Role of FcγRIIA in antibody-dependent enhancement of dengue virus infection

(FcγRIIAを介したデングウイルス感染における抗体依存性感染増強のメカニズムの解析)

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筑波大学大学院博士課程人間総合科学研究科

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博 士 （医 学） 学 位 論 文

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ABSTRACT

Dengue virus (DENV) causes a wide range of symptoms, from mild febrile illness, dengue fever (DF), to severe life threatening illness, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Subneutralizing concentrations of antibody to DENV enhance DENV infection in FcγR positive cells; this phenomenon is known as antibody-dependent enhancement (ADE). ADE is considered to be a risk factor to develop DHF and DSS. The objective of this research is to define the role of FcγRIIA in ADE of dengue virus infection. The mechanisms of ADE were first investigated with the introduction of a series of mutations in the cytoplasmic region of FcγRIIA. Disruption of the FcγRIIA conserved regions abrogated ADE; suggesting that the specific structure of FcγRIIA cytoplasmic domain is essential for the ability of FcγRIIA to mediate ADE. In addition, an ADE assay was developed using FcγRIIA-expressing BHK cells. Virus growth was directly quantified using standard plaque titration methods. This assay is capable of directly determining ADE activity, and thus, may present a valuable experimental system in defining the role of enhancing antibody in the pathogenesis of DENV infection. Next, to examine the protective capacity of antibody which better reflects *in vivo* conditions, neutralizing capacity of antibodies was tested using FcγRIIA-expressing BHK cells. Higher antibody concentration was required to neutralize DENV using FcγRIIA-expressing BHK cells demonstrating lower neutralizing antibody titers. Neutralizing antibody titers using FcγRIIA-expressing cells may better reflect protective capacity of antibodies, as the major target cells of DENV infection are FcγRIIA-positive cells. The data generated in this thesis extends our understanding on the role of enhancing antibodies and FcγRIIA in the pathogenesis of DENV infection. Presence of FcγRIIA-positive cells in experimental systems for ADE and neutralizing test may better reflect *in vivo* conditions, and may be helpful in finding better strategies for treatment and prevention.

ACKNOWLEDGEMENTS

I would like to give special thanks to my professor, Dr. Kurane Ichiro M.D., Ph.D. His advice, knowledge and guidance was invaluable throughout this entire research process. I would also like to thank Dr. Takasaki Tomohiko, M. D., Ph. D., and Dr. Lim Chang-Kweng D.V.M., Ph.D., Mr. Kotaki Akira, Dr. Omatsu Tsutomu D.V.M., Ph. D., Dr. Harada Fumio M.D., Ph. D., and members of Department of Virology 1, National Institute of Infectious Diseases of Japan for their advice, guidance and motivation. I would also like to acknowledge my family and friends for their kindness and motivation. Without their encouragement, this research would not be possible.

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ABBREVIATIONS

Ab	antibody
Ag	antigen
ADE	antibody dependent enhancement
BHK-21	baby hamster kidney-21 cell line
DENV	dengue virus
DF	dengue fever
DHF	dengue hemorraghic fever
DSS	dengue shock syndrome
ELISA	enzyme-linked immunosorbent assay
FcγR	Fc gamma receptor
MOI	multiplicity of infection
PRNT	plaque reduction neutralization test
RT-PCR	reverse transcriptase polymerase chain reaction

CHAPTER 1: INTRODUCTION

1.1. EPIDEMIOLOGY

Dengue virus (DENV) is found in most tropical and sub-tropical regions and has caused major outbreaks in Southeast Asia, South Asia and Latin America. Approximately 100 million people is affected by DF annually. DF epidemics in Brazil had resulted in 778, 037 cases in 2002, 341, 189 cases in 2003, 112, 851 cases in 2004 and 158, 553 cases in 2005. Areas of outbreak are expanding, with 15, 000 cases reported in Southern Taiwan in 2002. Dengue outbreaks had also occurred in Nepal and in a non-endemic country, Australia.

1.2. CLINICAL SYMPTOMS OF DENGUE FEVER

1.2.1. Dengue Fever (DF)

Most dengue patients show non-fatal, acute febrile symptoms accompanied by fever, skin rash, muscular and arthritic pain. Sudden onset of fever occurs after a 3 – 14 days of incubation period. Maculopapular rashes appear after 3 – 6 days after onset of the disease, which spreads from the chest and trunk to the extremities and face. These acute symptoms disappear in 7 – 10 days after onset of the disease, often without any complications.

1.2.2. Dengue Hemorrhagic Fever (DHF)

DENV causes DHF in 0.3 – 0.5% of dengue patients. Patients with DHF suffer extreme anxiety, excitation, heavy sweating, pleural effusion and ascites formation. Other symptoms include liver bloating, activated complements, decreased blood platelet count (100,000 mm^3 or less) and prolong blood clotting time. Petechia is observed in many cases, and nasal bleeding accompanied with throat hemorrhage, is observed in 10 – 20%. Cases involving plasma leakage that may develop into hypovolemic shock is known as dengue shock symdrome (DSS). DHF has a high mortality rate unless adequately treated.

1.3. LABORATORY DIAGNOSIS

1.3.1. Viral isolation in tissue culture

Several mammalian cells such as $LLCMK_2$ cell line, Vero cell line and BHK-21 cell line had been used to study DENV. DENV produces cytophatic effects on permissive cell lines and this feature had been used to quantify DENV.

1.3.2. Serological diagnosis

Two patterns of serological response can be observed in acute DENV infection: primary and secondary infection. A primary response is seen in individuals whom had not been previously exposed to flavivirus infection. A secondary response pattern occurs in an individual with an acute DENV infection and who was previously exposed to flavivirus infection. The plaque reduction neutralization test (PRNT) is a sensitive and specific serological assay for detection of anti-DENV antibodies. Enzyme-linked immunosorbent assays (ELISAs) for DENV antibody detection is rapid and simple to perform and had been proved useful for detection of flavivirus total antibodies. In comparison to PRNT, ELISA does not distinguish antibodies against specific DENV serotypes. Serological diagnosis of DENV is however, often complicated by cross-reactive antibodies towards four DENV serotypes and other flavivirus.

1.3.3. Molecular detection

The sensitivity, specificity and rapid detection of minute viral RNA in serum samples make PCR a very useful diagnostic tool for DENV. In comparison to serological diagnosis, PCR can only detect viral RNA during the acute phase of the disease.

1.4. PATHOGENESIS OF DENV INFECTION

1.4.1. Antibody dependent enhancement

Following primary DENV infection, life-long immunity develops to protect against homologous DENV infection. In Thailand, 99% of DHF cases occur in secondary infection. During secondary infection with a heterologous DENV serotype, antibodies to DENV at sub-neutralizing concentrations enhances

infection of FcγR-positive cells. DENV and immunoglobulin G (IgG) to DENV form virus-antibody complexes, and binding of these virus-antibody complexes to the FcγR via the Fc portion of IgG results in augmentation of DENV infection. Based on epidemiological and laboratory results, it has been hypothesized that antibodies to DENV and other serotype-cross-reactive-immune responses contribute to the pathogenesis of DHF.

1.4.2. Virulence of viral strains

DENV differs genotypically and the differences appear to be associated with disease severity. The first large outbreak in Cuba in 1981 coincided with the introduction of a new DENV-2 strain, which originated from South-east Asia, to this region. Introduction of this South-east Asian DENV-2 strain coincided with appearance of DHF in this region, while the original American genotype was only associated with DF. DENV strains were classified to 3 subtypes: (a) strains that may induce DSS, (b) strains that induce DF in primary infection but DHF in secondary infection, and, (c) strains that induce DF in both primary and secondary infection.

1.4.3. Complement activation

Certain non-cytokine, chemical mediators also induce symptoms as observed in DHF. These include platelet-activating factor (PAF), complement activation products C3a and C5a and histamine. Levels of C3a and C5a are highly elevated in DHF patients and correlation between disease severity and complement activation levels were observed.

1.4.4. T lymphocytes and cytokines

DENV infections of target cells, monocytes/ macrophages, is enhanced by ADE. The increased number of dengue virus-infected monocytes/macrophages results in increased T-cell activation, which in turn, causes the release of increased levels of cytokines and chemical mediators.

1.5. CELLS THAT SUPPORT DENV IN VIVO

Cells that mainly support DENV infection *in vivo* are monocytes, macrophages and other

cells of reticuloendothelial origin. Dendritic cells, namely Langerhans cells and dermal and interstitial dendritic cells, were reported to be more permissive to DENV than monocytes and macrophages. Although there were reports that fibroblasts, hepatocytes and B cells were infected with DENV, it is likely that dendritic cells and macrophages/ monocytes are the cells that mainly support DENV infection *in vivo*. Vascular endothelial cells can be infected with DENV *in vitro*, however no report demonstrated DENV infection of vascular cells in vivo. The main target organs of DENV are not known. DHF is frequently associated with moderate degrees of hepatic functional abnormalities and can show characteristics typical of acute hepatitis. In addition, the levels of aspartate transaminase (AST) and alanine transaminase (ALT) are significantly higher in patients with more severe grades of DHF.

1.6. DENGUE VIRUS REPLICATION

Infection with dengue virus begins when virus is introduced into the host via a mosquito bite. DENV binds to and using a receptor-mediated pathway, enters a permissive host cell. Upon internalization and acidification of the endosome, fusion of viral and vesicular membranes allows entry of the nucleocapsid into the cytoplasm and genome uncoating. Translation of the positive RNA strand and synthesis of the negative-strand templates for viral production then takes place. Successive rounds translation produces high levels of viral proteins, consisting of protein capsid (C), premembrane (prM), and envelope (E) proteins. Along with vRNA, the viral proteins are assembled into progeny virions, which are transported to Golgi compartment and secreted.

1.7. OBJECTIVES OF THESIS

Previous reports had shown that antibodies to DENV at sub-neutralizing concentrations enhances infection of FcγR-positive cells and contributes towards the pathogenesis of DHF/ DSS. It had been suggested that DENV and immunoglobulin G (IgG) to DENV form virus-antibody complexes, and binding of these virus-antibody complexes to the FcγR via the Fc portion of IgG results in augmentation of DENV infection. However, little is known on the role of FcγR structural components and signaling functions during DENV infection. In addition, complicated procedures of *in-vitro* systems for the analysis of the mechanisms of FcγR hampers the progress of DENV research. The objective of this thesis was to analyze the role of FcγR in antibody dependent enhancement of DENV infection.

The specific aims of this thesis are as follows:

1. To identify the significance of FcγRIIA structural component during DENV infection
2. To develop a simple and practical approach for the analysis of ADE activity *in vitro*
3. To analyze the effects of immune complexes on neutralization titers in the presence of FcγR

CHAPTER 2: Involvement of Fcγ receptor IIA cytoplasmic domain in antibody dependent enhancement of dengue virus infection

2.1. ABSTRACT

Subneutralizing concentrations of antibody to dengue virus enhance dengue virus infection of Fcγ receptor-expressing cells. This phenomenon, referred to as antibody dependent enhancement (ADE), has been hypothesized to be responsible for the pathogenesis of the severe form of dengue virus infection, including dengue hemorrhagic fever and dengue shock syndrome. To further analyze the mechanisms of ADE, we introduced a series of transmembrane and cytoplasmic mutants to human FcγRIIA. We then expressed the mutated FcγRIIA on COS-7 cells and examined whether these mutants could enhance dengue virus infection. Wild type FcγRIIA enhanced dengue virus infection, consistent with previous reports using FcγR-positive monocytes. Disruption of the immune tyrosine activation motif (ITAM) in the cytoplasmic domain of FcγRIIA or removing the sequences between the two ITAM regions abrogated ADE. These findings suggest that the specific structure of FcγRIIA cytoplasmic domain is essential for the ability of FcγRIIA to mediate ADE.

2.2. INTRODUCTION

Dengue fever ranks as the most important mosquito borne virus disease in the world and an estimated 2.5 billion people live in over 100 endemic countries and areas where dengue viruses can be transmitted. With up to 100 million infections occurring annually, dengue fever is a source of considerable economic loss to health authorities (Okanurak et al., 1997) as well as to patients (Anderson et al., 2007). DENV is characterized as a small (50 nm in diameter), enveloped virus containing a single strand positive RNA. The DENV genome expresses three structural proteins; envelope glycoprotein (E), core (C), and membrane (M). The viral envelope protein is thought to be responsible for initial viral attachment to cells and for mediating cellular entry of the virus. The E protein is a site for several important functions including hemagglutination, infectivity, antibody neutralization, and enhancement for the FcγR-expressing cells (Wang et al., 1995).

DENV exists in four distinct viral serotypes and each serotype can cause a spectrum of symptoms, ranging from mild febrile illness to severe life threatening dengue hemorrhagic fever (DHF). Epidemiological studies indicate that infection with a DENV serotype offers life-long protection against homologous infection by the same serotype. Immunity generated against a particular dengue serotype does not provide protection towards infection with a previously unexposed serotype in the long term. Instead, after a short period of cross-protection against heterologous serotypes, antibody generated against primary DENV infection is postulated to be one of the main factors for the severe form of dengue infection (Graham et al., 1999; Sangkawibha et al., 1984; Vaughn et al., 2000). Under the conditions of anti-DENV antibody cross-reactivity or concentration of where neutralization does not occur, virus-antibody complexes are taken up more readily than uncoated virus particles by cells expressing Fcγ receptors (FcγR), such as monocytes and macrophages (Kontny et al., 1988; Littaua et al., 1990). This effect, known as antibody dependent enhancement (ADE), has been demonstrated for both RNA and DNA viruses, and has been studied extensively with DENV (Littaua et al., 1990; Schlesinger et al., 1999). Interaction of FcγR and antigen-antibody complexes triggers an array of responses which includes phagocytosis, endocytosis, antibody dependent cell-mediated cytotoxicity,

superoxide generation, and release of inflammatory mediators, as well as immune complex clearance (An, 1982, Gessner et al., 1988; Indik et al., 1991, 1995a, b; Mero et al., 2006; Ravetch & Kinet, 1991). These responses are largely dependent upon interactions between FcγR and protein, and lipid signaling transduction moieties located in close proximity to the cytoplasmic and transmembrane regions of FcγR (Barabé et al., 2002; Booth et al., 2002; Garcia-Garcia & Rosales, 2002). Endocytosis of opsonized particles by FcγR involves lipid raft-induced receptor clustering which leads to signaling through immune tyrosine activation motifs (ITAMs) (Abdel Shakor et al.; 2004; Huang et al., 1992; Kwiatkowska et al., 2003; Sobota et al., 2005). When mutations are introduced into the tyrosine moieties located in the ITAM region of FcγRIIA (CD32A), kinase-mediated phagocytosis of opsonized particles was severely abrogated (Kim et al., 2003; Mitchell et al., 1994). In contrast, ability of the FcγR to associate with lipid rafts was disrupted by substitution of a cysteine residue located within the juxtamembrane region (Barnes et al., 2006). Transmembrane and cytoplasmic structures are, thus, required for FcγR functions and are likely to be involved in interaction with intermediate signal transduction elements that are components in immune complex internalization machinery.

However, the importance of such internalization machinery in FcγR mediated ADE is still obscure. FcγR could facilitate entry of DENV by directing the virus to the cell surface, and in turn, increases the probability of interactions between DENV and its unidentified viral receptor (Mady et al., 1991). In contrast, signaling systems triggered by FcγR, may lead to internalization of viral DENV-antibody immune complex, and enhanced infection.

In the present study, we evaluated the possible roles of the cytoplasmic and transmembrane regions of the FcγRIIA in facilitating DENV infection in the presence of antibody. We introduced a series of mutations in transmembrane and cytoplasmic domains of wild-type (WT) FcγRIIA and examined the capacity for ADE. Cytoplasmic domain of the receptor, including its palymitoylation site (Barnes et al., 2006) was required for ADE of DENV infection. The results indicate that FcγRIIA-mediated signal transduction is necessary for ADE.

2.3. METHODS & MATERIALS

2.3.1. Cell lines.

COS-7 cells, an African green monkey kidney-derived fibroblast cell line and Vero cells (ATCC CCL-81; American Type Culture Collection), an African green monkey kidney-derived epithelial cell line, were used. Cells were cultured in Eagle's Minimum Essential Medium (EMEM), (Sigma), supplemented with heat-inactivated 10% fetal calf serum, FCS (Sigma) without antibiotics at 37 °C in 5% CO_2.

2.3.2. Virus and antibodies.

Dengue virus type 1 (DENV-1), 01-44-1HuNIID strain (GenBank accession no. AB111070), isolated from Tahiti in 2001 (Ito et al., 2007), and dengue virus type 2 (DENV-2) TL-30 strain, isolated from East Timor in 2005 (Ito et al., unpublished data), were used. Virus was propagated on Vero cells. Titres of dengue virus (DENV) were determined by plaque assays in Vero cells. Virus dilution at volumes of 100 µl, was inoculated onto Vero monolayers in 12-well plates. The plates were incubated for 60 minutes at 37 °C in 5% CO_2. After virus absorption, the cells were overlaid with maintenance medium containing 1% methylcellulose (Wako Pure Chemical Industries). The plates were incubated at 37 °C in 5% CO_2 for 5 days and were fixed with neutral formalin for 60 minutes at room temperature. The cells were then stained with 0.3% methylene blue for 60 minutes at room temperature and washed with tap water. Plaques were counted and the virus infectivity titre expressed as plaque forming units (PFU) per ml. Human serum from a patient with dengue fever caused by DENV-3 was used as enhancing antibody. Dengue serotype-cross-reactive mouse monoclonal IgG antibody (MAbs, ATCC HB-112 D1-4G2-4-15) was used in immunofluorescent and flow cytometry assays.

2.3.3. FcγRIIA and mutant FcγRIIA plasmid constructions.

Human FcγRIIA cDNA (Brooks et al., 1989; GenBank accession no. M31932) was generously provided by Dr. Jeffrey V. Ravetch, Rockfeller University, NY, USA. The cDNA was subcloned into pcDNA3.1/neo+ (Invitrogen) and mutations were generated by

standard site directed mutagenesis method (QuikChange; Stratagene). The list of mutants is shown in table 2.1 and figure 2.1. Full length sequences for all constructs were verified by DNA sequence analysis.

2.3.4. Transient Expression of WT and mutated FcγRIIA in COS-7 cells.

Transfection of COS-7 cells with WT or mutated FcγRIIA cDNA was carried out with Lipofectamine LTX (Invitrogen), according to manufacturer's protocols. Cells were examined for surface expression of FcγRIIA by flow cytometry and standard immunoblot analysis at 48 hours after transfection.

2.3.5. Flow cytometry.

COS-7 transfectants were washed with PBS (Invitrogen) and stained with phycoerythrin (PE)-conjugated monoclonal antibody to human FcγRIIA (CD32A MAb, Clone 190723; R&D Systems, Minneapolis, MN, USA), according to manufacturer's instructions. Stained cells were analyzed by Guava EasyCyte Mini cytometer (Guava Techonologies). More than 5 000 cells were counted and the results were examined by FlowJo Version 7.5 software (Tree Star). For determining DENV infection, cells were fixed and permeabilized with 1:1 acetone/methanol mixture for 10 minutes and reacted with MAb ATCC HB-112 D1-4G2-4-15 at 37 °C for 60 minutes. Cells were then stained with Alexa Fluor 488-labeled, goat anti-mouse IgG (Invitrogen) and examined by flow cytometry.

2.3.6. Electrophoresis and Immunoblotting.

Cells were treated in Laemmli's sample buffer and separated under reducing conditions by 12.5% SDS-PAGE (Atto Corporation). Proteins were transferred to PVDF membranes (Millipore), blocked for 1 hour in 5% Immunoblock (Dainippon Sumitomo Pharma) in PBS with 0.01% Tween 20 (PBST) and were probed with anti FcγRIIA (goat anti-human FcγRIIA/ CD32a antibody; R&D Systems) at 1:500 dilution. After washing with PBST, the blots were probed with HRP-conjugated secondary antibodies (anti-goat IgG-HRP antibody; R&D Systems) at 1:2 000 dilution for 30 minutes and washed 3 times in PBST

for 10 minutes, prior to detection by chemiluminescence (Amersham Biosciences).

2.3.7. Infection of WT or mutant FcγRIIA transfected COS-7 cells with DENV alone or DENV-antibody complex.

DENV-antibody complex was prepared by mixing 25 µl of DENV-1 or 25 µl of DENV-2 at titers of $2x10^5$ PFU ml^{-1} with 25 µl of the dengue patient's serum at $1:10^4$ dilution for DENV-2 and $1:10^3$ for DENV-1 respectively. DENV mixtures were incubated at 37°C for 60 minutes with occasional agitation. For infection assay with DENV alone, viral mixture was prepared by mixing 25 µl of DENV-1 or 25 µl of DENV-2 at the titer of 2×10^5 PFU ml^{-1} with 25 µl EMEM and incubated at 37 °C for 60 minutes. COS-7 transfectants ($1x10^5$ cells/ well) were washed twice in 0.5 ml EMEM. The cells in 50 µl EMEM were incubated with 50 µl of DENV-antibody complex at a multiplicity of infection (MOI) of 0.1 PFU/ cell at 37 °C for 60 minutes, with occasional agitation. The cells were then washed twice with 0.5 ml PBS, and maintained in 0.5 ml EMEM supplemented with 10% FCS. DENV antigen positive cells were determined by immunofluorescent assay and flow cytometry at 72 hours after infection.

