H12. The mAbs were serially diluted tenfold. The following dilutions were used: a–1, b–1, and c–1, no dilution; a–2, b–2, and c–2, 1:10; a–3, b–3, and c–3, 1:100; a–4, b–4, and c–4, 1:1,000; a–5, b–5, and c–5, 1:10,000. Each of these tenfold serial dilutions was reacted with each dose of the serially diluted challenge virus (1×10^2- 1×10^6 PFU/ml). White bars indicate infection in the presence of mAb using FcγR-expressing BHK cells, and black bars indicate infection in the absence of mAb using FcγR-expressing BHK cells


(a) 6B6C
(b) 4G2
(c) 3H12
(a-I) No Dilution
(b-I) No Dilution
(c-I) No Dilution
(a-2) 1:10 Dilution
(b-2) 1:10 Dilution
(c-2) 1:10 Dilution
(a-3) 1:100 Dilution
(b-3) 1:100 Dilution
(c-3) 1:100 Dilution
(a-4) 1:1,000 Dilution
(b-4) 1:1,000 Dilution
(c-4) 1:1,000 Dilution
(a-5) 1:10,000 Dilution
(b-5) 1:10,000 Dilution
(c-5) 1:10,000 Dilution
Virus Titer (Log(10) PFU/ml)
Challenge virus dose (Log(10) PFU/ml)