2.3.8. Antibody dependent enhancement assay using BHK-21 cells and FcγRIIA-expressing BHK-21 cells

Mouse monoclonal antibody 4G2 (ATCC HB-112 D1-4G2-4-15) were diluted at 1:1 000 with EMEM supplemented with 10% FCS, to generate a concentration of antibody which enhances but does not neutralize DENV (Moi et al, in press). Virus-antibody mixture was prepared by mixing 25 µl of DENV-1 or DENV-2 at the titre of 2 500 PFU/ml with 25 µl of serially diluted antibodies or serum samples. Control virus samples were prepared by mixing 25 µl of DENV-1 and DENV-2 at the titre of 2 500 PFU/ml with 25 µl of EMEM supplemented with 10% FCS. Virus-antibody mixture was incubated at 37 °C for 1 hour. Fifty microliters of virus-antibody mixture was inoculated onto BHK-21 monolayers in 12-well plates. The plates were incubated for 1 hour at 37 °C in 5% CO_2. After virus absorption, the cells were washed twice with 1 ml of EMEM, and overlaid with

maintenance medium containing 1% methylcellulose (Wako Pure Chemical Industry, Osaka, Japan). The plates were incubated at 37 °C in 5% CO_2 for 5 days. After 5 days of incubation, the cells were fixed with 3.7% (v/v) formaldehyde for 1 hour at room temperature and washed with water. The cells were then stained with methylene blue solution for 1 hour at room temperature and washed with water. Plaques were counted by naked eye.

2.3.9. Capping of COS-7 cells expressing WT and mutant FcγRIIA.

COS-7 cells were seeded at a density of 4 x 10^4 cells on a 16-wells chamber slides (Nalge Nunc), incubated for 24 hours, transfected with either WT or a series of FcγRIIA mutants, and cultured for another 48 hours. The cells were washed twice with 0.1 ml EMEM, and reacted for 30 minutes on ice with 25 µl DENV-2-antibody complex or DENV-2 virus alone, prepared as described above. The cells were incubated for 10 minutes at 20 °C, and washed gently with 50 µl PBS. The cells were stained with PE-conjugated anti-FcγRIIA, mouse monoclonal antibody against human FcγRIIA (Clone 190723). After washing with 0.1 ml PBS twice, the cells were mounted in 50% glycerol and examined under fluorescence microscope (Olympus). Images were taken and were analyzed by QCapture Pro Version 5.1 (QImaging). The cells were identified as "cap positive", when distinct large conglomerates of crosslinked FcγRIIA formed at the cell margins (Kwiatkowska et al., 2002).

2.3.10. Phagocytosis Assay.

Opsonized E. coli was prepared by mixing 20 µl of E. coli bioparticles opsonizing reagent (Invitrogen) with 6x10^7 of succimidyl-ester (SE) labeled E. coli bioparticles (pHRODO E. coli Bioparticles; Invitrogen) in 1 ml EMEM supplemented with 10% FCS at 37 °C for 60 minutes and cooled on ice for 10 minutes. Fifty microliters of opsonized E. coli bioparticles mixture (2.5x10^6 of succimidyl-ester (SE) labeled E. coli bioparticles) was added to 1x10^5 COS-7 cells and incubated on ice for 15 minutes. The cells were then incubated at 37 °C for 45 minutes. After washing the cells twice, the cells were analyzed by flow cytometry and fluorescent microscopy.

2.3.11. Treatment with heparin and ammonium chloride

The mechanism of DENV entry was investigated using heparin and ammonium chloride. DENV-2-antibody complex was prepared with anti-DENV monoclonal antibody 4G2 (ATCC HB-112 D1-4G2-4-15) at a dilution of 1:1 000 as described above. DENV-2 alone and DENV-2-antibody complex were incubated with various concentrations (10 μg/ml or 100 μg/ml) of heparin for 1 hour at 37°C. The virus-heparin mixture was then added to BHK cell monolayers in 12-well plates for infection assay as described above (Methods 2.3.8) in the presence of 10 μg/ml or 100 μg/ml of heparin (Lin et al., 2002). For inhibition assays using a lysosomotropic agent, ammonium chloride, confluent BHK-21 cell monolayers (12-well plates) were treated with 2 mM or 10 mM of ammonium chloride for 2 hours at 37 °C before infection. Cells were infected with DENV-2 alone or DENV-2-antibody complex as described above (Methods 2.3.8) in the presence of 2 mM or 10 mM of ammonium chloride (Takano et al., 2008). Viral titre was determined by plaque assay as mentioned above.

2.4. RESULTS

2.4.1. Preparation of FcγRIIA receptor which contains mutations in signaling domains

Most hematopoietic cells express more than one FcγR (Daëron, 1997), and it has been difficult to define which FcγR mediates ADE in the absence of co-operative role from another FcγR. To define the role of FcγRIIA in ADE, the receptor was transfected into COS-7 cells, which lacks endogenous FcγR (Indik et al., 1991). The FcγRIIA cytoplasmic region tyrosine residues (Y281, Y288 and Y304), which are designated as P1, P2 and P3 respectively, contributes to receptor capability to undergo phagocytosis and capping (Kwiatkowska et al., 2003; Mitchell et al., 1994). The cysteine residue located in the transmembrane juxtapose of FcγRIIA (C241) is involved in raft localization of FcγRIIA and efficient receptor signaling (Barnes et al., 2006). We introduced a series of point and deletion mutations in residues in the cytoplasmic domain of FcγRIIA which involved in receptor signaling and phagocytosis of immune complexes (Fig. 2.1 and Table 2.1). The expression of each of the constructs in COS-7 cells was verified by immunoblotting (data not shown) and by flow cytometry (Fig. 2.1 and Table 2.1). More that 50% of the transfected cells constantly expressed mutant and wild-type (WT) FcγRIIA, except for dT (48 ± 5%) and Y3F (34 ± 10%).

2.4.2. Phagocytic activities of COS-7 cells expressing mutated FcγRIIA

To confirm that wild type (WT) and some of the mutated FcγRIIA maintain receptor biological function, we first measured phagocytic activity. Phagocytic activity is the most well studied biological function of FcγRIIA. (Indik et al., 1995a, b; Mitchell et al., 1994)

We adopted a quantitative fluorescence method that employed IgG-opsonized, succimidyl-ester (SE) labeled E. coli immuno-complex particles (Fig. 2.2a). COS-7 cells expressing WT and dT exhibited higher levels of phagocytic activity than those expressing other mutant FcγRIIA or those without FcγRIIA (Fig. 2.2b). Less than 5% of COS-7 cells expressing WT and dT were phagocytic when exposed to SE-labeled K-12 E. coli without IgG. The results suggest that the phagocytic activity of WT and dT-expressing cells is

FcγR-dependent, and that transfected FcγRIIA is functional.

2.4.3. Receptor clustering induced by the binding of DENV-antibody complex to FcγRIIA

Consequences of the binding of DENV-antibody complexes to wild type and mutant FcγRIIA were examined. DENV-1-antibody complex was added to COS-7 cells expressing each of the FcγRIIA mutants or WT, and cross-linking and capping was followed by immunofluorescene method as described in Methods. Capping occurred on COS-7 cells that expressed WT and dT (Fig. 2.3). Cross-linking, but not capping, occurred on those expressing dP3, dP2, dP1P2, dP2P3, dP1P2P3, CT, dISR, Y3F and C241A. Neither cross-linking nor capping occurred in WT-transfected COS-7 after inoculation with DENV-1 in the absence of antibody (Fig. 2.3 WT^{-Ab}).

2.4.4. Antibody-dependent enhancement of DENV infection in COS-7 cells expressing mutant FcγRIIA

Human serum from a DENV-3 infected patient was used to prepare DENV-1 or DENV-2-antibody complex for examining ADE. The human anti-DENV serum enhanced DENV-1 and DENV-2 infection of WT expressing COS-7 cells to the maximum levels at a final dilution of $1:10^3$ and $1:10^4$, respectively (Fig. 2.4). Based on these results, the serum was used at $1:10^3$ dilution for DENV-1, and at $1:10^4$ dilution for DENV-2 in the following experiments.

DENV-1 and DENV-2 were mixed with anti-dengue human serum at $1:10^3$ dilution and $1:10^4$ dilution, respectively, and DENV-antibody complex was prepared. DENV-antibody immune complex or DENV alone was inoculated to COS-7 cells expressing WT or mutant FcγRIIA. Presence of infected cells were confirmed by immunofluorescence assay (Fig. 2.5a), and the percentage of DENV-antigen positive cells was measured by flow cytometry (Fig. 2.5b and 2.5c). The percentage of antigen-positive cells was compared between transfected COS-7 cells infected with DENV-antibody-immune complex and those infected with DENV alone.

Antibody-dependent enhancement of DENV-1 and DENV-2 infection was detected with COS-7 cells expressing WT, dT and dP3, but not with those expressing other mutants of FcγRIIA or mock transfected COS-7 cells (Fig. 2.5b and 2.5c). The results indicate that the disruption of the ITAM motifs and the removal of the sequences between the two ITAM motifs abrogate the ability of FcγRIIA to mediate ADE. The results, thus, suggest that specific structure of FcγRIIA and signal transduction via FcγRIIA are required for ADE of dengue virus infection.

2.4.5. Absence of inhibitory effect of heparin on infection of FcγRIIA-expressing cells with DENV-antibody complex

Effect on heparin on ADE of dengue virus infection was examined. Heparin inhibited DENV-2 infection of BHK cells and FcγRIIA-expressing BHK cells in the absence of enhancing antibodies, at concentrations of 10 μg/ml and 100 μg/ml (figure 2.7a). Heparin partially inhibited infection of BHK cells with DENV-2-antibody complex, but did not inhibit that of FcγRIIA-expressing BHK cells with DENV-2-antibody complex (figure 2.7b). The results suggest that interaction between virus and cell surface components containing heparan sulphate is not necessary in ADE.

2.4.6. Inhibitory effect of lysomsomotropic agent on ADE

Whether acidification of endosomes is necessary for infection with DENV-2-antibody complex was investigated using a lysosomotropic agent, ammonium chloride. BHK-21 cells and FcγRIIA-expressing BHK cells were infected with DENV-2 or DENV-2-antibody complex in the presence of 2 mM or 10 mM of ammonium chloride. Ammonium chloride partially inhibited the infection of BHK-21 cells with DENV-2 and DENV-2-antibody complex (figure 2.7c). It also partially inhibited infection of FcγRIIA-expressing BHK cells with DENV-2 and DENV-2-antibody complex (figure 2.7d).

2.5. DISCUSSION

Antibody response is an important defense mechanism employed to control DENV infection. Anti-DENV antibodies at subneutralizing concentrations, however, enhance DENV infection via the FcγRI (Kontny et al., 1988) and FcγRII (Littaua et al., 1990). The ability of DENV to utilize FcγR for cell entry relies on the formation of virus-antibody complex. Thus, identification of the early steps of interactions between DENV-antibody and FcγR is important in the elucidation of the mechanism of ADE.

FcγR transfected in COS cells has proved to be useful for defining functions of FcγR in mediating receptor tyrosine phosphorylation, phagocytosis (Mitchell et al., 1994) and endocytosis (Davis et al., 1995). Specific structures of the transmembrane and cytoplasmic domain accounts for the ability of FcγRIIA to stimulate phagocytosis and tyrosine phosphorylation (Barnes et al., 2006; Garcia-Gracia & Rosales, 2007; Mitchell et al., 1994). Receptor phosphorylation are catalyzed by rafts, triggering signal pathways that target actin-based cytoskeleton reorganization, and in turn serves as a driving force for FcγRIIA-mediated phagocytosis and FcγRIIA capping (Kwiatkowska et al., 2003). These findings indicate that specific structures of FcγRIIA are crucial for triggering receptor-mediated signaling pathways and biological functions.

The present study was undertaken to determine whether modification of the conserved motifs of the cytoplasmic region of FcγRIIA affects the capability of the receptor to mediate ADE. Consistent with previous findings (Kwiatkowska et al., 2003; Mitchell et al., 1994), we found that native FcγRIIA (WT) mediated phagocytosis and receptor capping. In contrast, modification of the ITAM region and removal of sequences between the ITAM motifs abrogated phagocytosis and the ability of FcγRIIA to undergo capping. The specificity of FcγRIIA-mediated phagocytosis and receptor capping was confirmed by experiments in the absence of enhancing or opsonizing antibodies, and by using COS-7 cells without the FcγRIIA. DENV infection was enhanced by anti-DENV serum in COS-7 cells transfected with FcγRIIA (WT) and dT. The specific structure of the FcγRIIA is, thus, required for ADE. To examine whether phagocytosis and DENV-immune complex enhancement possess similar signal transduction mechanisms, we performed a linear

regression analysis and found a highly significant correlation (DENV-1, Fig. 2.6a and DENV-2, Fig. 2.6b) between the two processes. This suggests that similar signal transduction mechanisms may be in part involved in ADE and phagocytosis.

The enhancement of DENV infection by anti-DENV serum was absent with FcγRIIA/Y3F which carries mutations in the ITAM tyrosine moiety. The results differ from the conclusion of a recent study, which observed that tyrosine residues in the ITAM region do not play a role in FcγRIIA mediated ADE (Rodrigo et al., 2006). Differences in transfection method, infection method, variations in antibodies and virus strain may be the reason for the different results, but this should be further examined.

Studies indicated that cells of mononuclear phagocyte lineage (monocytes and macrophages) are the primary target cells *in vivo* (Jessie et al., 2004). Viral entry could be mediated by phagocytosis, macropinocytosis, clathrin-mediated endocytosis or caveolae-mediated endocytosis. The nature of cellular receptors for DENV has not been defined, although it is generally accepted that DENV gains entry to its target cell by receptor-mediated endocytosis in the absence of antibodies (van der Schaar et al., 2008). A number of different mammalian cell components have been proposed to be involved in DENV cellular invasion, including heparan sulfate (Chen et al., 1997, Germi et al., 2002), heat shock protein 70 (Hsp70) and Hsp90 (Chavez-Salinas et al., 2008, Reyes-del Valle et al., 2005), 37-kDa/ 67kDa high affinity laminin receptor (Thepparit & Smith, 2008), and DC-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintergrin (DC-SIGN) (Navarro-Sanchez et al., 2005, Tassaneetrithep et al., 2003).

Most of DENV target cells however, express FcγR, which enhances DENV infection in the presence of sub-neutralizing anti-DENV IgG antibodies. Two mechanisms are known to exist for viral entry via FcγR-expressing cells; via the trypsin-sensitive virus receptors and typsin-resistant immune complex receptors (FcγR) (Daughaday et al., 1981). The process of the entrance of DENV into the cells in FcγR mediated ADE has not been completely understood. FcγRIIA, in the presence immune complexes induce functional responses such as phagocytosis, respiratory burst, actin formation, and activation of Syk, phosphatidyinositol-3-kinase, and extracellular signal-regulated kinases 1 and 2. Upon

activation, several cytoplasmic domains of the FcγRIIA are involved in functional responses. Src family tyrosine kinase (SRTK), such as Hck, Fgr and Lyn, is capable of binding FcγR or other receptor subunits at low affinity before receptor stimulation. SRTK plays an important role in FcγR-induced formation of actin cups, and activation of Syk. Phosphorylated ITAMs then serve as docking site for the Src homology 2 (SH2)-containing signaling molecules, most notably Syk tyrosine kinase. After FcγR activation, phosphorylation of Syk provides a unique binding site for PI3K and Cbl. Binding of PI3K with Syk is essential for phagocytosis but not endocytosis of immune complex (Huang et al., 2006). Syk activation subsequently leads to activation of signaling cascades that involve a variety of molecules including Ca^{2+}, protein kinase C (PKC), phospholipase A2 (PLA2), phospholipase Cγ (PLCγ), phospholipase D (PLD), phosphatidyl inositol 3-kinase (PI3K), extracellular signal-regulated kinase (ERK), and GTPases of the Rho family, which are important for mediation of immune complex internalization. Antibody coated flavivirus has been reported to enter macrophages in aggregates of viral particles and increased infectivity could be due to a more efficient internalization process by a phagocytotic mechanism in the presence of enhancing antibodies (Gollins & Poterfield, 1985). Whether the Fc portion of the IgG is involved in ADE could be examined by removal of the Fc portion of enhancing antibodies or using anti-FcγRIIA antibody, MAb IV.3, to block the receptor Fc binding site (Mady et al., 1991). Protein kinase inhibitors such as SRTK inhibitor PP2, the Syk kinase-specific inhibitor piceatannol, and the phosphatidyl inositol 3-kinases (PI3K) inhibitor, wortmannin, are known to suppress phagocytosis but not endocytosis (Huang et al., 2006). In contrast, endocytosis of immune complexes by FcγRIIA depends on the presence of a ubiquitin conjugation system, that includes Cbl-mediated mono-ubiquitination of FcγRIIA and poly-ubiquitination of Syk (Huang et al., 2006, Mero et al., 2006). Preferential inhibition of phagocytosis indicates a differential requirement of phagocytosis and endocytosis for FcγRIIA signal transduction mechanisms. In the event of FcγR mediated viral entry via phagocytosis, introduction of protein kinase inhibitors such as PP2, piceatannol and wortmannin during infection could abrogate ADE. In contrast, indirect effect on receptor ubiquitylation, for example, depletion of free ubiquitin in the cell

by treatment with inhibitors such as clasto-lactacystin β-lactone, which inhibits endocytosis of immune complexes (Booth et al., 2002, Mero et al., 2006), could abrogate ADE mediated by immune complex endocytosis via the FcγR. Investigation on the molecular associations between Syk and PI3K using immunoprecipitation methods, of either phagocytosis or endocytosis of immune complex during ADE, could be further be elucidated by observing PI3K(p85)-Syk binding, as was observed in human monocytes during phagocytosis of large opsonized particles (Huang et al., 2006).

Alternatively, after binding of immune complexes to the FcγRIIA, the receptor forms clusters and is recruited to microdomains in the plasma membrane (Kwiatkowska et al., 2002). These glycolipid- and cholesterol-rich membrane microdomains, called lipid rafts, or detergent-resistant membranes (DRMs) concentrate GPI-anchored proteins, transmembrane proteins, SRTK and heterotrimeric GTP-binding proteins (Li et al., 1995, Pike et al., 1996). Local accumulation, clustering and accumulation into polar caps of FcγRIIA during binding of immune complexes occur by diffusion of the receptor in the plane of plasma membrane, which is promoted by ceramide and driven by actin cytoskeleton (Abdel Shakor et al., 2004). Enhancement of DENV infection could occur as the FcγRIIA focuses the virus on the cell membrane and permits it to bind to its receptor more efficiently; thus increasing its infectivity (Mady et al., 1991). ADE occurred in the presence of enhancing antibodies' without the constant region (Fc) of IgG as was performed with bispecific antibodies, which targets both DENV and FcγRIIA (Mady et al., 1991). However, ADE effect was lowered in the absence of the Fc portion of IgG. This suggests that in the absence of Fc portion of IgG, DENV may utilize other entry pathway(s) such as binding to other receptor located at close proximity to the FcγR or that DENV may require binding of the Fc portion of IgG to the FcγR for optimal enhancement. In respect to this, monoclonal antibody towards CD4 inhibited HIV infection and ADE, indicating that two receptors are required for infection of the cell and infection with and without antibodies is mediated by the same receptor (Takeda et al., 1990). In contrast, immune complex mediated activation of the FcγRIIA may play a role in ADE by modulating changes to the plasma membrane. Lipid rafts were required for DENV infection in the absence of

enhancing antibodies (Reyes-del Valle et al., 2005) and for hepatitis C virus (Kapadia et al., 2007). It is possible that in the presence of enhancing antibodies, DENV binds to FcγR and utilizes rafts or cellular components located in rafts to facilitate entry. The interactions between activated FcγRIIA and lipid rafts can be facilitated by ceramide. Abrogation of ceramide production under the influence of chloroquine and imipramine (Abdel Shakor et al., 2004) or methyl-γ-cyclodextrin, that sequesters cholesterol from plasma membrane (Reyes del Valle et al., 2005) would address the requirements of lipid rafts in ADE of DENV infection. In addition, isolation of virus and lipid rafts by immunoprecipitation would further clarify this issue, as was for mouse hepatitis virus (Choi et al., 2005).

Heparan sulphate has been reported to be an important host component for DENV entry which could increase DENV cell surface contact and in turn increase accessibility to DENV receptor. This process can be blocked by heparin in various human liver cell lines and baby hamster kidney cell lines (BHK-21 cell lines) (Lin et al., 2002, Thaisomboonsuk et al., 2005). In the absence of FcγR or enhancing antibodies, DENV binds to heparan sulphate on the cell membrane to efficiently enter the cells (Chen et al., 1997, Marks et al., 2001). The involvement of FcγRIIA in ADE of DENV infection has been reported but the necessity of heparan sulphate binding has not been determined. In preliminary studies (Fig. 2.7), infection of FcγRIIA-expressing cells by DENV-antibody complex was not blocked by heparin, suggesting that binding of DENV with heparan suphate is not necessary for ADE. On the other hand, heparin partially inhibited DENV infection in the absence of enhancing antibody. It is, thus, likely that the role of heparan sulphate on the cell membrane is different between DENV infection in the absence of enhancing antibody and FcγR-mediated infection in ADE. Although heparin has been reported as an important host component for DENV entry, infection was reduced but not abolished using DENV-2 in the absence of enhancing antibodies (Lin et al., 2002), suggesting that different cell components, other than heparan sulphate, may play an important role in DENV infection.

Viral entry is mediated by phagocytosis, macropinocytosis, clathrin-mediated endocytosis or caveolae-mediated endocytosis. In the absence of antibodies, clathrin-mediated endocytosis is the predominant viral entry pathway for DENV (Krishnan et al., 2007,

Suksanpaisan et al., 2009). Following receptor mediated endocytosis of DENV (van der Schaar et al., 2008), fusion of viral envelope and endosomal membrane occurs via a low pH-catalyzed reorganization of the E protein (Bressanelli et al., 2004, Kielian, 2006). Fusion process starts in early endosomes (van der Schaar et al., 2008) or in late endosomes, which requires microtubule trafficking (Acosta et al., 2008, Krishnan et al., 2007), according to structural features of the E-protein and microenvironment in the cells (Huang et al., 2010). An acidic pH in endosome is necessary for ADE in West Nile virus (Gollins & Poterfield, 1985) and feline infectious perintonitis virus (Takano et al., 2008). Further studies are however needed to determine whether endosomal acidification is required by DENV via internalization by the FcγRIIA, for example, by increasing lysosomal pH using ammonium chloride and quantification of virus RNA (Talarico & Damonte, 2007), or by visual examination using electron microscopy and fluorescence microscopy after virus-antibody intake.