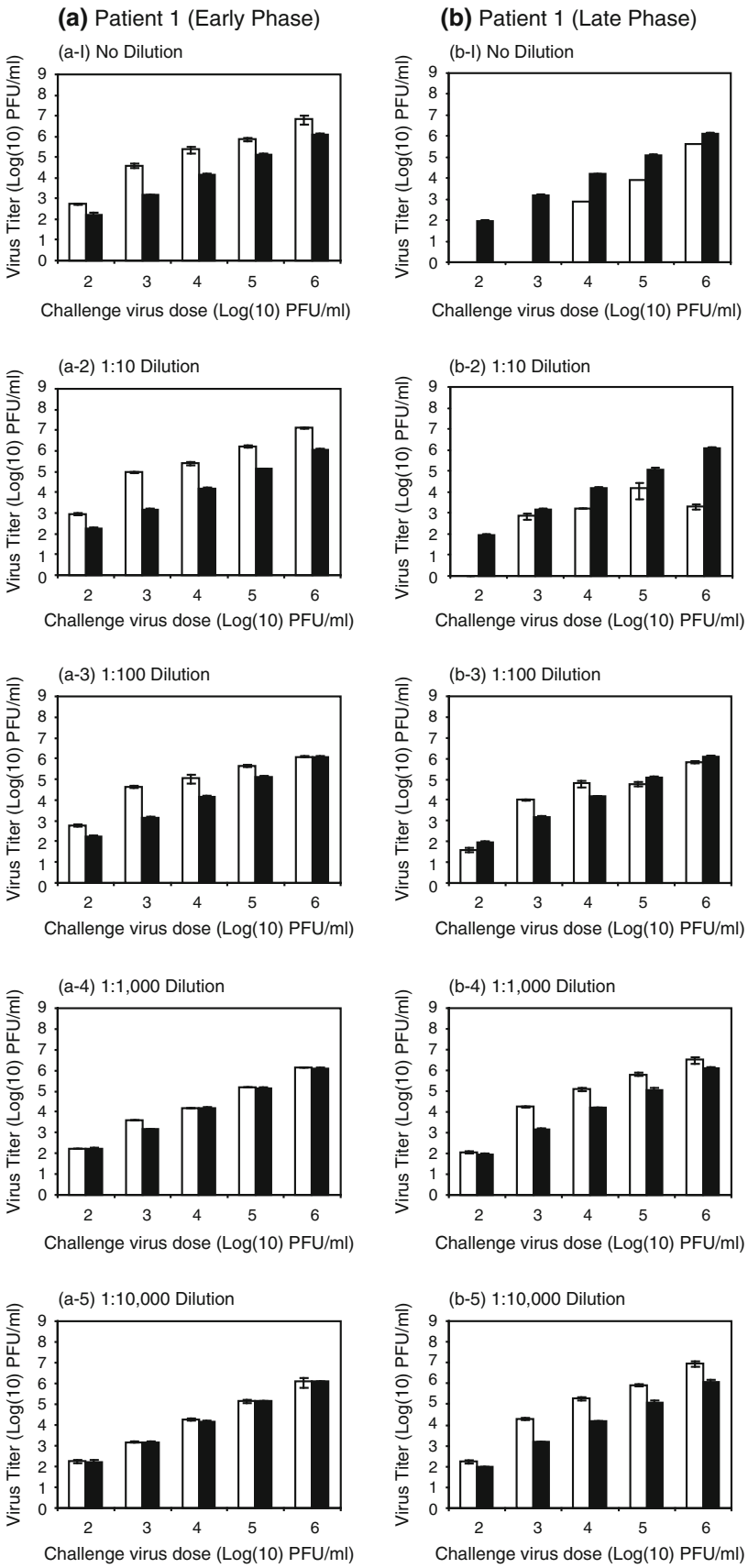


Fig. 2 Enhancement of DENV-2 infection by serum samples from patient 1. **a** Early-phase serum sample. **b** Late-phase serum sample. The serum samples were serially diluted tenfold. The following dilutions were used: a–1 and b–1, no dilution; a–2 and b–2, 1:10; a–3 and b–3, 1:100; a–4 and b–4, 1:1,000; a–5 and b–5, 1:10,000. Each of these tenfold serial dilutions was reacted with each dose of the serially diluted challenge virus (1×10^2-1×10^6 PFU/ml). White bars indicate infection in the presence of patient sera using FcγR-expressing BHK cells, and black bars indicate infection in the absence of sera using FcγR-expressing BHK cells

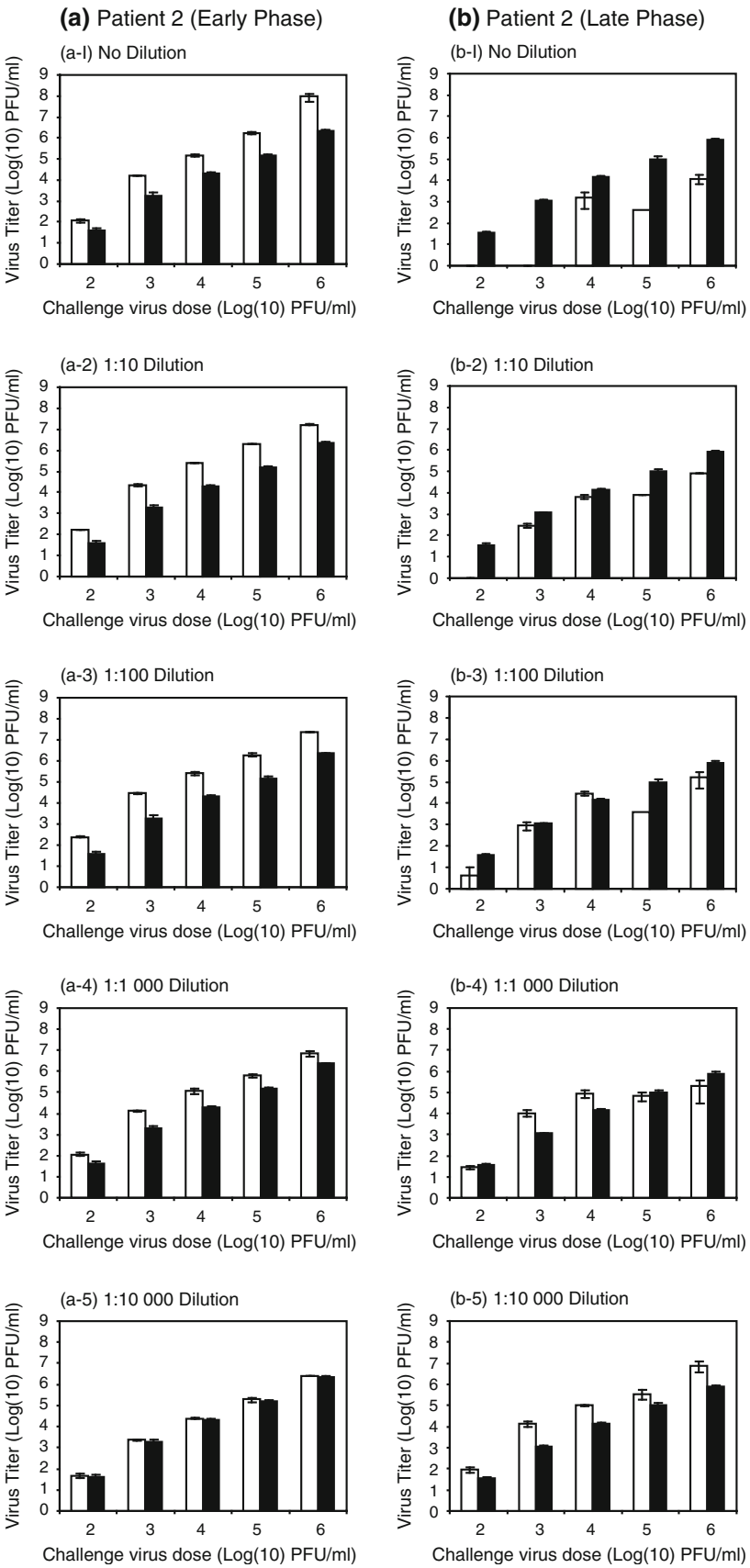


Fig. 3 Enhancement of DENV-2 infection by serum samples from patient 2. **a** Early-phase serum sample. **b** Late-phase serum sample. The serum samples were serially diluted tenfold. The following dilutions were used: a–1 and b–1, no dilution; a–2 and b–2, 1:10; a–3 and b–3, 1:100; a–4 and b–4, 1:1,000; a–5 and b–5, 1:10,000. Each of these tenfold serial dilutions was reacted with each dose of the serially diluted challenge virus (1×10^2-1×10^6 PFU/ml). White bars indicate infection in the presence of patient sera using FcγR-expressing BHK cells, and black bars indicate infection in the absence of sera using FcγR-expressing BHK cells

Table 4 Antibody-dependent enhancement activity detected in an early-phase serum sample (sample 1a) of DENV-2-infected patient 1

Virus dose/antibody dilution	FcγR-negative BHK cells		FcγR-expressing BHK cells	
	Virus titer (log10 PFU/ml)	Fold enhancement	Virus titer (log10 PFU/ml)	Fold enhancement
Patient 1 (Early)				
(a) 6 (log10) PFU/ml				
No dilution	5.60	0.25	6.83	5.67
10^1	5.78	0.38	7.10	10.50
10^2	5.86	0.45	6.10	1.04
10^3	6.03	0.68	6.13	1.13
10^4	6.08	0.75	6.08	1.00
No antibody	6.20	1.00	6.08	1.00
(b) 5 (log10) PFU/ml				
No dilution	4.56	0.31	5.81	5.29
10^1	4.94	0.76	6.25	12.06
10^2	4.96	0.79	5.60	3.24
10^3	5.08	1.03	5.18	1.15
10^4	5.05	0.97	5.18	1.00
No antibody	5.06	1.00	5.11	1.00
(c) 4 (log10) PFU/ml				
No dilution	3.38	0.22	5.37	16.39
10^1	4.13	1.00	5.40	17.50
10^2	3.94	0.69	5.06	8.06
10^3	4.05	0.75	4.18	1.06
10^4	4.08	0.87	4.26	1.25
No antibody	4.26	1.00	4.16	1.00
(d) 3 (log10) PFU/ml				
No dilution	2.51	0.26	4.60	27.40
10^1	3.15	1.13	4.96	63.01
10^2	3.12	1.06	4.64	30.14
10^3	3.17	1.19	3.60	2.74
10^4	3.06	0.94	3.16	1.00
No antibody	3.09	1.00	3.16	1.00
(e) 2 (log10) PFU/ml				
No dilution	0.60	0.03	2.72	3.07
10^1	2.13	0.97	2.93	5.00
10^2	2.18	1.09	2.78	3.47
10^3	2.23	1.20	2.21	0.95
10^4	2.11	0.91	2.26	1.07
No antibody	2.15	1.00	2.24	1.00

Serum samples from patient 1 were obtained at day 3 (serum sample 1a) and day 12 (serum sample 1b) after onset of disease. Serum samples from patient 2 were obtained at day 3 (serum sample 2a) and day 6 (serum sample 2b) after the onset of disease. Sample 1a was negative for anti-DENV IgM (Focus Diagnostics, Cypress, CA, USA) and positive for anti-DENV IgG (PanBio, Australia). The serum samples were examined for the presence of DENV viral RNA (vRNA) by RT-PCR, and anti-DENV IgM and IgG by enzyme-linked immunosorbent assay [34–36]. Serum samples 1b, 2a, and 2b were positive for anti-DENV IgM and IgG. The presence of secondary flavivirus infection was defined by a DENV IgM/IgG ratio of <1.2 [37]. Serum samples used in the experiments were heat-inactivated at 56 °C for 30 minutes.