The question remains on how DENV releases viral genome into the cytoplasm after endocytosis and whether these processes differ in FcγR mediated infection or non-FcγR mediated infection. Upon entry via an activated receptor, internalized particles are trafficked sequentially into early endosomes, late endosomes and degraded in lysosomes. Certain pathogens escape degradation by either blocking the maturation of endosomes, transformation of endosomes, targeting of endosomes to different locations, and endosomal escape. Successful DENV infection appears to require a transport regulator and marker of early endosome, Rab 5, but not the late endosomal marker, Rab 7 (Krishnan et al., 2007). Rab 5 is known to regulate processes such as delivery of plasma-membrane-derived vesicles to endosomes (van der Bliek et al., 2005). FcγR mediated endocytosis and phagocytosis of a macrophage-like cell line, RAW 264.7, is however, unaffected in the absence of Rab5 (Tse et al., 2003). It would be interesting to examine whether Rab5 regulates uptake of DENV, leading to successful infection and infection enhancement. In the event of FcγR mediated internalization, DENV could be targeted to different locations leading to enhanced infection. Examination of target cellular compartments using endosomal markers, such as Rab5 and Rab7 by RNAi to downregulate the gene expression

(Krishnan et al. 2007), would address this question. In addition to compartment targeting for effective infection, viruses such as the adenovirus have the capability of phagosomal escape. Uptake mechanisms via FcγR in adenovirus involve two distinct endocytic pathways, the phagocytic uptake of large clusters of viruses and early stages of clathrin-mediated uptake of single particles. Phagocytosis is responsible for the internalization of the FcγR-targeted adenovirus clusters, while clathrin is involved in breaking open the phagosomal membrane and delivering infectious endocytic virus particles to the cytosol. Breaking of the phagosomal membrane was found to be inhibited in clathrin knockdown cells (Meier et al., 2005). Whether similar mechanisms are involved in ADE of DENV could be examined by downregulating clathrin expression in FcγR-expressing cells.

The question of the role of DENV receptor in ADE could be further defined once the real DENV receptor is identified, prompting for the need of the identification of DENV receptor(s). Expression and screening of the receptors using cDNA library in DENV non-susceptible cells would address this question as was for measles virus (Tatsuo et al., 2000). We conclude that the specific structure of the FcγRIIA, when present on non-professional phagocytic cells, is crucial for mediating processes that promotes ADE and that the results provide a profound insight into the understanding of the mechanism of DENV entry into the cells in the presence of antibody.

Table 2.1. Characteristics of mutated FcγRIIA prepared in the study.

Mutants	Mutations					% positive cells* (mean ± SD)
	ITAM1	ITAM2	Y281	C241	ISR	
WT	+†	+	+	+	+	57 ± 9
dT	+	+	+	+	+	48 ± 5
dP3	+	-	+	+	+	61 ± 7
dP2	-‡	+	+	+	+	59 ± 1
dP1P2	-	+	-	+	+	60 ± 15
dP2P3	-	-	+	+	-	60 ± 11
dP1P2P3	-	-	-	+	-	87 ± 3
CT	-	-	-	-	-	55 ± 14
dISR§	+	+	+	+	-	97 ± 2
Y3F‖	Y304F	Y288F	Y281F	+	+	34 ± 10
C241A¶	+	+	+	C241A	+	80 ± 14

*: Surface expression of WT and mutated FcγRIIA was examined by flow cytometry. Results are shown as means and standard deviation of 3 experiments performed in triplicates; †: Plus sign (+) indicates that the region exists as wild type; ‡: Minus sign (-) indicates that the region was deleted; §: Deletion of 12 amino acids located between ITAM 1 and ITAM 2; ‖: Phenylalanine residue was substituted for tyrosine residue at the a.a. positions 281, 288 and 304; ¶: Alanine residue was substituted for cysteine residue at a.a. position 241.

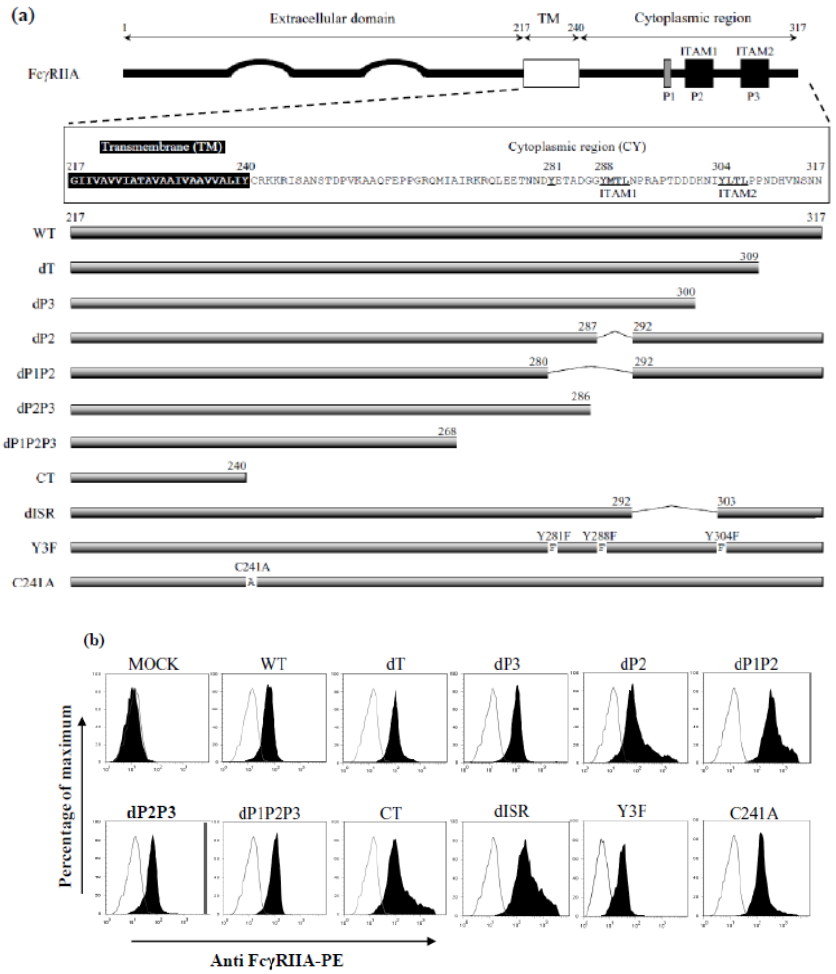

Fig. 2.1. Structure and expression of the mutated FcγRIIA constructs in COS-7 cells determined by flow cytometry. (a) The mutants were constructed by standard site directed mutagenesis methods, and introduced into pcDNA3.1(+) and expressed in COS-7 cells. The gene, FcγRIIA, is shown in the figure with the extracellular region (EX), transmembrane region (TM) and cytoplasmic region (CY). The FcγRIIA cDNA is numbered starting from +1. Shaded box represents a hydrophobic stretch of 24 amino acids presumed to span the membrane (Brooks *et al.*, 1989). Lines indicate deleted regions and the letters (A, F) represent substituted amino acids (a.a.) in the gene. (b) PE-labeled monoclonal antibody (mAb 16320) to human FcγRIIA was used to measure the percentage of COS-7 cells expressing WT and mutated FcγRIIA. Results were representative of more than 3 experiments performed in triplicate.

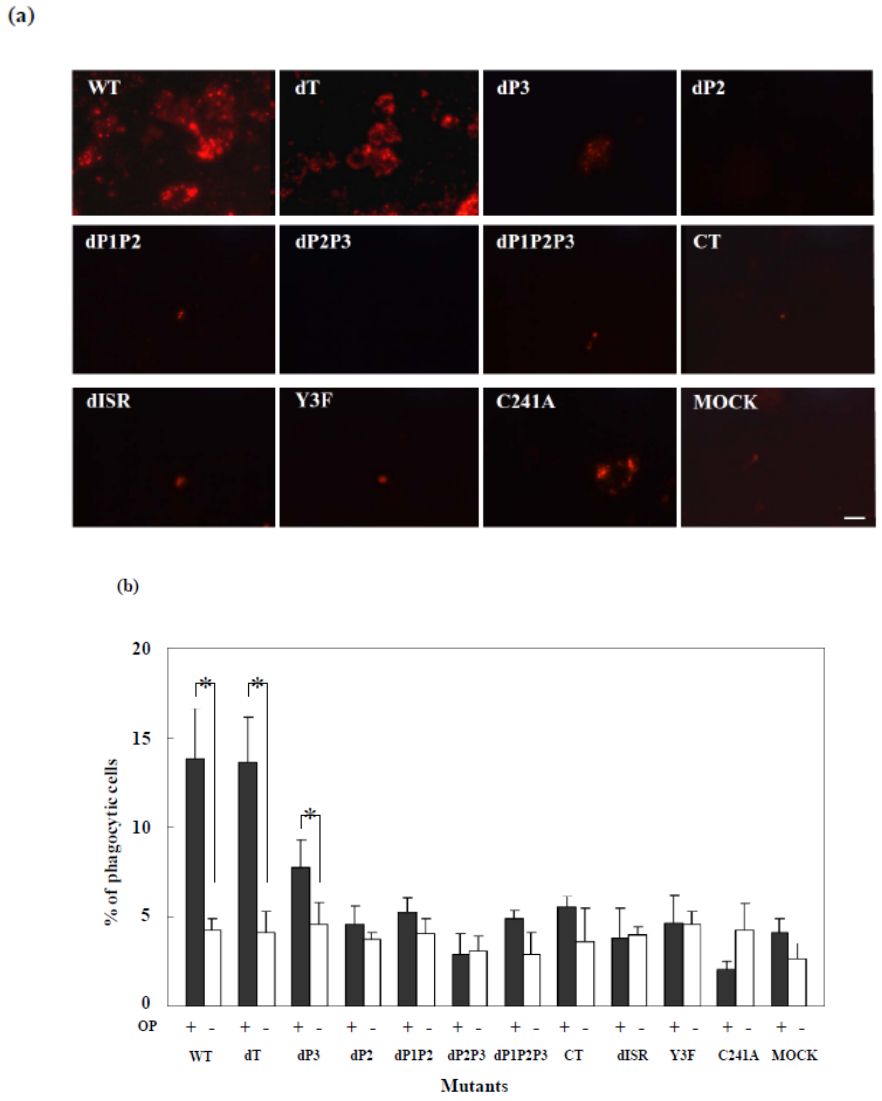

Fig. 2.2. Phagocytosis of opsonized SE-labeled K-12 E.coli by COS-7 cells expressing mutant and wild type FcγRIIA. (a) COS-7 cells expressing WT or mutated FcγRIIA were incubated with succinyl ester labeled-K-12 E. coli particles sensitized with rabbit IgG (OP) at 37°C for 45 minutes. Mock transfected COS-7 cells served as controls. Phagocytosis of E. coli by COS-7 cells was observed by immunofluorescent microscope. Bar, 20 μm. (b) Percentage of cells that phagocytosed E. coli was determined by flow cytometry. Solid bar indicates mean percentage of phagocytic cells in the presence of opsonizing reagent and closed bar indicates mean percentage of phagocytic cells in the absence of opsonizing reagent.

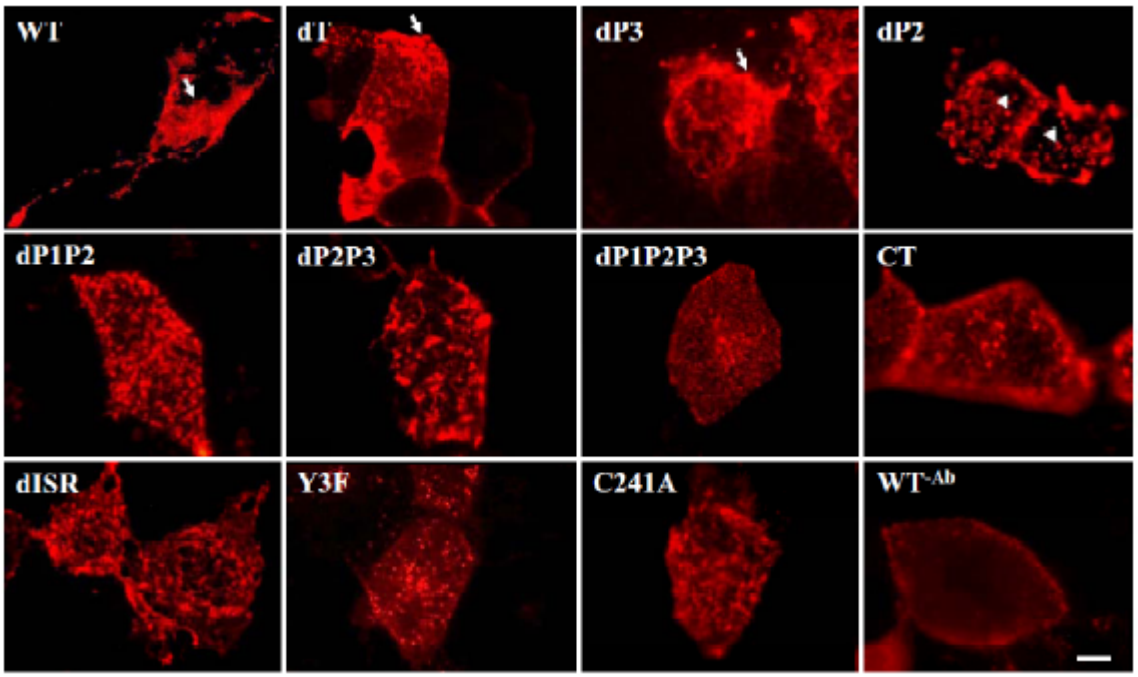

Fig. 2.3. Cross-linking and capping of anti-dengue serum-DENV-2 complex on COS-7 cells expressing WT and mutated FcγRIIA. COS-7 tranfectants were monitored for their ability to form cap-like structures upon binding of DENV-antibody complex. Capping of cross-linked cells was observed at 20 minutes after warming at 20°C. Each transfectant was stained with PE-labeled hFcγRIIA (mAb 16320). WT^{-Ab} represents cells stimulated with DENV in the absence of antibody. (→) Indicates capping and (►) indicates cross-linking. Bar, 5 μm.

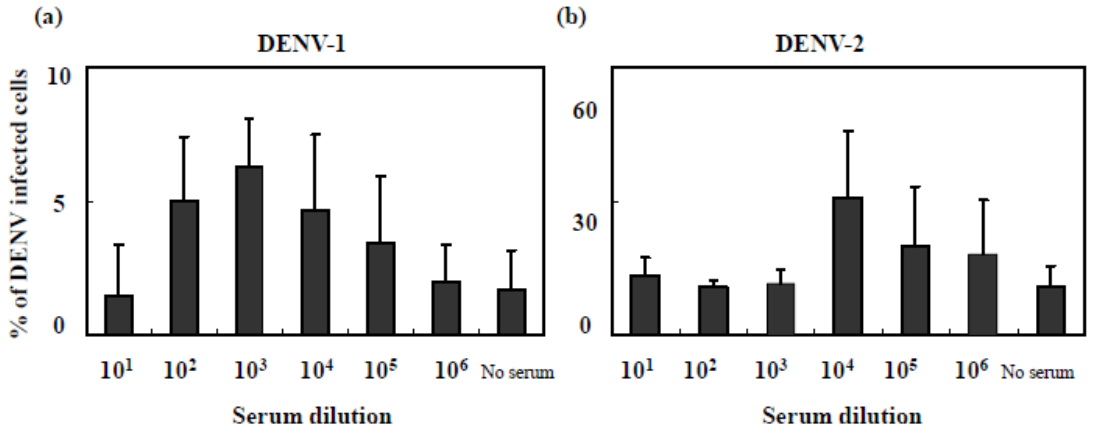

Fig. 2.4. Enhancement of DENV infection in COS-7 cells expressing WT-FcγRIIA. DENV-1 or DENV-2 pre-incubated with 10-fold diluted human anti-dengue-serum for 1 hour at 37°C. COS-7 cells expressing FcγRIIA were infected with DENV-antibody complex at 37°C for 72 hours. Percentage of infected cells was determined by flow cytometry after indirect immunostaining with anti-flavivirus monoclonal antibody, mAb 4G2. Results are representative of more than 2 experiments performed in triplicate.

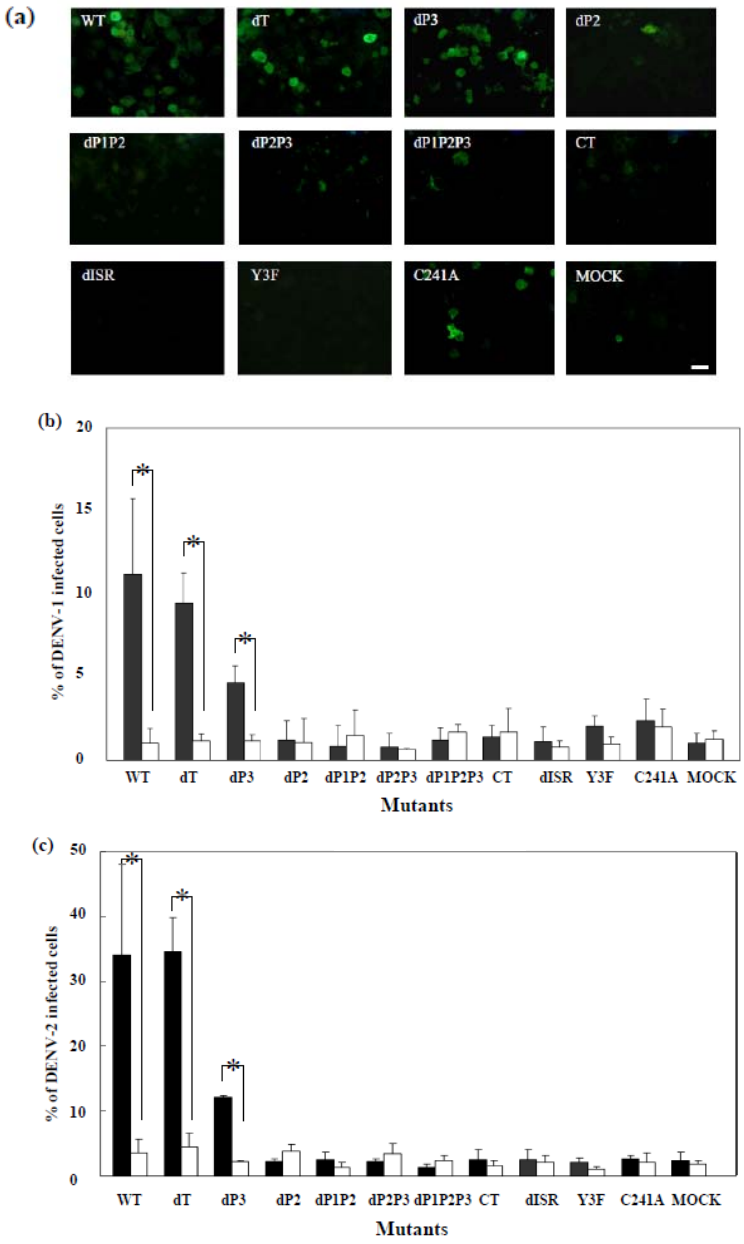

Fig. 2.5. ADE of DENV infection in COS-7 cells expressing WT and dT FcγRIIA. (a) DENV-antibody complex was prepared by incubation of DENV-2 and anti-DENV human serum diluted at $1:10^4$. COS-7 cells were infected with DENV-2-antibody complex. The cells stained with anti-flavivirus monoclonal antibody 4G2. Immunofluorescent photomicrographs shows DENV-2 antigen positive cells (green). Multiplicity of infection was 0.1. Bar, 20 μm. (b) The percentage of DENV-1 infected cells was quantified by flow

cytometry. Results are the mean and standard deviations of 3 experiments performed in duplicates or triplicates. Multiplicity of infection was 0.1. The solid bar indicates mean percentage of DENV-1 infected cells in the presence of enhancing antibody ($1:10^3$ dilution) and closed bar indicates mean percentage of DENV-1 infected cells in the absence of enhancing antibody. (c) Percentage of DENV-2 infected cells was quantified by flow cytometry. Results are the mean and standard deviations of 3 experiments performed in duplicates or triplicates. Multiplicity of infection was 0.1. Solid bar indicates mean percentage of DENV-2 infected cells in the presence of enhancing antibody ($1:10^4$ dilution) and closed bar indicates mean percentage of DENV-2 infected cells in the absence of enhancing antibody.

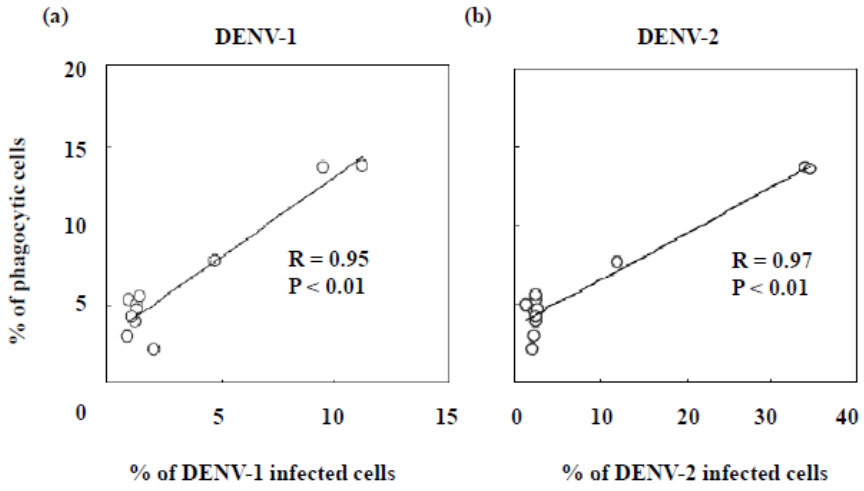

Fig. 2.6. Linear regression between ADE and phagocytic activities of COS-7 cells expressing WT and mutated FcγRIIA. The percentage of phagocytic and DENV infected COS-7 cells transfected with FcγRIIA is reduced with introduction of deletion and point mutation in the FcγRIIA cytoplasmic domain. Significant correlation between phagocytic and ADE capacities within the COS-7 cells transfected with FcγRIIA were observed. (a) DENV-1. (b) DENV-2.