Table 5 Antibody-dependent enhancement activity detected in an early-phase serum sample (sample 2a) of DENV-2-infected patient 2

Virus dose/antibody dilution	FcγR-negative BHK cells		FcγR-expressing BHK cells	
	Virus titer (log10 PFU/ml)	Fold enhancement	Virus titer (log10 PFU/ml)	Fold enhancement
Patient 2 (Early)				
(a) 6 (log10) PFU/ml				
No dilution	5.64	0.30	7.96	40.35
10^1	6.16	0.97	7.72	22.81
10^2	6.17	0.99	7.38	10.61
10^3	6.13	0.91	6.86	3.16
10^4	6.18	1.02	6.40	1.11
No antibody	6.24	1.00	6.36	1.00
(b) 5 (log10) PFU/ml				
No dilution	4.96	0.47	6.23	11.05
10^1	5.09	0.63	6.31	13.42
10^2	5.11	0.65	6.28	12.63
10^3	5.25	0.90	5.81	4.21
10^4	5.17	0.76	5.28	1.26
No antibody	5.29	1.00	5.18	1.00
(c) 4 (log10) PFU/ml				
No dilution	3.92	0.51	5.15	7.00
10^1	4.24	1.05	5.40	12.60
10^2	4.31	1.24	5.41	13.00
10^3	4.26	1.10	5.08	6.00
10^4	4.31	1.24	4.38	1.20
No antibody	4.21	1.00	4.30	1.00
(d) 3 (log10) PFU/ml				
No dilution	2.90	0.36	4.21	8.72
10^1	3.21	0.75	4.34	11.70
10^2	3.24	0.78	4.46	15.32
10^3	3.15	0.64	4.12	7.02
10^4	3.27	0.85	3.37	1.26
No antibody	3.34	1.00	3.27	1.00
(e) 2 (log10) PFU/ml				
No dilution	1.08	0.23	2.06	2.90
10^1	1.60	0.77	2.21	4.10
10^2	1.68	0.92	2.39	6.10
10^3	1.56	0.69	2.06	2.90
10^4	1.68	0.92	1.68	1.20
No antibody	1.72	1.00	1.60	1.00

Preparation of virus-antibody immune complex

Heat-inactivated undiluted serum samples were prepared with EMEM supplemented with 10 % FBS and serially diluted tenfold from $1{:}10^1$ to $1{:}10^4$. Twenty-five microliters of DENV at concentrations of 1×10^6 PFU/ml, 1×10^5 PFU/ml, 1×10^4 PFU/ml, 1×10^3 PFU/ml, and 1×10^2 PFU/ml was added to 25 µl of serially diluted serum samples. For infection in the absence of patient serum, 25 µl of virus suspension was added to 25 µl of 10 % FBS EMEM. The diluent for these assays consisted of EMEM supplemented with 10 % FBS. The virus-antibody mixture was incubated at 37 °C for 1 hour.

Infection assay

After incubation, virus mixtures were serially diluted tenfold and 50 µl of the serially diluted virus-antibody mixtures was added to the FcγR-expressing BHK cells and FcγR-negative BHK cells in 12-well plates. The plates were incubated for

Table 6 Antibody-dependent enhancement activity detected in a late-phase serum sample (sample 1b) of DENV-2-infected patient 1

Virus dose/antibody dilution	FcγR-negative BHK cells		FcγR-expressing BHK cells	
	Virus titer (log10 PFU/ml)	Fold enhancement	Virus titer (log10 PFU/ml)	Fold enhancement
Patient 1 (Late)				
(a) 6 (log10) PFU/ml				
No dilution	4.11	0.01	5.60	0.32
10^1	4.51	0.03	3.30	<0.01
10^2	4.60	0.04	5.83	0.55
10^3	5.90	0.74	6.51	2.58
10^4	6.09	1.15	6.94	7.10
No antibody	6.03	1.00	6.09	1.00
(b) 5 (log10) PFU/ml				
No dilution	3.97	0.11	3.90	0.07
10^1	4.38	0.29	4.20	0.14
10^2	4.70	0.60	4.78	0.52
10^3	4.92	1.00	5.81	5.52
10^4	5.00	1.19	5.90	6.90
No antibody	4.92	1.00	5.06	1.00
(c) 4 (log10) PFU/ml				
No dilution	NT[a]	-[b]	2.90	0.05
10^1	NT	-	3.20	0.10
10^2	3.68	0.33	4.81	4.10
10^3	4.11	0.89	5.09	7.95
10^4	4.08	0.83	5.26	11.79
No antibody	4.16	1.00	4.19	1.00
(d) 3 (log10) PFU/ml				
No dilution	NT	-	NT	-
10^1	NT	-	2.86	0.47
10^2	2.64	0.28	4.00	6.58
10^3	3.06	0.74	4.25	11.58
10^4	3.15	0.90	4.30	13.16
No antibody	3.19	1.00	3.18	1.00
(e) 2 (log10) PFU/ml				
No dilution	NT	-	NT	-
10^1	NT	-	NT	-
10^2	NT	-	1.60	0.43
10^3	0.90	0.11	2.06	1.26
10^4	1.30	0.28	2.23	1.83
No antibody	1.86	1.00	1.96	1.00

[a] NT indicates that virus was below the detection limit

[b] - indicates that fold enhancement values could not be determined

60 minutes at 37 °C in 5 % CO_2. One milliliter of maintenance medium consisting of 2 % FBS/ MEM and 1 % methylcellulose was added to the cells after virus absorption. The plates were incubated at 37 °C in 5 % CO_2 for 5 days. After 5 days of incubation, cells were fixed with 5 % paraformaldehyde for 60 minutes at room temperature and washed with water. Methylene blue was used for staining, and plaques were counted. Viral titers were expressed as plaque-forming units per milliliter (p.f.u./ml) and were determined by the following formula: (mean number of plaques per well) × (dilution) / (inoculum volume). The fold enhancement values were determined by the following formula: (mean number of plaques in the presence of antibodies or serum sample) / mean number of plaques in the absence of antibodies or serum sample). The mean plus two standard deviations (SD) of the negative control was used as a cutoff

Table 7 Antibody-dependent enhancement activity detected in a late-phase serum sample (sample 2b) of DENV-2 infected patient 2

Virus dose/antibody dilution	FcγR negative BHK cells		FcγR-expressing BHK cells	
	Virus titer (log10 PFU/ml)	Fold enhancement	Virus titer (log10 PFU/ml)	Fold enhancement
Patient 2 (Late)				
(a) 6 (log10) PFU/ml				
No dilution	4.08	0.01	4.08	0.02
10^1	4.38	0.02	4.90	0.10
10^2	4.90	0.08	5.20	0.20
10^3	4.90	0.08	5.30	0.25
10^4	5.88	0.73	6.88	9.50
No antibody	6.02	1.00	5.90	1.00
(b) 5 (log10) PFU/ml				
No dilution	3.88	0.08	2.60	<0.01
10^1	4.39	0.26	3.90	0.08
10^2	4.08	0.13	3.60	0.04
10^3	4.08	0.13	4.83	0.68
10^4	4.90	0.83	5.56	3.60
No antibody	4.98	1.00	5.00	1.00
(c) 4 (log10) PFU/ml				
No dilution	NT[a]	-[b]	3.20	0.11
10^1	3.29	0.17	3.81	0.46
10^2	3.78	0.52	4.45	2.00
10^3	4.15	1.21	4.94	6.29
10^4	4.02	0.90	5.00	7.14
No antibody	4.06	1.00	4.15	1.00
(d) 3 (log10) PFU/ml				
No dilution	NT	-	NT	-
10^1	NT	-	2.45	0.24
10^2	2.64	0.32	2.96	0.79
10^3	2.94	0.65	4.02	8.97
10^4	3.13	1.00	4.12	11.38
No antibody	3.13	1.00	3.06	1.00
(e) 2 (log10) PFU/ml				
No dilution	NT	-	NT	-
10^1	0.61	0.11	NT	-
10^2	0.61	0.11	0.61	0.11
10^3	0.61	0.11	1.45	0.78
10^4	1.30	0.56	1.94	2.44
No antibody	1.56	1.00	1.56	1.00

[a] NT indicates that virus was below the detection limit

[b] - indicates that fold enhancement values could not be determined

to differentiate enhancing and non-enhancing activity [21, 38]. Each sample was tested in duplicate assays.