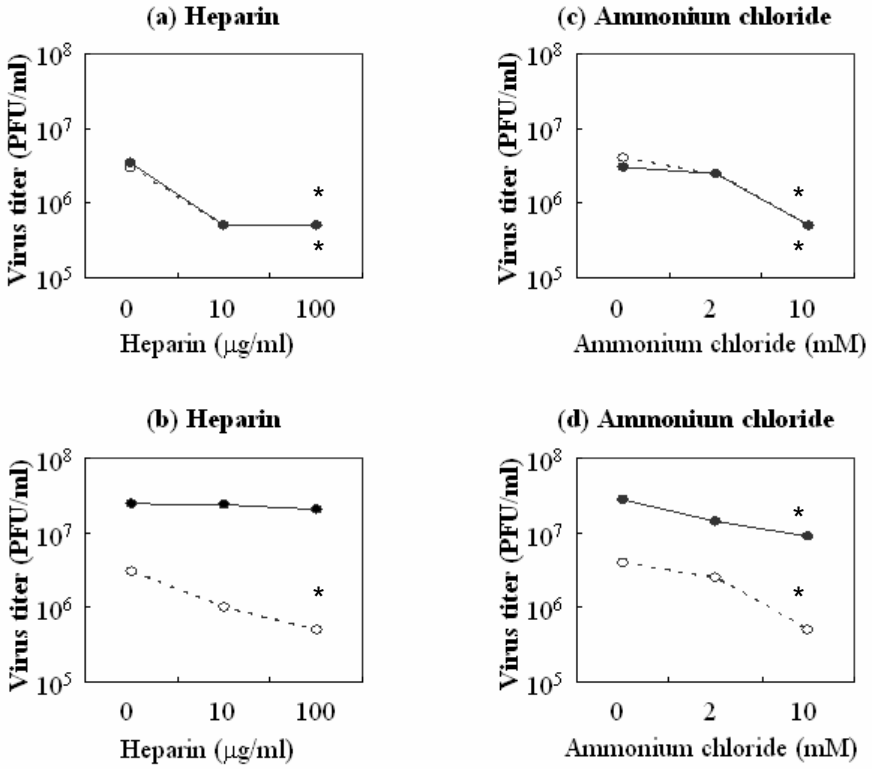

Fig. 2.7. Effects of heparin and ammonium chloride on ADE of DENV-2 infection in BHK cells and FcγRIIA-expressing BHK cells. BHK-21 cells and FcγRIIA-expressing BHK cells were infected with DENV or DENV-antibody complex in the presence of heparin or ammonium chloride. Infection with DENV-2 alone (○) or DENV-2 and 4G2 at 1:1 000 dilution (●) at 37 ^{o}C for 1 h. After 5 days, virus titer was measured by direct plaque count (n = 4). (a, c) BHK-21 cell lines; (b, d) FcγRIIA-expressing BHK-21 cell lines; (a, b) heparin treatment; (c, d) ammonium chloride treatment. *P<0.05 compared to medium without heparin. *P<0.05 compared to medium without ammonium chloride.

CHAPTER 3 Development of antibody-dependent enhancement assay for dengue virus using BHK cell lines that express stably FcγRIIA

3.1. ABSTRACT

Dengue virus (DENV) causes a wide range of symptoms, from mild febrile illness, dengue fever (DF), to severe life threatening illness, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Subneutralizing concentrations of antibody to DENV enhance DENV infection of FcγR positive cells. This phenomenon is known as antibody-dependent enhancement (ADE). ADE is considered to be a risk factor for DHF and DSS. To develop an ADE assay for DENV, two BHK cell lines that express stably FcγRIIA were established. The FcγRIIA-expressing BHK cell lines were used in ADE assay with monoclonal antibody (4G2) to DENV, and DENV antibody-positive human sera. Virus growth was quantified directly in FcγR-expressing BHK cells by standard plaque assay procedure. ADE was detected with monoclonal antibody (4G2) to DENV. ADE was also detected with DENV antibody-positive human sera, but not with DENV antibody-negative human sera. The new ADE assay using FcγR-expressing BHK cells is simple and practical, and is useful for defining the role of ADE in the pathogenesis of DENV infection.

3.2. INTRODUCTION

Dengue virus (DENV) infection occurs in most tropical and subtropical regions of the world, including Asia, Africa and South America, with up to an estimated 100 million people infected worldwide annually. Each of the four serotypes of DENV (DENV-1, DENV-2, DENV-3 and DENV-4) causes a spectrum of symptoms, ranging from mild febrile illness, dengue fever (DF), to severe life threatening illness, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Infection with one DENV serotype offers life-long protection against infection by the same serotype. At the same time, immunity induced against one dengue serotype does not provide protection towards infection with serotypes not encountered previously. Antibody induced in primary DENV infection is postulated to be one of the risk factors for DHF/ DSS in secondary infection with the serotype different from those causing primary infection (Graham et al., 1999; Sangkawibha et al., 1984).

In secondary DENV infection, DENV-antibody complexes are formed and taken up more readily than uncoated virus particles by cells expressing Fcγ receptors (FcγR), such as monocytes and macrophages (Kontny et al., 1988; Littaua et al., 1990). This effect, known as antibody-dependent enhancement (ADE), leads to higher levels of progeny virus production. This possible ADE activity of antibody poses a challenge for the development of a dengue vaccine, and therefore additional investigation into the comparative nature of neutralizing and immune enhancing antibodies stimulated by natural infection and vaccination is required. Current DENV plaque neutralization assays, performed conventionally in mammalian cells without FcγR (Vero, LLC-MK_2, BHK), (Morens et al., 1985; Roehrig, 2007) measure selectively the neutralizing activity of antibodies without ADE activity. Consequently, it is likely that discrepancy may occur in correlation between protection and neutralizing antibody titres generated by these current assay, when the principal target cells of DENV, FcγR expressing monocytes (Kou et al., 2008), are used for measurement of neutralizing antibody titres.

In the present study, BHK cells which express stably FcγRIIA were established. It was then determined whether enhancing antibodies could be measured in BHK cells expressing

FcγRIIA, using conventional plaque assay. Human sera positive for DENV IgG and monoclonal anti DENV IgG enhance DENV-2 infection using the new BHK cells expressing FcγRIIA. The new ADE assay is simple, and is useful for determining the role of antibody in DENV infection.

3.3. MATERIALS AND METHODS

3.3.1. Cell Lines

BHK-21 cells, a hamster kidney cell line, and Vero cells (Vero 9013, Japan Health Science Research Resources Bank), an African green monkey kidney-derived epithelial cell line, were used. Cells were cultured in Eagle's Minimum Essential Medium (EMEM), (Sigma, St. Louis, MO, USA), supplemented with heat inactivated 10% FCS (Sigma) without antibiotics at 37 °C in 5% CO_2.

3.3.2. Virus

Dengue virus type-1 (DENV-1), 01-44-1HuNIID strain (GenBank accession no. AB111070), dengue virus type-2 (DENV-2) TL-30 strain (GenBank accession no. AB219135), and DENV-2 S16803 strain, dengue virus type-3 (DENV-3) TL-18 strain (GenBank accession no. AB214879), and dengue virus type-4 (DENV-4) TVP-360 strain were used. DENV-1 01-44-1HuNIID strain was isolated in Tahiti in 2001 (Ito et al., 2007). DENV-2 (S16803) and DENV-4 (TVP-360) were reference laboratory strains (Roehrig, 2007). Virus was propagated on Vero cells. DENV-2 TL-30 strain was harvested at the 5th culture passage, and virus from this single passage was used in all the infection enhancement experiments.

3.3.3. Serum specimens and antibody

Serum specimens were heat inactivated at 56 °C for 30 minutes and used in the experiments. All the sera used in the present study had been tested for DENV antibody by IgG ELISA and IgM ELISA. Dengue serotype-cross-reactive mouse monoclonal IgG antibody (MAbs, ATCC MAb HB-112 D1-4G2-4-15) was also used in ADE assay.

3.3.4. FcγRIIA plasmid construction

Human FcγRIIA cDNA (Brooks et al., 1989, GenBank accession no. M31932) was generously provided by Dr. Jeffrey V. Ravetch, Rcokfeller University, NY, USA. The

cDNA was subcloned into pcDNA 3.1/neo+ (Invitrogen, Calsbad, CA, USA) and full length sequence of the construct was verified by DNA sequence analysis.

3.3.5. Stable Expression of FcγRIIA in BHK-21 cells

Transfection of BHK-21 cells with pcDNA3.1/neo+ vector and FcγRIIA cDNA was carried out with Lipofectamine LTX reagent (Invitrogen) and Nupherin-neuron reagent (Biomol Research Laboratories, Plymouth Meeting, PA), according to the manufacturers' protocols. Transfected cells were selected with 0.5 mg/ml neomycin (G418, PAA Laboratories GmbH, Austria) for 2 weeks and then further selected by the limiting dilution method. Cells stably transfected with the neomycin resistant vector were maintained in EMEM, 0.5 mg/ml neomycin supplemented with 10% FCS (Sigma) at 37 °C in 5% CO_2.

3.3.6. Flow cytometry

FcγRIIA transfected BHK-21 cells were examined for surface expression of FcγRIIA by flow cytometry. Cells were washed in PBS (Invitrogen) and stained with phycoerythrin (PE)-conjugated monoclonal antibody to human FcγRIIA (CD32 MAb, Clone 190723; R&D Systems, Minneapolis, MN, USA), according to manufacturer's instructions. Stained cells were analyzed by Guava EasyCyte Mini cytometer (Guava Technologies, Millipore, MA, USA). More than 5,000 cells were counted and the results were examined by FlowJo software (Tree Star, Ashland, OA, USA).

3.3.7. Preparation of virus-antibody complex

Each serum specimen was serially diluted 10 folds from $1:10^1$ to $1:10^6$ with EMEM supplemented with 10% FCS. Mouse monoclonal antibody 4G2 (ATCC HB-112 D1-4G2-4-15) was serially diluted 10 folds from 2.6 mg/ml ($1:10^1$) to $1:10^6$ with EMEM supplemented with 10% FCS. DENV-antibody complex was prepared by mixing 0.05 ml of DENV-2 TL-30 strain at titres of 1,250 PFU/ml, 125 PFU/ml or 1.25 PFU/ml with 0.05 ml of diluted serum samples or 0.05 ml of diluted antibody. For infection with DENV-2 TL-30 strain alone, viral mixture was prepared by mixing 0.05 ml of DENV-2 at titres of 1,250

PFU/ml, 12.5 PFU/ml or 1.25 PFU/ml with 0.05 ml EMEM supplemented with 10% FCS. Virus-antibody mixture was incubated at 37 °C for 60 minutes, and used in infection experiments.

3.3.8. Virus infection

One-tenth milliliter of virus-antibody mixture was inoculated on BHK-21 monolayers in 12-well plates. The plates were incubated for 60 minutes at 37 °C in 5% CO_2. After virus absorption, the cells were washed with 1 ml of EMEM and overlaid with maintenance medium containing 1% methylcellulose (Wako Pure Chemical Industry, Osaka, Japan). The plates were incubated at 37 °C in 5% CO_2 for 5 days.

3.3.9. Plaque visualization

After 5 days of incubation, the cells were fixed with neutral formalin for 60 minutes at room temperature and washed with tap water. The cells were then stained with 0.3% methylene blue for 60 minutes at room temperature and washed with tap water. Plaques were counted by naked eye and the virus infectivity titre was expressed as plaque forming units (PFU) per ml.

3.4. RESULTS

3.4.1. Establishment of BHK cell lines that express stably FcγRIIA

BHK-21 cell line was selected for transfection with FcγRIIA, because it forms plaques upon DENV infection. Four BHK-21 cell lines stably expressing the FcγRIIA were established (data not shown), and two of the cell lines, BHK-FcγRIIA/2 and BHK-FcγRIIA/4 were selected for further studies. The expression of FcγRIIA was verified by flow cytometry (Table 3.1 and Fig. 3.1). More than 50% of the transfected cells express FcγRIIA for as long as 18 continuous passages, during a span of 5 months (Table 3.1).

3.4.2. Efficiency of DENV plaque formation in BHK cells expressing FcγRIIA

Four serotypes of DENV were quantified in the absence of DENV antibody using two BHK expressing FcγRIIA cell lines, parent BHK cells and Vero cells (Table 3.2). The titres of DENV were similar among these 4 cell lines. The results indicate that efficiency of DENV plaque formation in BHK expressing FcγRIIA cell lines were similar to that of Vero and parent BHK cell lines.

3.4.3. Antibody-dependent enhancement of DENV-2 infection using mouse monoclonal antibody, 4G2

Mouse monoclonal antibody 4G2 (ATCC HB-112 D1-4G2-4-15), which reacts with a broad range of flaviviruses, was used to prepare virus-antibody complex for examining ADE. The 4G2 antibody, enhanced DENV-2 infection of FcγRIIA-expressing BHK-21 cells at a final dilutions of $1:10^2$ to $1:10^4$ (Fig. 3.2). ADE was not detected in BHK-21 cells and BHK-21 cells transfected with empty vector (pcDNA3.1/neo+) (Fig. 3.2B). ADE in FcγRIIA-expressing BHK-21 cell lines was consistently observed until passage 18 using monoclonal antibody 4G2 at $1:10^3$ dilution (Table 3.1).

3.4.4. Antibody-dependent enhancement of DENV-2 infection using DENV antibody-positive human sera

Ten DENV IgG-positive serum samples were tested for their capacity to enhance DENV-2 infection in BHK-FcγRIIA/2 and BHK-FcγRIIA/4. ADE activity was detected with all the dengue IgG-positive serum samples (Fig. 3.3, No. 1-10). DENV IgG-negative serum samples did not enhance DENV-2 infection in either FcγRIIA expressing cell line (Fig. 3.3, No. 11-15). The results indicate that BHK-FcγRIIA/2 and BHK-FcγRIIA/4 are suitable for detecting ADE activity of DENV antibody-positive human sera.

3.5. DISCUSSION

An assay was established to quantify and compare the in vitro ADE activity of human serum specimens. The plaque method described employs BHK cells that were transfected with human FcγRIIA, an IgG Fc-receptor that is distributed among cells of macrophage/monocyte lineage (Daëron, 1997), and which was known to mediate ADE (Littaua et al., 1990; Mady et al., 1991). Similarly, BHK cells transfected with FcγRIIA have been proved useful for functional studies of the FcγRIIA, such as phagocytosis and IgG complex clearance (Kwiatkowska et al., 2002; Sobota et al., 2005).

Infection enhancement of DENV-2 TL-30 strain complexed with 4G2, an IgG2a flavivirus group-reactive monoclonal mouse antibody, was reproducible in FcγRIIA-expressing BHK cells and provided an internal assay control. This antibody had been reported to enhance DENV infection of FcγRIIA-expressing K562 cells (Guy et al., 2004; Littaua et al., 1990; Mady et al., 1991).

Immune serum may possess two effects on DENV growth in the presence of FcγR expressing cells: neutralization and enhancement. In immune complex mediated DENV infection enhancement, interaction between DENV-antibody complexes and FcγR may suppress intracellular innate response against DENV, resulting in increased viral replication (Chareonsirisuthigul et al., 2007; Ubol et al., 2008). Although it is likely that the ADE is governed by similar intracellular innate responses in FcγR-expressing BHK cells, it remains to be seen whether this phenomenon holds true for FcγR-expressing BHK cells. Sera from symptomatic dengue cases, exhibited high levels of neutralizing activity in BHK cells, which lacks FcγR, in assays that measures selectively neutralizing activity but not enhancing activity. In contrast, sera with low levels of neutralizing activity enhanced DENV-2 infection using BHK cells expressing FcγR. ADE activity, abolished at higher antibody concentrations in some serum samples, indicates that the FcγR-expressing BHK cells is capable of detecting both neutralization and enhancement activity. Enhancing activity was detected for all the serum specimens that were positive for anti DENV IgG using BHK cells expressing FcγRIIA. None of the DENV-IgG-negative sera demonstrated ADE activity. The new assay, therefore, consistently detects ADE activity of human sera

ADE assays that use FcγR-expressing COS-7 and CV-1 cells have been described previously (Rodrigo et al., 2006, 2009a, 2009b). In these reports, plaques were detected by indirect immunostaining method using anti-NS1 protein monoclonal antibody. In comparison, the DENV plaque detection method adopted in the present study is a widely used conventional titration method (Roehrig, 2007), in which cells were stained directly by vital stain, and plaques were counted by naked eye.

The newly established BHK-FcγRIIA cell lines, constantly demonstrated ADE of DENV with anti-DENV antibody positive human sera. The ADE activity of serum samples detected by conventional plaque formation assay offers several advantages over ADE assays using hematopoeitic origin non-adherent cell lines. First, in the non-adherent cell lines, virus titers in the culture supernatant need to be assayed using surrogate plaque titration assays. Second, the conventional plaque assay used in the present study makes it possible to visualize plaque size and introduce flexibility into both incubation times and experimental workflow. Recent studies have suggested that ADE infection not only facilitates the entry process, but also modifies innate and adaptive intracellular antiviral mechanisms, resulting in unrestricted DENV replication, using THP-1 cells-derived macrophages or peripheral blood mononuclear cells (PBMCs) (Chareonsirisuthigul et al., 2007; Ubol et al., 2008). Most hematopoietic cells express more than one FcγR or other myeloid-specific receptors, that might influence biological function (Daeron, 1997; Lund-Johansen et al., 1992; Ziegler et al., 1980), and thus it has been difficult to determine the exact roles of FcγR and/or other myeloid-specific receptors that may co-operatively mediate ADE in the presence of co-operative role from other FcγR and myeloid-specific receptors.

The new ADE assay using non-hematopoeitic origin BHK-21 cells, which lack endogenous FcγR and other myeloid-specific receptors is suitable for determining the role of FcγRIIA in ADE including virus initiation, replication, and host innate immunity. BHK-FcγRIIA cell lines are suitable for determining the enhancing activity of DENV in the sera of patients, and provides a valuable tool for defining the role of antibody in the pathogenesis of DENV infection.

Table 3.1. Characteristics of FcγRIIA-expressing BHK cell lines.

Cell line	Passage Number	% of FcγRIIA expressing cells[a]	Fold increase[b]
BHK-FcγRIIA/2	2	85	ND[c]
	4	74	14
	5	66	ND
	9	69	15
	18	56	15
BHK-FcγRIIA/4	2	83	ND
	4	68	13
	5	65	ND
	9	64	13
	18	71	14

[a] The percentage of FcγRIIA expressing cells was determined by flow cytometer as described in Materials and Methods; [b] Fold increase was calculated by the formula: (DENV-2 titre in the presence of mouse monoclonal antibody 4G2 at 1:1000 dilution)/ (DENV-2 titre in the absence of anti dengue serum); [c] ND indicates not determined.

Table 3.2. DENV titre in Vero and BHK cells

Cell Line	Virus Titres (PFU/ ml)				
	DENV-1[a]	**DENV-2[b]**	**DENV-2[c]**	**DENV-3[d]**	**DENV-4[e]**
Vero	$6.3x10^7$	$4.0x10^6$	$2.5x10^5$	$3.2x10^4$	$1.2x10^6$
BHK	$3.2x10^7$	$2.5x10^6$	$2.0x10^5$	$1.0x10^4$	$1.0x10^6$
BHK-FcγRIIA/2	$4.0x10^7$	$3.2x10^6$	$1.6x10^5$	$2.5x10^4$	$1.1x10^6$
BHK-FcγRIIA/4	$4.0x10^7$	$2.5x10^6$	$1.3x10^5$	$2.0x10^4$	$1.2x10^6$

Strains used were DENV-1[a] (01-44-01 HuNIID), DENV-2[b] (TL-30), DENV-2[c] (S16803), DENV-3[d] (TL-18), DENV-4[e] (TVP-360). Virus titres were shown as the mean of two readings.