Results

Detection of DENV-2 infection-enhancement activity of monoclonal antibodies 6B6C, 4G2 and 3H12

Three DENV-reactive antibodies, 6B6C, 4G2 and 3H12, were tested for their capacity to enhance DENV-2 infection. ADE activity was detected for the three antibodies, when FcγR-expressing BHK cells were used, but not when FcγR-negative BHK cells were used (Tables 1, 2, 3). ADE activity was examined by adding undiluted and 1:10 serially diluted monoclonal antibodies to each of the virus titers (1×10^6 to 1×10^2 PFU/ml). We analyzed whether

ADE activity was determined by the concentration of monoclonal antibodies or by the ratio of challenge DENV titer to antibody. ADE activity was constantly detected at defined dilutions of monoclonal antibodies, irrespective of the challenge DENV titers: mAb 6B6C at $1:10^3$–$1:10^4$, 4G2 at $1:10^3$–$1:10^4$, and 3H12 at $1:10^3$-$1:10^4$ (Tables 1, 2, 3, Fig. 1). The results indicate that antibody concentration, rather than DENV-antibody ratio, determines the presentation of ADE activity.

Detection of DENV-2 infection-enhancement activity of early-phase patient serum samples without dilution and at low dilutions.

Heat-inactivated serum samples obtained from two patients at the early and late phases of secondary DENV-2 infection were used. The serum samples from patient 1 obtained on day 3 after the onset of disease exhibited ADE activity (3.07- to 27.40-fold enhancement) without dilution (Table 4, Fig. 2a). The level of enhancement of DENV-2 was higher (5.00- to 63.01-fold) at 1:10 dilution than other serum dilutions. The serum samples from patient 2 obtained on day 3 of infection constantly exhibited ADE activity (2.90- to 40.35-fold enhancement) without dilution and at 1:10-$1:10^2$ dilutions (Table 5, Fig. 3a). Thus, ADE activity was detected without dilution and at 1:10 dilution for early serum samples from patient 1, and without dilution and at 1:10 dilution for early serum samples from patient 2, using any of the challenge DENV titers of 1×10^2-1×10^6 PFU/ml. The results indicate that in the presence of FcγR, the concentration of reactive antibody, but not the DENV-antibody ratio, determines the presentation of ADE activity of human serum.

Detection of DENV-2 infection-enhancement activity of late-phase patient serum samples at high dilutions

No ADE activity (enhancement of up to 0.32-fold) was detected for late-phase undiluted serum samples (serum samples obtained from day 12 after onset of disease and day 6 after onset of disease, respectively) from patient 1 (Table 6) and patient 2 (Table 7) using FcγR-expressing cells. ADE activity was constantly detected only at higher serum dilutions ($1:10^3$–$1:10^4$) for either samples, using any of the challenge DENV titers of 1×10^2-1×10^6 PFU/ml (Fig. 2b and Fig. 3b). The results provide further evidence that the concentration of reactive antibody, but not the DENV-antibody ratio, determines the presentation of ADE activity.

Discussion

In the present study, assay parameters were defined for the *in vitro* ADE activity of monoclonal antibodies and human serum samples by a conventional plaque titration assay, using FcγR-expressing BHK cells at viral titers of 1×10^2 PFU/ml -1×10^6 PFU/ml. The plaque titers detected in heat-inactivated serum samples were <100 PFU/ml for patient 1 and patient 2. In comparison, virus titers in untreated serum samples were 3.4×10^6 PFU/ml for patient 1 and 7.5×10^6 PFU/ml for patient 2. The results indicate that the heat-inactivated serum samples were not a source of infectious virus in the assays.

A major strength of this method is the ability to detect ADE activity of all four DENV serotypes at a given virus concentration by conventional plaque assays [29, 39]. Here, ADE assessment using an FcγR-expression- based plaque assay makes it possible to visualize plaques and introduce flexibility into incubation times and experimental workflow, and also to determine virus growth directly in infected cells. ADE assays using non-adherent FcγR-expressing cells have been described previously and share some of the features described in the present study in terms the ability to detect ADE activity in undiluted human sera [21]. In the present study, ADE activity was detected using monoclonal antibodies and undiluted and diluted human serum samples, irrespective of challenge DENV titers (10^2-10^6PFU/ml). Thus, when using FcγR-positive BHK cells in ADE assays, the concentration of antibodies is a major parameter, whereas the DENV-antibody ratio is a comparatively minor parameter. These results suggest that appropriate titers of challenge DENV could be selected for the ADE assay with only a minimal effect on ADE activity.