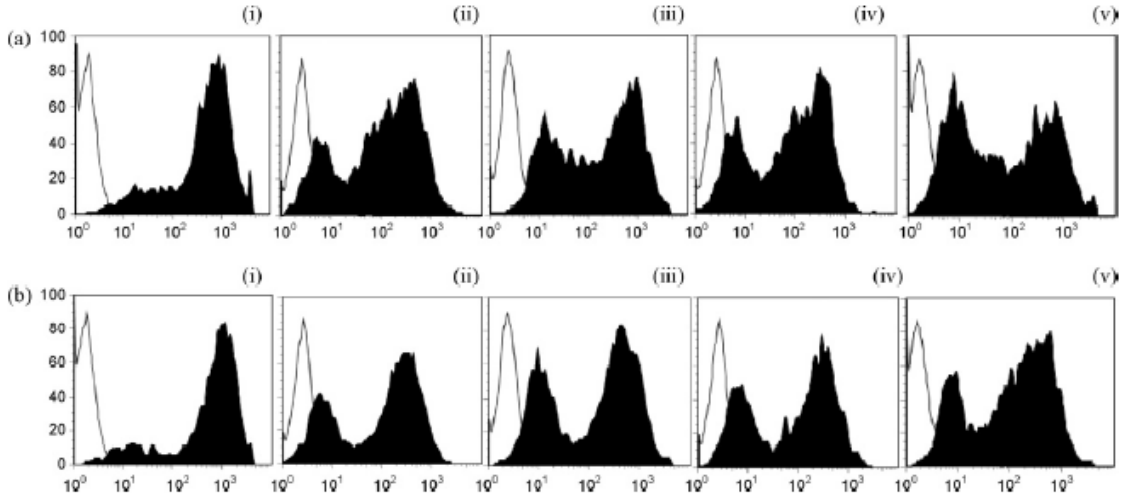

Fig. 3.1. Expression of FcγRIIA in BHK cells as determined by flow cytometry

PE-labeled monoclonal (mAb 16320) to FcγRIIA was used to measure the percentage of BHK cells expressing FcγRIIA. Solid black graph shows BHK cells transfected with FcγRIIA and open graph shows untransfected parent BHK cells. **3.1a:** FcγRIIA transfected BHK cell line 2 (BHK-FcγRIIA/2), (i) passage 2, (ii) passage 4, (iii) passage 5, (iv) passage 9, and (v) passage 18. **3.1b:** FcγRIIA transfected BHK cell line 4 (BHK-FcγRIIA/4). (i) passage 2, (ii) passage 4, (iii) passage 5, (iv) passage 9, and (v) passage 18.

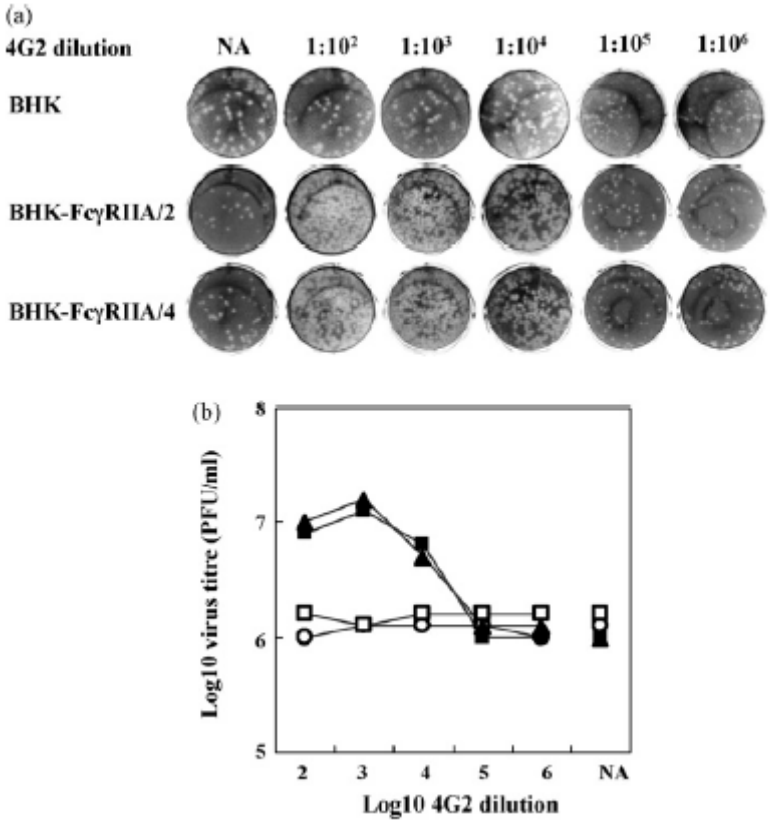

Fig. 3.2. Figures of plaques developed by DENV-2-4G2 antibody complex

3.2a: Mouse monoclonal antibody 4G2 was 10-fold serially diluted, starting from $1:10^2$. Untransfected BHK cells did not demonstrate infection enhancement. In contrast, 2 cell lines transfected with FcγRIIA showed DENV-2 infection enhancement at antibody dilutions from $1:10^2$ to $1:10^4$. NA indicates no antibody. **3.2b:** Virus titres as determined by plaque assay. (○) Untransfected BHK cells, (□) BHK cells transfected with empty vector, (▲) BHK-FcγRIIA/2 and (■) BHK-FcγRIIA/4. NA indicates no antibody.

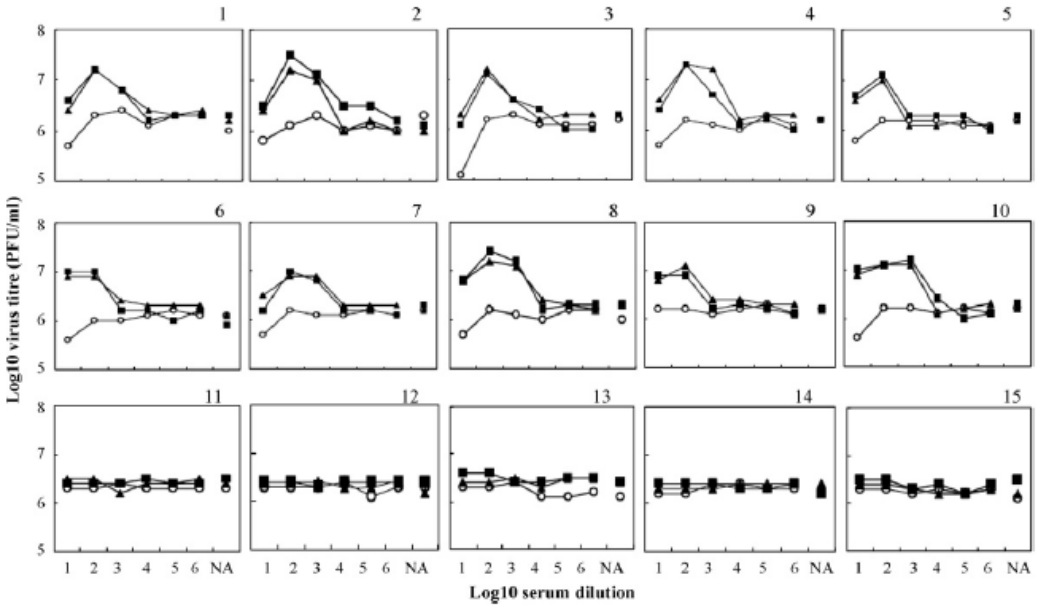

Fig. 3.3. Enhancement of DENV-2 infection by DENV antibody-positive human sera

DENV-2 was reacted with human serum samples, 10-fold serially diluted from 1:10 to $1:10^6$. Figures were presented according to serum sample number. Sera 1-10 were anti-DENV IgG positive and sera 11-15 were anti-DENV IgG negative. (○) Untransfected BHK cells, (▲) BHK-FcγRIIA/2 and (■) BHK-FcγRIIA/4. NA indicates no human serum

CHAPTER 4: Discrepancy in Neutralizing Antibody Titres between Plaque Reduction Neutralizing Tests FcγR-negative and FcγR-expressing BHK cells.

4.1. ABSTRACT

Protective immunity against dengue virus (DENV) is best reflected by the presence of neutralizing antibodies. Conventional plaque reduction neutralizing test (PRNT) is performed using FcγR-negative assay cells. Because FcγR plays a key role in antibody-dependent enhancement, we examined neutralizing antibody titers of mouse monoclonal antibodies and human serum samples in PRNT assays using FcγRIIA-negative and FcγRIIA-expressing BHK cells. There was discrepancy in neutralizing antibody titers between PRNT using FcγRIIA-negative and FcγRIIA-expressing BHK cells. Neutralizing antibody titers to DENV-1 and DENV-2 of monoclonal antibodies and most of the tested human serum samples were higher in assays using BHK cells than those using FcγRIIA-expressing BHK cells. The results suggests that neutralizing antibody titres determined using FcγRIIA-expressing cells may better reflect the protective capacity of anti-DENV antibodies, as the major target cells of DENV infection are FcγR-positive cells.

4.2. INTRODUCTION

Dengue virus (DENV), members of the family Flaviviridae, represents a major health problem in tropical and sub-tropical regions of the world. There are four serotypes, dengue virus types 1−4 (DENV-1−DENV-4). DENV causes a wide range of symptoms, from mild febrile illness, dengue fever (DF), to severe life threatening illness, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Infection with one serotype induces life-long protection against homologous serotype, but protection against other serotypes is short-lived. In secondary infection, cross-reactive, non-neutralizing antibodies bind to DENV. DENV-antibody complexes are taken-up more efficiently by FcγR expressing cells, and higher level of viremia is developed (Kontny et al., 1988; Littaua et al., 1990; Rodrigo et al., 2006; Schlesinger et al., 1999). This phenomenon, known as antibody-dependent enhancement (ADE) is considered to be a risk factor for DHF and DSS.

Protective immunity against DENV is best reflected by the presence of neutralizing antibody. High neutralizing antibody levels induced by primary infection is considered central in offering life-long protective immunity against homologous serotype. Thus, vaccine against DENV infection is expected to induce high levels of neutralizing antibody to all the four serotypes. Plaque reduction neutralizing test (PRNT) is a widely accepted approach to measure neutralizing activity of antibodies (Roehrig, 2007). PRNT, which employs Vero, LLC-MK2 or BHK cells as assay cells (Morens et al., 1985; Roehrig, 2007) is, however, limited to measure neutralizing activity on viral infectivity in the absence of FcγR (Daeron, 1997). It is possible that neutralizing antibody titers of anti-DENV antibodies induced by natural infection or by vaccine may differ when assayed in the presence of enhancing activity. The neutralizing antibody titers determined using FcγR-expressing BHK cells may better reflect the protective immunity, because the principal target cells of DENV are FcγR-expressing cells such as monocytes (Kou et al., 2008). In the present study, we sought to determine if neutralizing antibody titres were at the same or different levels, using BHK cells and those expressing FcγR as assay cells.

4.3. METHODS & MATERIALS

4.3.1. Cell Lines

BHK-21 cells, a hamster kidney cell line, and Vero cells (Vero 9013, Japan Health Science Research Resources Bank), an African green monkey kidney-derived epithelial cell line, were used. Establishment of FcγRIIA-expressing BHK-21 cells was reported elsewhere (Moi et. al., 2010b). BHK-21 and Vero cells were cultured in Eagle's Minimum Essential Medium (EMEM), (Sigma, St. Louis, MO, USA), supplemented with heat inactivated 10% FCS (Sigma), without antibiotics at 37 ˚C in 5% CO_2. FcγRIIA expressing BHK-21 cells were cultured in Eagle's Minimum Essential Medium (EMEM), (Sigma), supplemented with heat inactivated 10% FCS (Sigma) and 0.5 mg/ml neomycin (G418, PAA Laboratories GmbH, Austria) at 37 °C in 5% CO_2.

4.3.2. Virus

Dengue virus type-1 (DENV-1), 01-44-1HuNIID strain (GenBank accession number AB111070; 4) and dengue virus type-2 (DENV-2), D2/Hu/OPD030NIID/2005 strain (GenBank accession number AB219135), dengue virus type-3 (DENV-3), CH53962 strain and dengue virus type-4 (DENV-4) TVP-360 strain were used. DENV-3 (CH53962 strain) and DENV-4 (TVP-360 strain) were reference laboratory strains (Roehrig, 2007).

4.3.3. Monoclonal antibodies

Flavivirus-cross-reactive mouse monoclonal IgG2a antibody (ATCC MAb HB-112 D1-4G2-4-15) and DENV-2 serotype-specific mouse monoclonal IgG1 antibody, ATCC MAb HB-46 3H5-1 (Henchal et al., 1982), used in the assays were purchased from American Culture Type Collection (Manassas, VA, USA).

4.3.4. Serum specimens

Serum specimens were heat inactivated at 56 °C for 30 minutes and used in the experiments. All the serum samples had been tested for the presence of DENV by reverse

transcriptase-polymerase chain reaction (RT-PCR) and, anti-DENV antibody by IgG ELISA and IgM ELISA. Serum samples 1 and 3, and, 2 and 4, were paired serum samples, respectively, obtained from two cases of primary DENV infections. Serum samples designated as early samples were collected on a range of 1-3 days after the onset of the disease; therefore, early samples did not demonstrate neutralizing activities in the experiments. Serum samples designated as late phase samples were obtained in a range of 6-14 days after the onset of the disease.

4.3.5. Plaque reduction neutralizing assays

Mouse monoclonal antibody 4G2 (ATCC HB-112 D1-4G2-4-15; 1.3 mg/ml) and mouse monoclonal antibody 3H5 (ATCC HB-46 3H5-1; 2.5 mg/ml) were serially diluted 2 folds from 1:10 to 1:5120 with EMEM supplemented with 10% FCS. Human serum samples were serially diluted 2 folds from 1:5 to 1:2560 with EMEM supplemented with 10% FCS. Virus-antibody mixture was prepared by mixing 25 µl of DENV-1 or DENV-2 at titres of 2500 PFU/ml with 25 µl of serially diluted antibodies or serum samples. Control virus samples were prepared by mixing 25 µl of DENV-1 and DENV-2 at titres of 2500 PFU/ml with 25 µl of EMEM supplemented with 10% FCS. Virus-antibody mixture was incubated at 37 °C for 1 hour. Fifty microliters of virus-antibody mixture was inoculated onto BHK-21 monolayers in 12-well plates. The plates were incubated for 1 hour at 37 °C in 5% CO_2. After virus absorption, the cells were washed with 1 ml of EMEM and overlaid with maintenance medium containing 1% methylcellulose (Wako Pure Chemical Industry, Osaka, Japan). The plates were incubated at 37 °C in 5% CO_2 for 5 days. After 5 days of incubation, the cells were fixed with 3.7% (v/v) formaldehyde for 1 hour at room temperature and washed with water. The cells were then stained with methylene blue solution for 1 hour at room temperature and washed with water. Plaques were counted by naked eye. Neutralization titer was defined as the highest serum dilution which reduced the number of plaques by 50% (Takasaki et al., 2003). No plaque reduction, or increase in the number of plaques were considered as “0%”, in percentage of plaque reduction.

4.4. RESULTS

4.4.1. Neutralizing titers of mouse monoclonal antibody, 4G2 and 3H5, determined by assays using parent BHK and those expressing FcγRIIA cell lines

Neutralizing titres of mAb 4G2 and 3H5 were examined against DENV-1, DENV-2, DENV-3 and DENV-4, using BHK cells, and BHK-FcγRIIA/2 and BHK-FcγRIIA/4 which stably express FcγRIIA (Fig 4.1, Fig 4.2). The mAb 4G2 demonstrated neutralizing titers of 1:320 to 1:40 to all the 4 serotypes of DENV, using parent BHK cells. However, when BHK-FcγRIIA/2 and BHK-FcγRIIA/4 were used as assay cells, no neutralizing activity was detected to 3 of the 4 serotypes, and neutralizing titer as low as 10 was detected to DENV-3 (Table 4.1).

DENV-2 serotype specific mAb 3H5 demonstrated a neutralizing titre of 1:40 only to DENV-2, using BHK cells. Neutralizing activity to DENV-2 was not detected, when FcγRIIA/2 and FcγRIIA/4 were used as assay cells (Table 4.1). The results indicate that there is a discrepancy in neutralizing activities of two mAbs between assays using Fcγ-negative BHK cells and those using FcγRIIA-positive cells.

4.4.2. Neutralizing antibody titres to DENV-1 of human serum samples determined by assays using BHK cells and Fcγ-expressing BHK cells

Fourteen DENV IgG-positive serum samples were tested for their neutralizing titres to DENV-1, using BHK cells, BHK-FcγRIIA/2 and BHK-FcγRIIA/4 (Fig 4.3). Serum samples # 6, # 10, # 11, # 12, # 13, and #14 demonstrated neutralizing antibody titres of 1:160-1:640 when Fcγ-negative BHK cells were used as assay cells; however, they demonstrated neutralizing antibody titres of <1:5-1:40, when FcγR-positive cells were used (Table 4.2). Interestingly, serum samples # 3 and # 10 demonstrated similar levels of neutralizing antibody titers in assays using FcγR-negative and FcγR-positive BHK cells. DENV-antibody negative samples (# 15-18) did not show any neutralizing activity in BHK, BHK-FcγRIIA/2 and BHK-FcγRIIA/4 cells. The results indicate that neutralizing antibody titres of human serum samples from dengue patients were different between assays using

FcγR-negative and FcγR-positive BHK cells.

4.4.3. Neutralizing antibody titres to DENV-2 of human serum samples determined by assays using BHK cells and FcγR-expressing cells BHK cells

Fourteen DENV-IgG-positive serum samples were also tested for their neutralizing titres to DENV-2, using BHK, BHK-FcγRIIA/2 and BHK-FcγRIIA/4 cells (Fig 4.4). Serum samples # 5, # 6, # 9, # 10, # 13 and # 14 demonstrated neutralizing titres of 1:20-1:320 when BHK cells were used; however, they did not demonstrate detectable levels of neutralizing activity using FcγR-expressing BHK cells (Table 4.3). Serum samples # 4 and # 12 demonstrated neutralizing titers of 40 and 80 respectively in BHK cells; and 10 and 5, respectively, in FcγR-expressing BHK cells. These results were consistent with those shown in Tables 4.1 and 4.2, and indicate that neutralizing titers were higher, when determined by assays using FcγR-negative cells than when using Fcγ-expressing BHK cells.

4.5. DISCUSSION

Plaque reduction neutralizing test (PRNT) is a widely accepted method to measure neutralizing capacity of antibodies against DENV. Conventional PRNT assays employ Vero, LLC-MK2 or BHK cells that do not express FcγR (Daeron, 1997; Morens et al., 1985; Roehrig, 2007). Thus, the assay using these cells lines measure the effects on viral infectivity in the absence of FcγR, and antibody enhancement activity measurements is excluded. In the present study, we compared DENV neutralizing titres between FcγRIIA-expressing BHK cells and parent FcγR-negative BHK cells to examine the influence of FcγR on DENV neutralization. The assay using FcγRIIA-expressing BHK cells in this study was developed to examine neutralizing antibody titres of anti-DENV antibody present in serum samples obtained from DENV patients from various stages of the disease. The assay had been proved useful in studies on the role on antibodies in ADE of DENV infection using human serum samples. The ability to enhance DENV using flavivirus group reactive monoclonal antibody by FcγRIIA-expressing BHK cell line-2 and FcγRIIA-expressing BHK cell line-4 were at similar levels (Moi et al.,2010b).

Flavivirus group reactive monoclonal mouse antibody 4G2 neutralized all 4 DENV-serotypes in BHK cells. In contrast, 4G2 did not neutralize 3 of the 4 serotypes, and neutralized DENV-3 at as low as antibody 1:10 dilution, when FcγR-expressing BHK cells were used. The antibody 3H5, neutralized only DENV-2 but neutralizing activity was not detected using FcγR-expressing BHK cells. The absence of neutralization using FcγR-expressing BHK cells suggest that presence of ADE lowers neutralizing activity of monoclonal antibody. Human serum samples from dengue patients demonstrated similar results. The neutralizing antibody titers of most of the tested samples were higher when determined using FcγR-negative BHK-cells than when determined by FcγR-expressing cell lines; BHK-FcγRIIA/2 and BHK-FcγRIIA/4.

Antibodies posses two competing effects on DENV growth in the presence of FcγR: neutralization and infection enhancement. In the presence of FcγR, infection enhancing effect may hamper neutralization. DENV-1 immune complexes formed with DENV-1 antibodies, or DENV-2 immune complexes formed with DENV-2 antibodies (homologous

DENV-immune complexes), were susceptible to neutralization in both FcγRIIA-expressing BHK cells and parent BHK cells. Primary infection with one DENV serotype usually induces long-term protective immunity against homologous serotype (Endy et al., 2004). Neutralization of heterologous DENV in assays using FcγR-expressing cells, thus, strongly reflects the effect of ADE activity. Some serum samples from primary infection also demonstrated higher neutralizing antibody titers when determined using BHK cells than when determined using FcγR-expressing BHK cells. It is possible that some ADE activity exist in neutralizing assay, even against homologous serotypes. DENV immune complexes formed with heterologous antibodies were less susceptible to neutralization in the presence of FcγRIIA-expressing BHK cells, is consistent with and extends earlier findings using FcγRIIA-expressing CV-1 cells by others (Rodrigo et al., 2009a & b). In the present study, a conventional plaque detection method was used by directly staining vital cells. Serum samples used in this study was obtained from primary or secondary DENV 1-4 patients, at both early phase and late phase of the disease, and thus, offers insights into individual immulogical response during various stages of the disease.

PRNT assays using FcγRIIA-expressing BHK sastifies the criteria for an acceptable alternative to conventional neutralization assays: the assay detects the sum of neutralizing and enhancing activity as neutralizing titers in the presence of FcγRIIA. At the same time, the simplicity and ease of performance using the cell lines in the present study meet or exceed those of previous studies (Martin et al., 2006; Rodrigo et al., 2006; Rodrigo et al., 2009a & b). The results suggest that PRNT using FcγRIIA-expressing BHK cells could be a feasible alternative to the detection of neutralizing titers of DENV. In addition, the assay holds potential in assessing protective capacity against heterologous DENV challenge using *in vivo* animal models. However, subsequent studies will be needed to determine whether FcγRIIA-expressing BHK cells PRNT will better demonstrate the correlation between PRNT of serum samples and protective capacity against DENV in vivo.

TABLE 4.1. Neutralizing titres of anti-DENV monoclonal antibodies determined by assays using BHK, BHK- FcγRIIA/2 and BHK- FcγRIIA/4.

Monoclonal antibodies	Challenge virus	Neutralizing titres*		
		BHK	FcγRIIA/2	FcγRIIA/4
4G2				
	DENV-1	320	<10	<10
	DENV-2	160	<10	<10
	DENV-3	80	10	10
	DENV-4	40	<10	<10
3H5				
	DENV-1	<10	<10	<10
	DENV-2	40	<10	<10
	DENV-3	<10	<10	<10
	DENV-4	<10	<10	<10

* $PRNT_{50}$ was determined as described in Materials and Methods.