Most DENV neutralizing antibodies are speculated to possess ADE activity *in vitro* at subneutralizing concentrations [40, 41]. The FcγR-expressing BHK cells detected ADE activity using an inoculation dose of as little as 5 PFU per reaction and using both diluted and undiluted serum samples. Additionally, virus growth was detected in cell culture supernatants using as low as 0.1 PFU per inoculation dose with an antibody that possesses ADE activity [39]. These results suggest that the FcγR-expressing BHK cells could provide a useful tool for the elucidation of the ADE mechanism in DENV infection using antibodies induced by either natural infection or vaccination, because the cells confer the ability to detect ADE activity over a wide range of virus concentrations.

The DENV infection-enhancement activity could be influenced by three conditions: virus concentration, antibody concentration, and virus serotype [40]. Although monocyte-lineage cells have proven useful in ADE studies, these cells require different virus titers for each DENV strain to elicit infection, and this may limit the utility of these assays to study ADE mechanisms. Previous studies have shown that ADE is present in undiluted sera during the early phase, but not during the late phase of the disease [21, 27]. Our study confirms the demonstration of ADE activity in the early serum samples without dilution [21, 43]. The FcγR-expressing BHK cells also detected

infection-enhancement activity in sera that exhibited ADE activity in THP cells [42]. Additionally, ADE activity was detected at wide ranges of DENV concentrations (Tables 4, 5, 6, 7). Maternal antibodies have been reported to play a significant role in dengue pathogenesis in infants. In FcγR-positive monocytes, maternal dengue antibodies exhibit both virus neutralizing and infection-enhancement activity. These antibodies provide protection against DENV. However, as the antibodies decay, protective activity disappears and the remaining antibodies render infants susceptible to enhanced DENV infection [21, 44]. The DHF hospitalization curve among infants also resembles the titration curve for enhancing antibodies *in vitro* [45]. Using this schema, in the present study, antibodies were diluted to reflect *in vivo* antibody decay during DENV infection. Although samples from DHF patients were not included to demonstrate antibody decay *in vivo*, our study demonstrated that serum samples from patients during the late phase of infection have infection-enhancing activity when diluted, that undiluted serum samples from acute DENV patients have DENV infection-enhancing activity, and that our assay is potentially useful for the detection of infection-enhancing activity of serum samples from DENV patients. The degree of enhancement was also lower when low virus titers were used (Table 4e, Table 5e). These results suggest that antibody concentrations in excess may abrogate ADE, and this highlights the utility of the present assay in ADE studies that require low virus titers or an excess of antibody.

In summary, concentration of antibody rather than DENV-antibody ratio was determined to be the important parameter for the demonstration of DENV infection-enhancement activity using FcγR-expressing BHK cells. The results suggest that the precise definition of DENV titer is not needed in our ADE assay system. Additionally, advantages of the ADE assay based on FcγR-expressing BHK cells include (i) the ability to measure ADE activity by a direct plaque titration method, (ii) the ability to determine ADE activity in undiluted serum samples, (iii) the limited differences in background growth or similar infection threshold using different virus serotypes, and (iv) the ability to detect ADE activity irrespective of challenge DENV titers [39]. Thus, this assay is useful for defining ADE activity in antibody responses in DENV infections.

Acknowledgements We thank Dr. Eiji Konishi for generously providing us with the 3H12 monoclonal antibody. We also thank Dr. Jeffrey V. Ravetch (Rockefeller University, NY, USA) for generously providing us with the FcγRIIA cDNA, and Dr. Susheela Tridandapani (Ohio State University College of Medicine, Columbus, OH, USA) for assistance in obtaining the FcγRIIA cDNA. This work was supported by grants from Research on Emerging and Re-emerging Infectious Diseases (H23-shinkou-ippan 010) from the Ministry of Health, Labour and Welfare, Japan, a grant-in-aid for Scientific Research (Wakate B No. 23790515) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a grant-in-aid from the Cooperative Research Grants of the Institute of Tropical Medicine, Nagasaki University (NEKKEN), 2012 (H24-ippan-16).

Conflicts of interest None to declare.

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