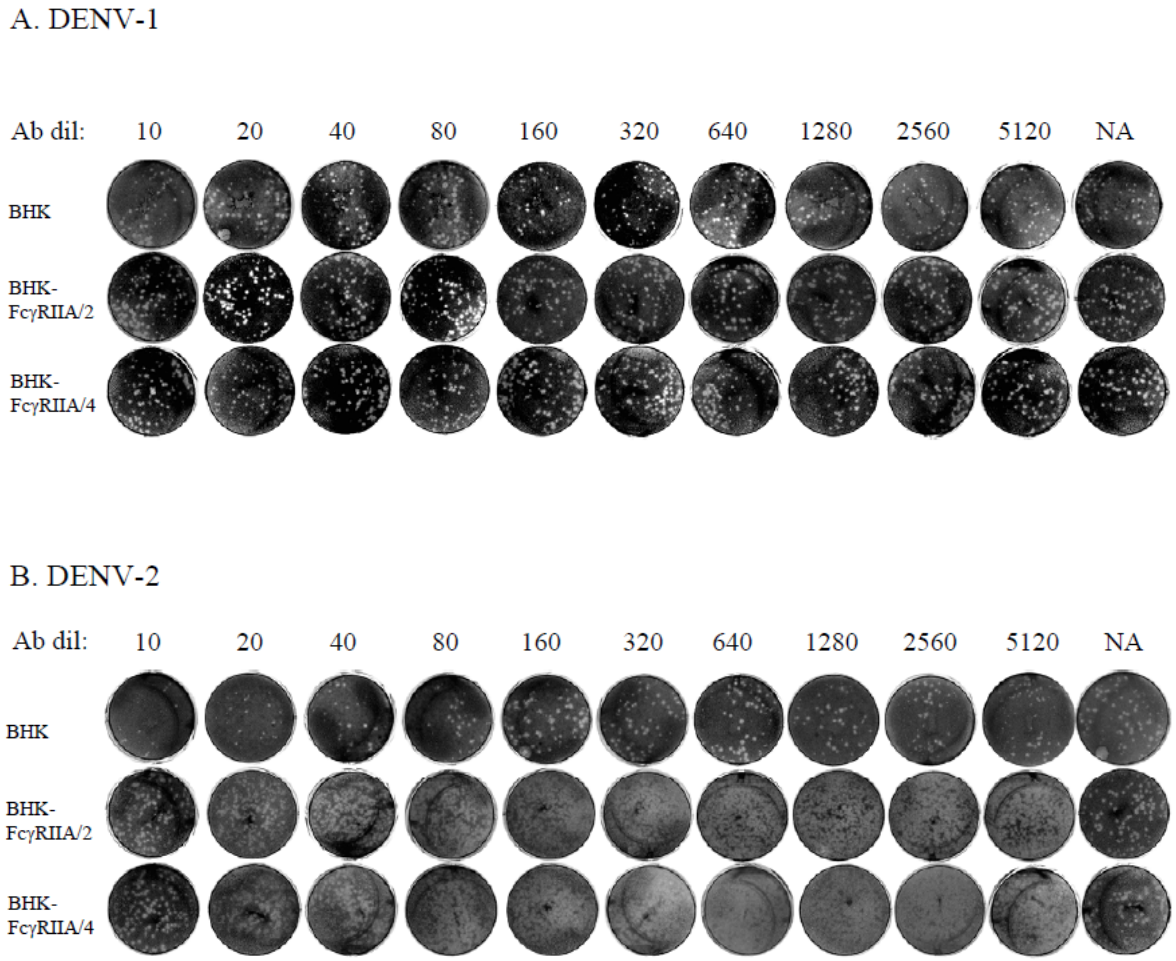

Fig. 4.1. Plaque reduction neutralizing assays using BHK cells and FcγRIIA-expressing BHK cells. DENV-1 and DENV-2 were reacted with serially diluted mouse monoclonal antibody 3H5 in PRNT assays using BHK and FcγRIIA-expressing BHK cells in 12-well plates.

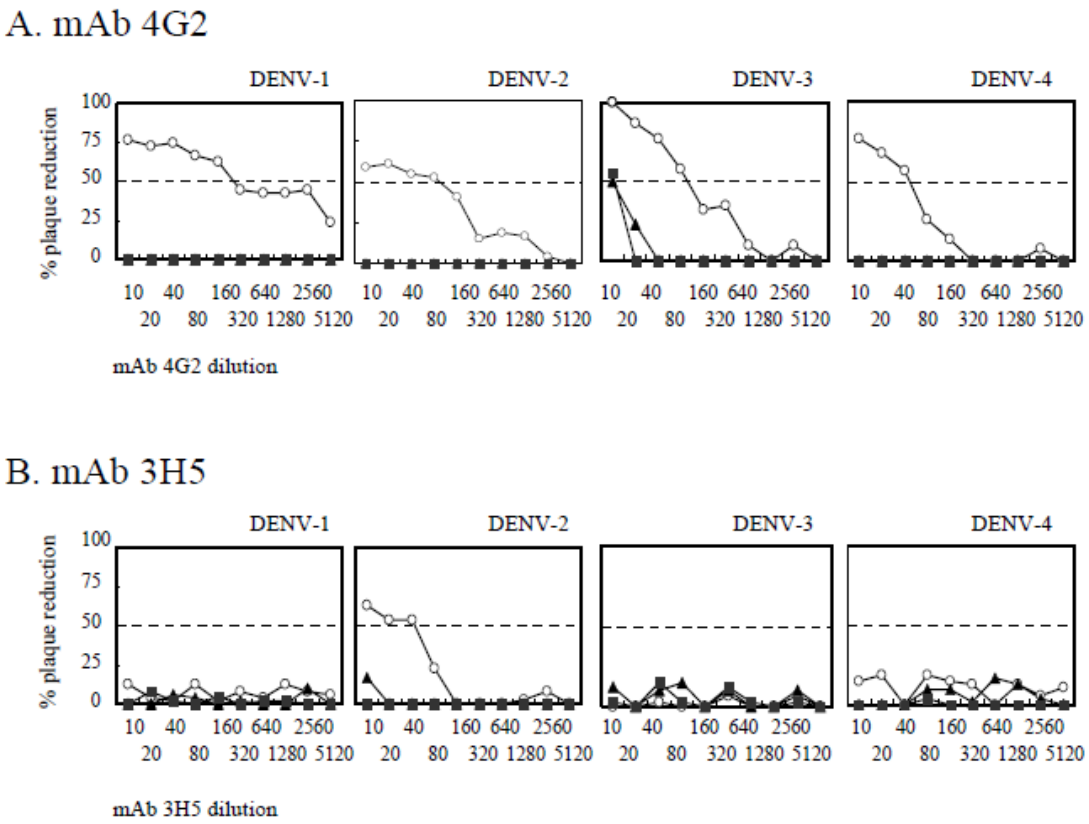

Fig. 4.2. Patterns of plaque reduction against DENV in neutralization assays. (**A**) 4G2 and (**B**) 3H5. (○) Untransfected BHK cells, (▲) BHK-FcγRIIA/2 and (■) BHK-FcγRIIA/4. Each curve is the mean of duplicate experiments.

TABLE 4.2. Neutralizing antibody titres of human serum samples against DENV-1 as determined using BHK, BHK- FcγRIIA/2 and BHK- FcγRIIA/4.

	Neutralizing titers to DENV-1		
Serum Number	**BHK**	**FcγRIIA/2[a]**	**FcγRIIA/4[b]**
Primary DENV infection			
Early phase[e]			
1. (DENV-1)	<5	<5	<5
2. (DENV-2)	<5	<5	<5
Late phase[f]			
3. (DENV-1)	160	160	160
4. (DENV-2)	<20[d]	<20[d]	<20[d]
5. (DENV-3)	20	<5	<5
6. (DENV-4)	320	10	10
Secondary DENV infection			
Early phase[e]			
7. (DENV-1)[c]	10	<5	<5
8. (DENV-1)	<5	<5	<5
9. (DENV-2)	10	<5	<5
10. (DENV-3)	320	160	160
Late phase[f]			
11. (DENV-1)	640	40	40
12. (DENV-2)	320	20	20
13. (DENV-3)	160	<5	<5
14. (DENV-4)	640	10	10
DENV-IgG negative			
15	<5	<5	<5
16	<5	<5	<5
17	<5	<5	<5
18	<5	<5	<5

[a] FcγRIIA-expressing BHK cell line-2.

[b] FcγRIIA-expressing BHK cell line-4.

[c] DENV serotype during early phase of infection.

[d] Serum was serially diluted 2-folds from 1:20.

[e] Days 1-3 after onset of the disease

[f] Days 6-14 after onset of the disease.

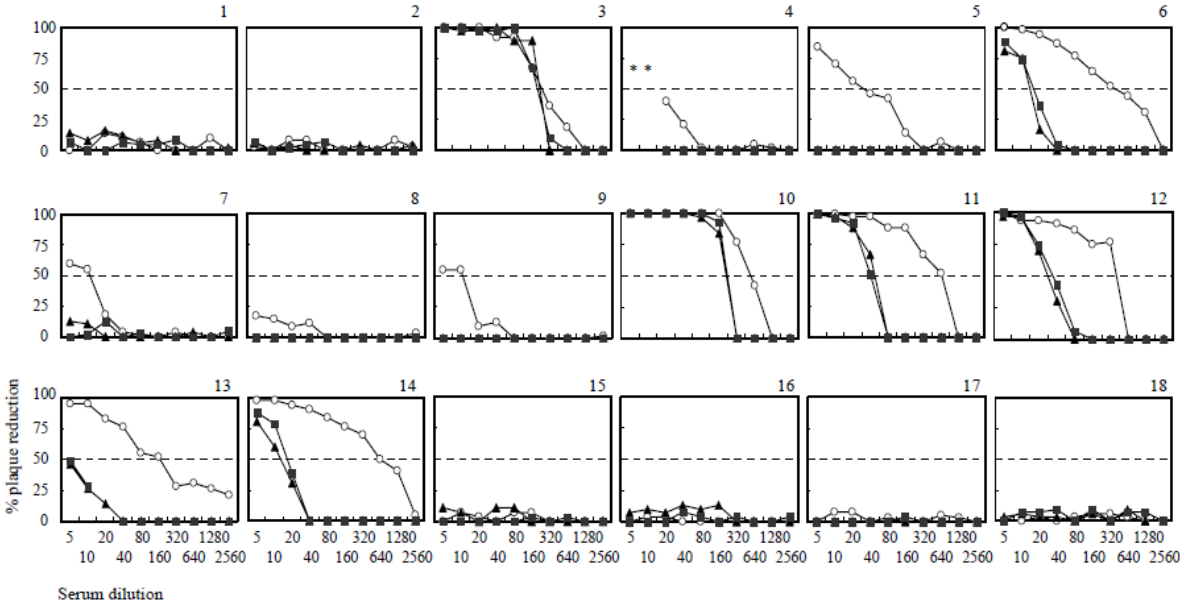

Fig. 4.3. Patterns of plaque reduction against DENV-1 in neutralization assays of human serum samples. DENV-1 was reacted with human serum samples, 2-fold serially diluted from 1:5 to 1:2560. Figures were presented according to serum sample number. Characterization of samples are described in Tables 5.2 and 5.3. (○) Untransfected BHK cells, (▲) BHK-FcγRIIA/2 and (■) BHK-FcγRIIA/4. Each curve is the mean of duplicate experiments. DENV-1 was reacted with human serum samples, 2-fold serially diluted from 1:5 to 1:2560. Figures were presented according to serum sample number. Characterization of samples are described in Tables 5.2 and 5.3. (○) Untransfected BHK cells, (▲) BHK-FcγRIIA/2 and (■) BHK-FcγRIIA/4. Each curve is the mean of duplicate experiments.

TABLE 4.3. Neutralizing titres of human serum samples against DENV-2 as determined using BHK, BHK-FcγRIIA/2 and BHK-FcγRIIA/4.

	Neutralizing titers to DENV-2		
Case number (DENV serotype)	**BHK**	**FcγRIIA/2[a]**	**FcγRIIA/4[b]**
Primary DENV infection			
Early phase[d]			
1. (DENV-1)	<5	<5	<5
2. (DENV-2)	<5	<5	<5
Late phase[e]			
3. (DENV-1)	80	<5	<5
4. (DENV-2)	40	10	10
5. (DENV-3)	20	<5	<5
6. (DENV-4)	40	<5	<5
Secondary DENV infection			
Early phase[d]			
7. (DENV-1)[c]	5	<5	<5
8. (DENV-1)	10	<5	<5
9. (DENV-2)	40	<5	<5
10. (DENV-3)	20	<5	<5
Late phase[e]			
11. (DENV-1)	<5	<5	<5
12. (DENV-2)	80	5	5
13. (DENV-3)	40	<5	<5
14. (DENV-4)	320	<5	<5
DENV-IgG negative			
15	<5	<5	<5
16	<5	<5	<5
17	<5	<5	<5
18	<5	<5	<5

[a] FcγRIIA-expressing BHK cell line-2.

[b] FcγRIIA-expressing BHK cell line-4.

[c] DENV serotype during acute phase of infection.

[d] Days 1-3 after onset of the disease

[e] Days 6-14 after onset of the disease

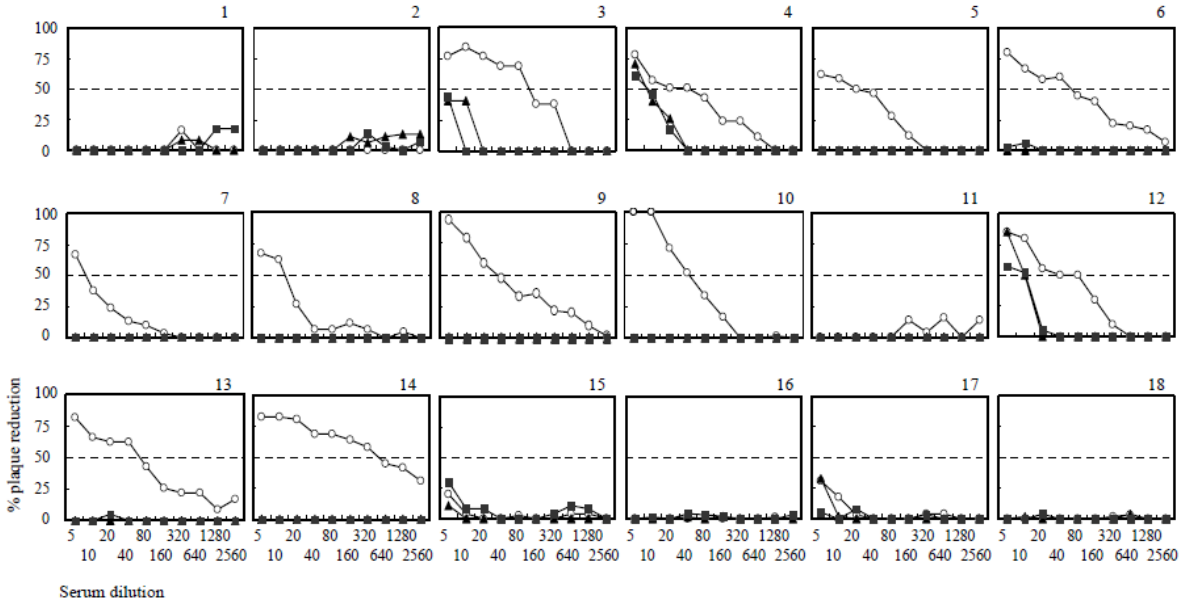

Fig. 4.4. Patterns of plaque reduction against DENV-2 in neutralization assays of human serum samples. DENV-2 was reacted with human serum samples, 2-fold serially diluted from 1:5 to 1:2560. Figures were presented according to serum sample number. Characterization of samples are described in Tables 5.2 and 5.3. (○) Untransfected BHK cells, (▲) BHK-FcγRIIA/2 and (■) BHK-FcγRIIA/4. Each curve is the mean of duplicate determinants.

CHAPTER 5: SUMMARY

The results have provided a better understanding of the mechanisms by which DENV ultilizes sub-neutralizing antibodies to enhance infection of FcγR-expressing cells. The important observations of this thesis can be summarized as follows:

1. Specific structure of the FcγRIIA, when present on non-professional phagocytotic cells is crucial for mediating processes that promote ADE of dengue virus infection.

2. A new ADE assay capable of determining the sum of ADE activity and neutralization antibody titers had been established.

3. Neutralization of heterologous DENV is lowered in the presence FcγRIIA, suggesting presence of ADE activity in serum samples when tested in circumstances which better reflect in vivo conditions.

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LIST OF PUBLICATIONS

Parts of this dissertation have been partly presented in the following publications:

Moi, M. L., Lim, C. K., Takasaki, T. and Kurane, I. 2010. Involvement of the Fcγ receptor IIA cytoplasmic domain in antibody dependent enhancement of dengue virus infection. *J Gen Virol* 91, 103-111.

Moi, M. L., Lim C. K., Kotaki A., Takasaki T., & Kurane, I. 2010. Development of an antibody-dependent enhancement assay for dengue virus using stable BHK-21 cell lines expressing FcγRIIA. *J Virol Meth* 163(2), 205-209.

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Journal of General Virology (2010), **91**, 103–111 DOI 10.1099/vir.0.014829-0

Involvement of the Fcγ receptor IIA cytoplasmic domain in antibody-dependent enhancement of dengue virus infection

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Received 10 July 2009
Accepted 20 September 2009

Sub-neutralizing concentrations of antibody to dengue virus (DENV) enhance DENV infection of Fcγ receptor-expressing cells. This phenomenon, referred to as antibody-dependent enhancement (ADE), has been hypothesized to be responsible for the severe form of DENV infection, including dengue haemorrhagic fever and dengue shock syndrome. To analyse further the mechanisms of ADE *in vitro*, this study introduced a series of cytoplasmic mutants into human FcγRIIA. The mutated FcγRIIA was then expressed on COS-7 cells to see whether these mutants could enhance DENV infection. Wild-type FcγRIIA enhanced DENV infection, consistent with previous reports using FcγR-positive monocytes. Disruption of the immune tyrosine activation motif (ITAM) in the cytoplasmic domain of FcγRIIA or removing the sequences between the two ITAM regions eliminated ADE. These findings suggest that the specific structure of the FcγRIIA cytoplasmic domain is essential for the ability of FcγRIIA to mediate ADE.

INTRODUCTION

Dengue fever ranks as the most important mosquito-borne virus disease in the world, and an estimated 2.5 billion people live in over 100 endemic countries and areas where dengue viruses (DENVs) can be transmitted. With up to 100 million infections occurring annually, dengue fever is a source of considerable economic loss to health authorities (Okanurak *et al.*, 1997), as well as to patients (Anderson *et al.*, 2007).

DENV exists in four distinct viral serotypes (DENV-1 to -4), and each serotype can cause a spectrum of symptoms, ranging from mild febrile illness to severe life-threatening dengue haemorrhagic fever. Epidemiological studies have indicated that infection with a DENV serotype offers life-long protection against homologous infection by the same serotype. Immunity generated against a particular dengue serotype does not provide protection towards infection with a previously unexposed serotype in the long term. Instead, after a short period of cross-protection against heterologous serotypes, antibodies generated against primary DENV infection are postulated to be one of the main factors contributing to the severe form of DENV infection (Graham *et al.*, 1999; Sangkawibha *et al.*, 1984; Vaughn *et al.*, 2000). Under the conditions of anti-DENV antibody cross-reactivity or at concentrations where neutralization does not occur, virus–antibody complexes are taken up more readily than uncoated virus particles by cells expressing Fcγ receptors (FcγRs), such as monocytes and macrophages (Kontny *et al.*, 1988; Littaua *et al.*, 1990). This effect, known as antibody-dependent enhancement (ADE), has been demonstrated for both RNA and DNA viruses, and has been studied extensively with DENV (Littaua *et al.*, 1990; Schlesinger & Chapman, 1999).

Interaction of FcγR and antigen–antibody complexes triggers an array of responses, which include phagocytosis, endocytosis, antibody-dependent cell-mediated cytotoxicity, superoxide generation and release of inflammatory mediators, as well as immune-complex clearance (An, 1982; Gessner *et al.*, 1998; Indik *et al.*, 1991, 1995a, b; Mero *et al.*, 2006; Ravetch & Kinet, 1991). These responses are largely dependent upon interactions between FcγR and protein, and lipid signalling transduction moieties located in close proximity to the cytoplasmic and transmembrane regions of FcγR (Barabé *et al.*, 2002; Booth *et al.*, 2002; García-García & Rosales, 2002). Endocytosis of opsonized particles by FcγRIIA (CD32A) involves lipid raft-induced receptor clustering, which leads to signalling through the Ig gene family tyrosine activation motif (ITAM: E-X_8-D-X_2-YXXL-X_{12}-YXXL), observed in the cytoplasmic domains of several Ig gene family receptors (Abdel Shakor *et al.*, 2004; Huang *et al.*, 1992; Indik *et al.*, 1991; Kwiatkowska *et al.*, 2003; Sobota *et al.*, 2005). When mutations are introduced into the tyrosine moieties located in the ITAM region of

FcγRIIA, kinase-mediated phagocytosis of opsonized particles is severely reduced (Kim *et al.*, 2003; Mitchell *et al.*, 1994). In contrast, the ability of FcγR to associate with lipid rafts is disrupted by substitution of a cysteine residue within the juxtamembrane region (Barnes *et al.*, 2006). Transmembrane and cytoplasmic structures are thus required for FcγR functions and are likely to be involved in interactions with intermediate signal transduction elements that are components in the immune-complex internalization machinery.

However, the importance of such internalization machinery in FcγR-mediated ADE remains obscure. FcγR could facilitate the entry of DENV by directing the virus to the cell surface and, in turn, increasing the probability of interactions between DENV and its unidentified viral receptor (Mady *et al.*, 1991). In contrast, signalling systems triggered by FcγR may lead to internalization of the viral DENV–antibody immune complex and thus enhanced infection.

In the present study, we evaluated the possible roles of the cytoplasmic and transmembrane regions of FcγRIIA in facilitating DENV infection in the presence of antibody. We introduced a series of mutations in the cytoplasmic domains of wild-type (WT) FcγRIIA and examined the capacity for ADE. The cytoplasmic domain of the receptor, including its palmitoylation site (Barnes *et al.*, 2006), was found to be required for ADE of DENV infection. The results indicate that FcγRIIA-mediated signal transduction is necessary for ADE.

RESULTS

Preparation of FcγRIIA receptor containing mutations in signalling domains

It has been reported that FcγRIIA mediates ADE using K562 cells, which express only FcγRIIA (Littaua *et al.*, 1990). To define the requirement for the cytoplasmic domain in FcγRIIA-mediated ADE, the receptor with or without mutations was transfected into COS-7 cells, which lack endogenous FcγR (Indik *et al.*, 1991). The FcγRIIA cytoplasmic region tyrosine residues (Y281, Y288 and Y304), designated P1, P2 and P3, respectively, contribute to the ability of receptors to undergo phagocytosis and capping (Kwiatkowska *et al.*, 2003; Mitchell *et al.*, 1994). The cysteine residue within the juxtamembrane region of FcγRIIA (C241) is involved in raft localization of FcγRIIA and efficient receptor signalling (Barnes *et al.*, 2006). We introduced a series of point and deletion mutations of residues in the cytoplasmic domain of FcγRIIA that are involved in receptor signalling and phagocytosis of immune complexes (Fig. 1a, Table 1). The expression of each of the constructs in COS-7 cells was verified by immunoblotting (data not shown) and flow cytometry (Fig. 1b, Table 1). More than 50 % of the transfected cells constantly expressed mutant and WT FcγRIIA, except for the mutants dT (48 ± 5 %) and Y3F (34 ± 10 %).

Phagocytic activities of COS-7 cells expressing mutated FcγRIIA

To confirm that the WT and mutated FcγRIIA maintained the biological function of the receptors, we first measured phagocytic activity. Phagocytic activity is the best-studied biological function of FcγRIIA (Indik *et al.*, 1995a, b; Mitchell *et al.*, 1994). We adopted a quantitative fluorescence method that employed anti-*Escherichia coli* polyclonal antibody (pAb)-opsonized, succinimidyl ester (SE)-labelled *E. coli* immunocomplex particles (Fig. 2a). With anti-*E. coli* pAb, COS-7 cells expressing WT, dT and dP3 exhibited higher levels of phagocytic activity (13.8 ± 2.9 %, 13.6 ± 2.6 % and 7.7 ± 1.5 %, respectively) than those expressing the other FcγRIIA mutants or those without FcγRIIA (Fig. 2b). Less than 5 % of COS-7 cells expressing WT, dT and dP3 were phagocytic when exposed to SE-labelled *E. coli* strain K-12 without anti-*E. coli* pAb (Fig. 2b). The results suggested that the phagocytic activity of WT-, dT- and dP3-expressing cells is FcγR-dependent and that the transfected FcγRIIA is functional.

Receptor clustering induced by binding of DENV–antibody complex to FcγRIIA

The consequences of the binding of DENV–antibody complexes to WT and mutant FcγRIIA were examined. The occurrence of cross-linking and capping was monitored by immunofluorescence as described in Methods. Capping occurred only on COS-7 cells that expressed WT, dT and dP3 (Fig. 3). Cross-linking, but not capping, occurred on cells expressing dP2, dP1P2, dP2P3, dP1P2P3, CT, dISR, Y3F and C241A (Fig. 3). Neither cross-linking nor capping occurred in WT-transfected COS-7 cells after inoculation with DENV-1 in the absence of antibody (Fig. 3, WT^{-AB}). The results indicated that the ability of FcγRIIA to cluster after the binding of DENV–antibody complex varies depending on the induced mutations.

ADE of DENV infection in COS-7 cells expressing mutant FcγRIIA

Human serum from a DENV-3-infected patient was used to prepare DENV-1– or DENV-2–antibody complexes to examine ADE. COS-7 cells that expressed WT were infected with the human serum–DENV-1 or –DENV-2 complex and the cells were stained with dengue serotype-cross-reactive mAb 4G2 and examined by flow cytometry. The results indicated that the human anti-DENV serum enhanced DENV-1 and DENV-2 infection of WT-expressing COS-7 cells to the maximum levels at a final dilution of 1 : 1000 and 1 : 10 000, respectively (Fig. 4). Based on these results, the serum was used at a 1 : 1000 dilution for DENV-1 and a 1 : 10 000 dilution for DENV-2 in the following experiments.

To evaluate the incubation period of DENV-infected cells, COS-7 cells transfected with WT FcγRIIA were infected with DENV-1 with or without human serum treatment,

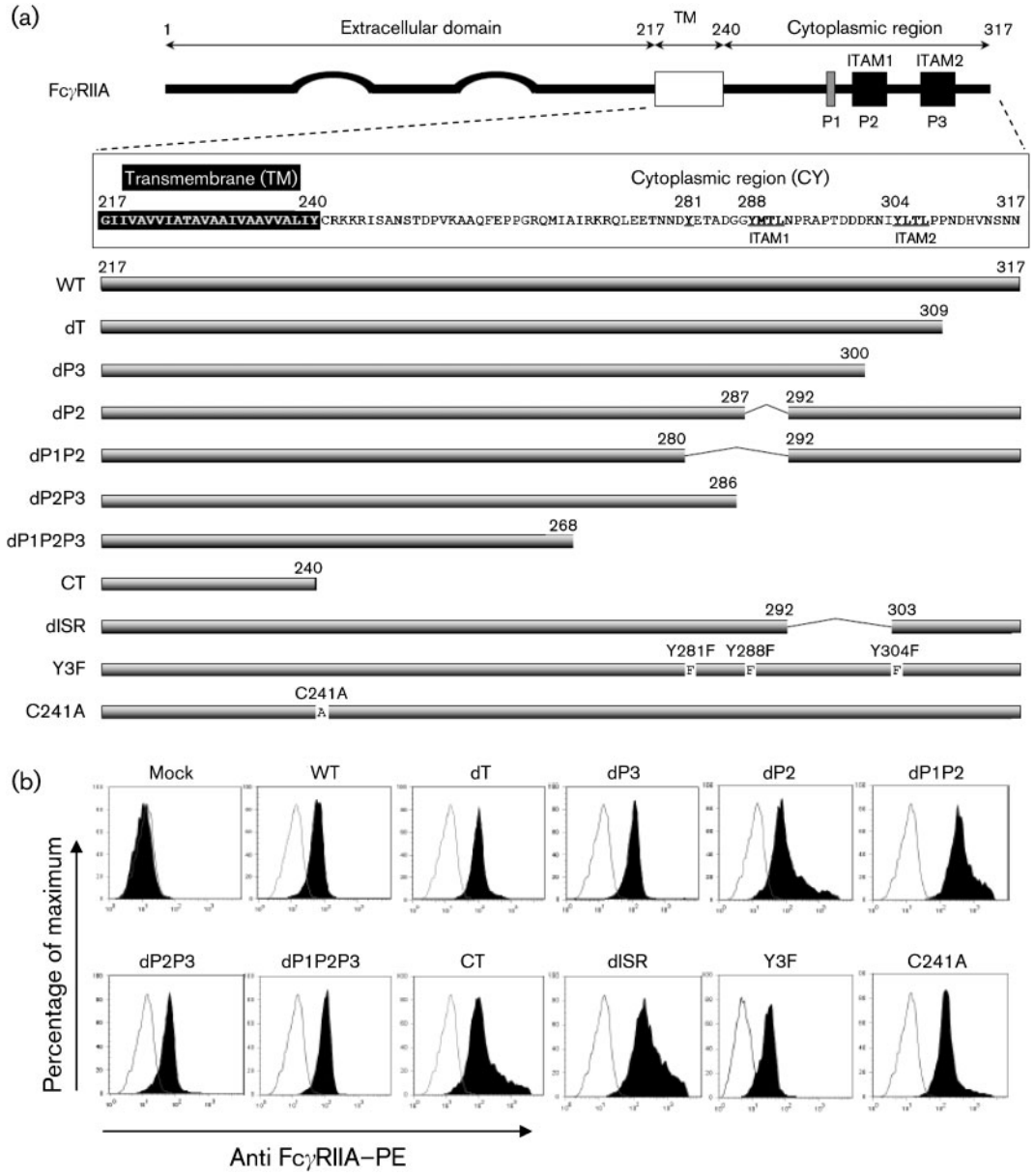

Fig. 1. Structure of the mutated FcγRIIA constructs, and expression in COS-7 cells as determined by flow cytometry. (a) The mutants were constructed by standard site-directed mutagenesis methods, introduced into pcDNA3.1(+) and expressed in COS-7 cells. The FcγRIIA gene is shown in the figure with the extracellular region, transmembrane region (TM) and cytoplasmic region (CY) indicated. The FcγRIIA cDNA is numbered starting from +1. The filled box represents a hydrophobic stretch of 24 aa presumed to span the membrane. Lines indicate deleted regions, and the letters A and F represent substituted amino acids in the gene. (b) The percentage of COS-7 cells expressing WT and mutated FcγRIIA was determined by flow cytometry, using PE-labelled mAb 16320 against human FcγRIIA. Results are representative of four or more experiments performed in triplicate.

and the presence of DENV antigen-positive cells was examined by flow cytometry at different time points. The proportions of infected cells after DENV-1 infection with or without antibody were 0.4 ± 0.3 and 0.3 ± 0.03 % at 48 h, 4.0 ± 1.5 and 0.6 ± 0.2 % at 72 h, 12.2 ± 6.0 and 3.3 ± 2.6 % at 96 h, and 15.9 ± 6.9 and 3.7 ± 2.2 % at 120 h, respectively. The percentage of infected cells increased rapidly after DENV-1 infection with enhancing antibody, suggesting that the cells infected with DENV-1–antibody complex released progeny virus as efficiently as the cells infected with DENV-1 alone. We used the incubation period of 72 h after inoculation to assay the enhancement of DENV infection in primary infection.

Table 1. Characteristics of the mutated FcγRIIA prepared in this study

+ indicates that the region exists as in the wild type; − indicates that the region was deleted.

Mutant	Mutation					Positive cells (%)*
	ITAM1	ITAM2	Y281	C241	ISR	
WT	+	+	+	+	+	57±9
dT	+	+	+	+	+	48±5
dP3	+	−	+	+	+	61±7
dP2	−	+	+	+	+	59±1
dP1P2	−	+	−	+	+	60±15
dP2P3	−	−	+	+	−	60±11
dP1P2P3	−	−	−	+	−	87±3
CT	−	−	−	−	−	55±14
dISR†	+	+	+	+	−	97±2
Y3F‡	Y304F	Y288F	Y281F	+	+	34±10
C241A§	+	+	+	C241A	+	80±14

*Surface expression of WT and mutated FcγRIIA was examined by flow cytometry. Results are shown as means±SD of three experiments performed in triplicate.

†Deletion of 12 aa located between ITAM1 and ITAM2.

‡A phenylalanine residue was substituted for tyrosine at aa 281, 288 and 304.

§An alanine residue was substituted for cysteine at aa 241.

DENV-1 and DENV-2 were mixed with anti-dengue human serum at a 1 : 1000 dilution and 1 : 10 000 dilution, respectively, and the DENV–antibody complex was prepared. COS-7 cells expressing WT or mutant FcγRIIA were inoculated with the DENV–antibody immune complex or DENV. The presence of infected cells was confirmed by an immunofluorescence assay (Fig. 5a), and the percentage of DENV antigen-positive cells was measured by flow cytometry (Fig. 5b, c). The percentage of antigen-positive cells was compared between transfected COS-7 cells infected with the DENV-1–antibody immune complex and those infected with DENV-1 alone. ADE of DENV-1 was detected in COS-7 cells expressing WT (11.2±4.5 % after infection with immune complex and 1.1±0.8 % after infection with virus alone), dT (9.5±1.8 and 1.2±0.4 %) and dP3 (4.7±1.0 and 1.2±0.3 %), but not in those expressing the other mutants of FcγRIIA: dP2 (1.3±1.1 and 1.2±1.4 %), dP1P2 (0.9±1.2 and 1.5±1.5 %), dP2P3 (0.9±0.8 and 0.6±0.1 %), dP1P2P3 (1.3±0.7 and 1.8±0.4 %), CT (1.5±0.7 and 1.8±1.4 %), dISR (1.2±0.9 and 0.9±0.3 %), Y3F (2.1±0.7 and 1.0±0.4 %), C241A (2.4±1.3 and 2.1±1.0 %) and mock transfected (1.1±0.6 and 1.3±0.5 %) (Fig. 5b). ADE of DENV-2–antibody immune complexes was also detected in cells expressing WT (34.1±13.9 and 3.5±0.9 %), dT (34.6±5.3 and 4.4±2.1 %), and dP3 (12.0±0.3 and 2.2±0.2 %), but not in cells expressing the other mutants: dP2 (2.2±0.4 and 3.8±1.0 %), dP1P2 (2.4±1.2 and 1.3±0.7 %), dP2P3 (2.2±0.4 and 3.4±1.6 %), dP1P2P3 (1.3±0.5 and 2.3±0.9 %), CT (2.5±1.6 and 1.6±0.8 %), dISR (2.4±1.6 and 2.1±1.1 %), Y3F (2.1±0.7 and 1.1±0.4 %), C241A (2.6±0.5 and 2.0±1.4 %) and mock transfected (2.4±1.3 and 1.8±0.6 %) (Fig. 5c). The results indicated that disruption of the ITAM motifs and removal of the sequences between the two ITAM motifs eliminated the ability of FcγRIIA to mediate ADE. The results thus suggest that the specific structure of FcγRIIA, and signal transduction via FcγRIIA, are both required for ADE during dengue virus infection.

DISCUSSION

Following entry of DENV into the bloodstream, the virus enters a target cell where it replicates, after which it can exist in several forms based on the level of viraemia and host response to the viraemia (Noisakran & Perng, 2008). Antibody response is an important defence mechanism employed to control DENV infection. Anti-DENV antibodies at sub-neutralizing concentrations, however, enhance DENV infection via FcγRI (Kontny *et al.*, 1988) and FcγRII (Littaua *et al.*, 1990). DENV-infected cells in turn stimulate specific T lymphocytes, resulting in a rapid increase in inflammatory mediators. The mediators generated as a result of immune responses contribute towards progression of severe DENV infection, causing plasma leakage, shock and haemorrhagic manifestations (Kurane & Ennis, 1992). Circulation DENV immune complexes have been observed in 80 % of dengue haemorrhagic fever cases (Ruangjirachuporn *et al.*, 1979). The ability of DENV to utilize FcγR for cell entry relies on the formation of a virus–antibody complex. Thus, identification of the early steps of interactions between the DENV–antibody complex and FcγR is important in elucidation of the mechanism of ADE.

FcγRIIA-transfected COS cells have proved useful for determining the functions of FcγR in mediating receptor tyrosine phosphorylation, phagocytosis (Mitchell *et al.*, 1994) and endocytosis, when 15–30 % of the cells expressed the transfected receptors (Davis *et al.*, 1995). Specific structures of the transmembrane and cytoplasmic domain account for the ability of FcγRIIA to stimulate phagocytosis and tyrosine phosphorylation (Barnes *et al.*, 2006; García-García & Rosales, 2002; Mitchell *et al.*, 1994). Receptor phosphorylation is catalysed by rafts (Kwiatkowska *et al.*, 2003), triggering signal pathways that target actin-based cytoskeleton reorganization, and this in turn serves as a driving force for FcγRIIA-mediated phagocytosis and FcγRIIA capping (Kwiatkowska *et al.*, 2003). In order for the receptor to form, FcγR needs to cross-link, which in turn triggers FcγR clustering and receptor phosphorylation (Huang *et al.*, 1992). Tyrosine phosphorylation of FcγR and accompanying proteins facilitates clustering of FcγR, thereby permitting efficient binding of particles and immune complexes (Sobota *et al.*, 2005). These findings

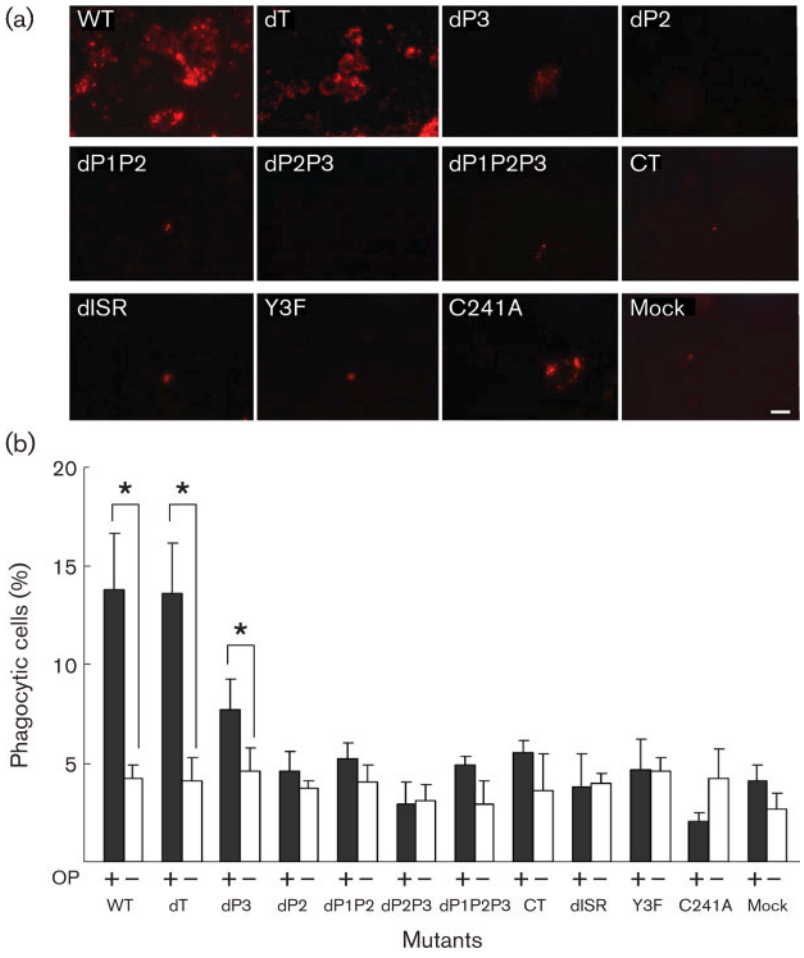

Fig. 2. Phagocytosis of opsonized SE-labelled *E. coli* K-12 by COS-7 cells expressing mutant and WT FcγRIIA. (a) COS-7 cells expressing WT or mutated FcγRIIA were incubated with SE-labelled *E. coli* K-12 particles sensitized with anti-*E. coli* pAb at 37 °C for 45 min. Mock-transfected COS-7 cells served as controls. Phagocytosis of *E. coli* by COS-7 cells was observed by fluorescence microscopy. Bar, 20 μm. (b) The percentage of cells (mean ± SD) that phagocytosed *E. coli* was determined by flow cytometry. Opsinization (OP): the presence (+) or absence (−) of anti-*E. coli* pAb is indicated. **P*<0.05.

indicate that specific structures of FcγRIIA are crucial for triggering receptor-mediated signalling pathways and biological functions.

The present study was undertaken to determine whether modification of the conserved motifs of the cytoplasmic region of FcγRIIA affects the ability of the receptor to mediate ADE. The specificity of FcγRIIA-mediated phagocytosis and receptor capping was confirmed by experiments in the absence of enhancing or opsonizing antibodies, and by using COS-7 cells without FcγRIIA. Consistent with previous findings (Kwiatkowska *et al.*,

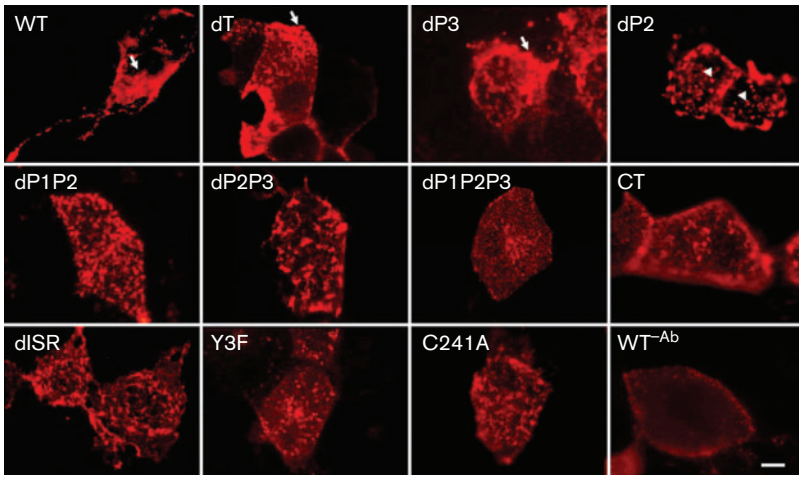

Fig. 3. Cross-linking and capping of anti-dengue serum–DENV-1 complexes on COS-7 cells expressing WT and mutated FcγRIIA. COS-7 transfectants were monitored for their ability to form cap-like structures following binding of the DENV–antibody complex. Capping of cross-linked cells was observed 10 min after warming at 20 °C. Each transfectant was stained with PE-labelled anti-hFcγRIIA (mAb 16320). WT^{-Ab} represents cells stimulated with DENV in the absence of antibody. Arrows indicate capping, whilst arrowheads indicate cross-linking. Bar, 5 μm.

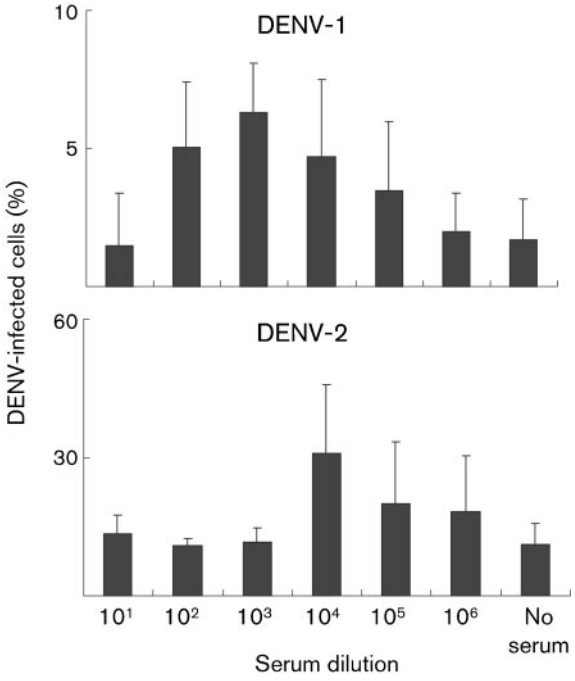

Fig. 4. Enhancement of DENV infection in COS-7 cells expressing WT-FcγRIIA. DENV-1 or DENV-2 was pre-incubated with tenfold-diluted human anti-dengue serum for 1 h at 37 °C. COS-7 cells expressing FcγRIIA were infected with the DENV–antibody complex at 37 °C for 72 h. The percentage of infected cells was determined by flow cytometry after indirect immunostaining with dengue serotype-cross-reactive mAb 4G2. Results are representative of three or more experiments performed in triplicate.

2003; Mitchell *et al.*, 1994), we found that native FcγRIIA (WT) and the dT mutant mediated phagocytosis and receptor capping. DENV infection was enhanced by anti-DENV serum in COS-7 cells transfected with FcγRIIA (WT) and dT. A low but significant level of phagocytosis was detected in COS-7 cells expressing mutant dP3, which has a deletion downstream from K301 including ITAM2 ($P<0.05$) (Fig. 2). Capping and a low level of ADE were detected in COS-7 cells transfected with dP3 ($P<0.05$) (Figs 3 and 5). In contrast, other modifications of the ITAM region and removal of sequences between the ITAM motifs eliminated phagocytosis and the ability of FcγRIIA to undergo capping, indicating that the specific structure of FcγRIIA is required for ADE. To examine whether phagocytosis and DENV immune complex enhancement require similar signal transduction mechanisms, we performed a linear regression analysis and found a highly significant correlation ($P<0.01$) between the two processes (Fig. 6). The results suggest that the structure of the ITAM motif of FcγRIIA is, in part, involved in both ADE and phagocytosis. Signal transduction was not analysed in the present study, and further studies are needed to determine whether similar signal transduction mechanisms are involved in ADE and phagocytosis.

Although the cross-linking of receptor was observed by using anti-DENV serum in COS-7 cells transfected with Y3F, which carries mutations in the ITAM tyrosine moiety, capping, receptor-induced phagocytosis and enhancement of both DENV-1 and DENV-2 infection by anti-DENV serum were absent from FcγRIIA/Y3F. These results differ from the conclusion of a recent study, which observed that tyrosine residues in the ITAM region do not play a role in FcγRIIA-mediated ADE (Rodrigo *et al.*, 2006). Differences in the transfection and infection methods, as well as variations in antibodies and virus strain, may be the reason for the different results, but this should be investigated further.

We conclude that the specific structure of FcγRIIA, when present on non-professional phagocytic cells, is crucial for mediating processes that promote ADE. The results provide a profound implication for our understanding of the mechanism of DENV entry into cells in the presence of antibody.

METHODS

Cell lines. COS-7 cells, an African green monkey kidney-derived fibroblast cell line, and Vero cells (ATCC CCL-81), an African green monkey kidney-derived epithelial cell line, were used. Cells were cultured in Eagle's minimum essential medium (EMEM; Sigma), supplemented with heat-inactivated 10 % fetal calf serum (FCS; Sigma) without antibiotics at 37 °C in 5 % CO_2.

Virus and antibodies. DENV-1 strain 01-44-1HuNIID (GenBank accession no. AB111070), isolated from Tahiti in 2001 (Ito *et al.*, 2007), and DENV-2 strain D2/Hu/OPD030NIID/2005 (TL-30) (GenBank accession no. AB219135), isolated from East Timor in 2005, were used. Virus was propagated on Vero cells. Titres of DENV were determined by plaque assay in Vero cells. Virus dilutions in volumes of 100 µl were inoculated on Vero cell monolayers in 12-well plates. The plates were incubated for 60 min at 37 °C in 5 % CO_2. After virus absorption, the cells were overlaid with maintenance medium containing 1 % methylcellulose (Wako Pure Chemical Industries). The plates were incubated at 37 °C in 5 % CO_2 for 5 days and fixed with neutral formalin for 60 min at room temperature. The cells were then stained with 0.3 % methylene blue for 60 min at room temperature and washed with tap water. Plaques were counted and the virus infectivity titre was expressed as p.f.u. ml^{-1}. Human serum from a dengue fever patient caused by DENV-3 was used as the enhancing antibody. Dengue serotype-cross-reactive mouse IgG monoclonal antibody (mAb) 4G2 (ATCC HB-112), which recognizes the E protein, was used in immunofluorescent and flow cytometry assays.

FcγRIIA and mutant FcγRIIA plasmid constructions. Human FcγRIIA cDNA (Brooks *et al.*, 1989, GenBank accession no. M31932) was generously provided by Dr Jeffrey V. Ravetch, Rockfeller University, NY, USA. The cDNA was subcloned into pcDNA3.1/neo+ (Invitrogen) and mutations were generated by standard site-directed mutagenesis (QuikChange; Stratagene). The list of mutants is shown in Table 1 and Fig. 1(a). Full-length sequences for all constructs were verified by DNA sequence analysis.

Transient expression of WT and mutated FcγRIIA in COS-7 cells. Transfection of COS-7 cells with WT or mutated FcγRIIA cDNA was carried out with Lipofectamine LTX (Invitrogen),

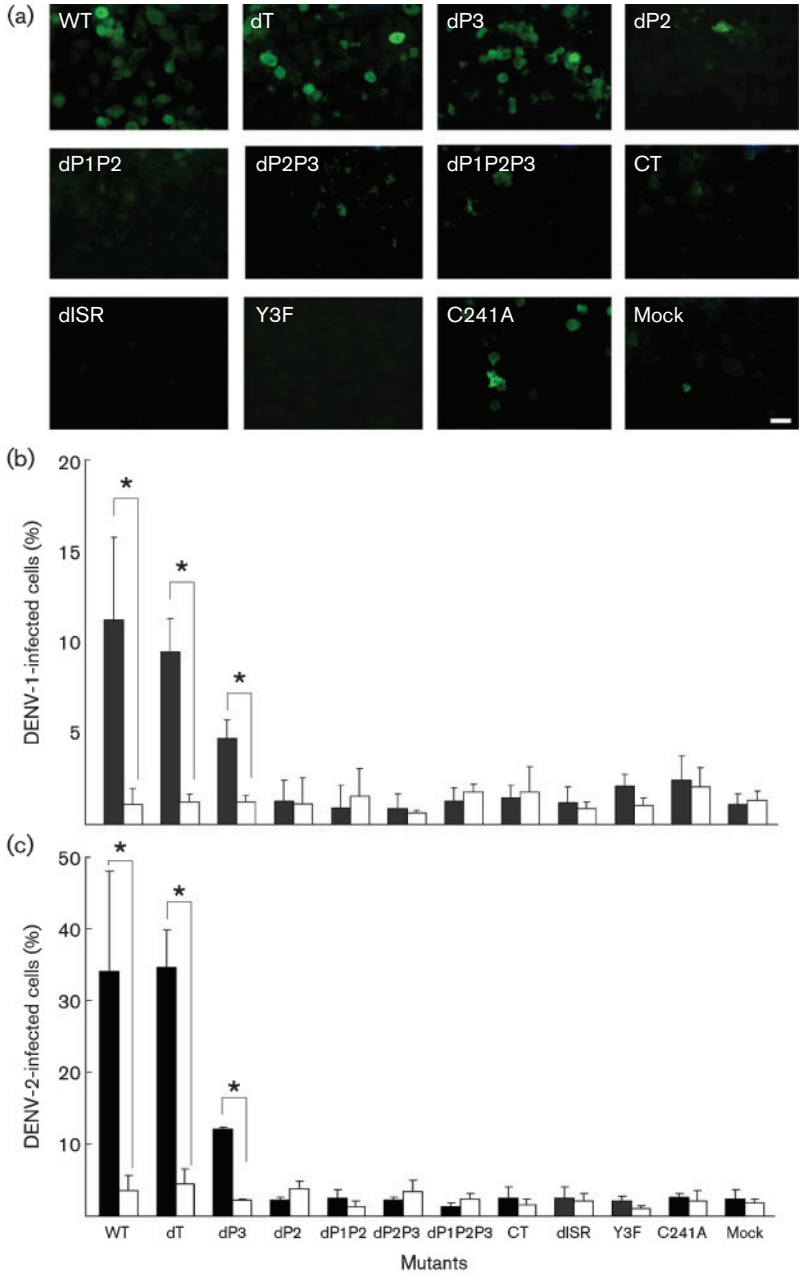

Fig. 5. ADE of DENV infection against COS-7 cells expressing WT and mutated FcγRIIA. (a) Immunofluorescence staining of COS-7 cells expressing WT and mutated FcγRIIA after infection with DENV–antibody complex. DENV–antibody complex was prepared by incubation of DENV-2 and anti-DENV human serum diluted 1 : 10 000. COS-7 cells were infected with the DENV-2–antibody complex and stained with mouse mAb 4G2. The immunofluorescence photomicrographs show DENV-2 antigen-positive cells (green). The m.o.i. was 0.1. Bar, 20 μm. (b, c) The percentage of DENV-1-infected (b) or DENV-2-infected (c) cells was quantified by flow cytometry. Results are the means ± SD of three experiments performed in triplicate. The m.o.i. was 0.1. Filled bars show the mean percentage of infected cells in the presence of enhancing antibody at a 1 : 1000 dilution (b) or a 1 : 10 000 dilution (c), and open bars indicate the mean percentage of infected cells in the absence of enhancing antibody. *$P<0.05$.

according to the manufacturer's protocol. Cells were examined for surface expression of FcγRIIA by flow cytometry and standard immunoblot analysis at 48 h after transfection.

Flow cytometry. COS-7 transfectants were washed with PBS (Invitrogen) and stained with phycoerythrin (PE)-conjugated mAb to human FcγRIIA (CD32A mAb, clone 190723; R&D Systems), according to the manufacturer's instructions. Stained cells were analysed using a Guava EasyCyte Mini cytometer (Millipore). More than 5000 cells were counted and the results were analysed using FlowJo Version 7.5 software (Tree Star). For determining DENV infection, cells were fixed and permeabilized with 1 : 1 acetone : methanol mixture for 10 min and reacted with mAb 4G2 at 37 °C for 60 min. Cells were then stained with Alexa Fluor 488-labelled goat anti-mouse IgG (Invitrogen) and examined by flow cytometry.

Electrophoresis and immunoblotting. Cells were treated in Laemmli's sample buffer and separated under reducing conditions by 12.5 % SDS-PAGE (Atto Corp.). Proteins were transferred to PVDF membranes (Millipore), blocked for 1 h in 5 % Immunoblock (Dainippon Sumitomo Pharma) in PBS with 0.01 % Tween 20 (PBST) and probed with anti-FcγRIIA (goat anti-human FcγRIIA/CD32a antibody; R&D Systems) at a 1 : 500 dilution. After washing with PBST, the blots were probed with horseradish peroxidase-conjugated anti-goat secondary antibodies (R&D Systems) at a 1 : 2000 dilution for 30 min and washed three times in PBST for 10 min, prior to detection by chemiluminescence (GE Healthcare).

Infection of WT- or mutant FcγRIIA-transfected COS-7 cells with DENV alone or DENV–antibody complex. DENV–antibody complex was prepared by mixing 25 μl DENV-1 or DENV-2 at titres

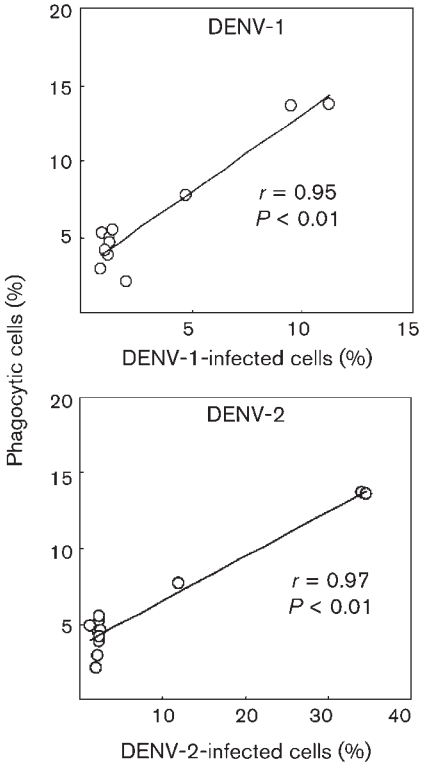

Fig. 6. Linear regression between ADE and phagocytic activities of COS-7 cells expressing WT and mutated FcγRIIA. The percentage of phagocytic and DENV-infected COS-7 cells transfected with FcγRIIA was reduced with the introduction of deletions and point mutations in the FcγRIIA cytoplasmic domain. A significant correlation between phagocytic and ADE capacities within the COS-7 cells transfected with FcγRIIA was observed with DENV-1 and DENV-2.

of 2×10^5 p.f.u. ml^{-1} with 25 µl of the dengue patient's serum at a 1 : 1000 dilution for DENV-1 and 1 : 10 000 dilution for DENV-2, respectively. DENV mixtures were incubated at 37 °C for 60 min with occasional agitation. For the infection assay with DENV alone, virus mixture was prepared by mixing 25 µl DENV-1 or DENV-2 at titres of 4×10^5 p.f.u. ml^{-1} with 25 µl EMEM and incubated at 37 °C for 60 min. COS-7 transfectants (1×10^5 cells per well) were washed twice in 0.5 ml EMEM. The cells in 50 µl EMEM were incubated with 50 µl DENV–antibody complex at an m.o.i. of 0.1 at 37 °C for 60 min, with occasional agitation. The cells were then washed twice with 0.5 ml PBS and maintained in 0.5 ml EMEM supplemented with 10 % FCS. DENV antigen-positive cells were determined by an immunofluorescence assay and flow cytometry at 72 h after infection.

Capping of COS-7 cells expressing WT and mutant FcγRIIA. COS-7 cells were seeded at a density of 4×10^4 cells on 16-well chamber slides (Nalge Nunc), incubated for 24 h, transfected with WT or the series of FcγRIIA mutants, and cultured for another 48 h. To induce cross-linking of FcγRIIA (patching), the cells were washed twice with 0.1 ml EMEM and reacted for 30 min on ice with 25 µl DENV-1–antibody complex or DENV-1 alone, prepared as described above. Subsequently, the cells were incubated for 10 min at 20 °C to induce the formation of FcγRIIA caps and washed gently with 50 µl PBS (Kwiatkowska *et al.*, 2003). The cells were stained with PE-conjugated anti-FcγRIIA, a mouse mAb against human FcγRIIA (clone 190723). After washing with 0.1 ml PBS twice, the cells were mounted in 50 % glycerol and examined under a fluorescence microscope (Olympus). Images were taken and analysed by QCapture Pro version 5.1 (QImaging). Distinct large conglomerates formed in a polar fashion at the cell margins (Kindzelskii *et al.*, 1994; Kwiatkowska *et al.*, 2003) were scored as cap positive, patching was scored as cross-linking, and other results were considered negative.

Phagocytosis assay. Opsonized *E. coli* was prepared by mixing 20 µl anti-*E. coli* rabbit pAb IgG (*E. coli* BioParticles opsonizing reagent; Invitrogen) with 6×10^7 SE-labelled *E. coli* BioParticles (pHrodo *E. coli* BioParticles; Invitrogen) in 1 ml EMEM supplemented with 10 % FCS at 37 °C for 60 min and cooled on ice for 10 min. Fifty microlitres of opsonized *E. coli* BioParticles mixture (2.5×10^6 SE-labelled *E. coli* BioParticles) was added to 1×10^5 COS-7 cells and incubated on ice for 15 min. The cells were then incubated at 37 °C for 45 min. After washing twice, the cells were analysed by flow cytometry and fluorescence microscopy.

ACKNOWLEDGEMENTS

We thank Dr Jeffrey V. Ravetch (Rockfeller University, NY, USA) for generously providing the FcγRIIA cDNA. We gratefully acknowledge Dr Susheela Tridandapani (Ohio State University College of Medicine, Columbus, OH, USA) for her assistance in obtaining the FcγRIIA cDNA for this work. This work was supported by grants from Research on Publicly Essential Drug and Medical Devices from the Japan Health Sciences Foundation (KH53333 and KHC3332), and Research on Emerging and Re-emerging Infectious Diseases (H20-shinkou-ippan-013 and H20-shinkou-ippan-015) by the Ministry of Health, Labour and Welfare, Japan.

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Journal of Virological Methods 163 (2010) 205–209

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Development of an antibody-dependent enhancement assay for dengue virus using stable BHK-21 cell lines expressing FcγRIIA

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Article history:
Received 9 July 2009
Received in revised form
10 September 2009
Accepted 15 September 2009
Available online 23 September 2009

Keywords:
Dengue virus
Antibody-dependent enhancement (ADE)
Fc gamma receptor
FcγRIIA (CD32a)
Stable expression
DF/DHF/DSS

A B S T R A C T

Dengue virus (DENV) causes a wide range of symptoms, from mild febrile illness, dengue fever (DF), to severe life threatening illness, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Subneutralizing concentrations of antibody to DENV enhance DENV infection in FcγR positive cells. This phenomenon is known as antibody-dependent enhancement (ADE). ADE is considered to be a risk factor for DHF and DSS. To develop an ADE assay for DENV, two stable BHK-21 cell lines were established that express FcγRIIA (BHK-FcγRIIA). The BHK-FcγRIIA cell lines were used in an ADE assay with monoclonal antibody (4G2) to DENV, and DENV antibody-positive human sera. Virus growth was quantified directly in BHK-FcγRIIA cells with a standard plaque assay procedure. ADE was detected with monoclonal antibody (4G2) to DENV. ADE was also detected with DENV antibody-positive human sera, but not with DENV antibody-negative human sera. The new ADE assay using BHK-FcγRIIA cells is simple and practical, and is useful for defining the role of ADE in the pathogenesis of DENV infection.

1. Introduction

Dengue virus (DENV) infection occurs in most tropical and subtropical regions of the world, including Asia, Africa and South America, with up to an estimated 100 million people infected worldwide annually. Each of the four serotypes of DENV (DENV-1, DENV-2, DENV-3 and DENV-4) cause a spectrum of symptoms, ranging from mild febrile illness, dengue fever (DF), to severe life threatening illness, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Infection with one DENV serotype offers life-long protection against infection by the same serotype. At the same time, immunity induced against one dengue serotype does not provide protection against infection with serotypes not encountered previously. Antibody induced in primary DENV infection is postulated to be one of the risk factors for DHF/DSS in secondary infection with serotypes different from those causing the primary infection (Graham et al., 1999; Sangkawibha et al., 1984).

In secondary DENV infection, DENV–antibody complexes are formed and taken up more readily than uncoated virus particles by cells expressing Fcγ receptors (FcγR), such as monocytes and macrophages (Kontny et al., 1988; Littaua et al., 1990). This effect, known as antibody-dependent enhancement (ADE), leads to higher levels of progeny virus production. This possible ADE activity of antibodies to DENV poses a challenge for the development of a dengue vaccine, and therefore additional investigation into the comparative nature of neutralizing and immune enhancing antibodies stimulated by natural infection and vaccination is required. Current DENV plaque neutralization assays, performed conventionally in mammalian cells without FcγR (Vero, LLC-MK_2, and BHK-21) (Morens et al., 1985; Roehrig, 2007) measure selectively the neutralizing activity of antibodies without ADE activity. Consequently, it is likely that discrepancy may occur between protection and neutralizing antibody titers generated by these current assays, and when the principal target cells of DENV, FcγR expressing monocytes (Kou et al., 2008), are used for measurement of neutralizing antibody.

In the present study, stable BHK-21 cell lines expressing FcγRIIA (CD32a) (BHK-FcγRIIA) were established. It was then determined whether enhancing antibodies could be measured in BHK-FcγRIIA cell lines, using conventional plaque assay. Human serum positive for DENV IgG and monoclonal anti-DENV IgG enhanced DENV-2 infection using the new BHK-FcγRIIA cell lines. The new ADE assay

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doi:10.1016/j.jviromet.2009.09.018

is simple, reproductive, and thus useful for determining the role of antibody in DENV infection.

2. Materials and methods

2.1. Cell lines

BHK-21 cells, a hamster kidney cell line, and Vero cells (Vero 9013, Japan Health Science Research Resources Bank), an African green monkey kidney-derived epithelial cell line, were used. Cells were cultured in Eagle's minimum essential medium (EMEM) (Sigma, St. Louis, MO, USA), supplemented with heat inactivated 10% FCS (Sigma) without antibiotics at 37 °C in 5% CO_2.

2.2. Virus

Dengue virus type-1 (DENV-1), 01-44-1HuNIID strain (GenBank accession no. AB111070), dengue virus type-2 (DENV-2) D2/Hu/OPD030NIID/2005 strain (TL-30; GenBank accession no. AB219135), DENV-2 S16803 strain, dengue virus type-3 (DENV-3) D3/Hu/TL018NIID/2005 strain (TL-18; GenBank accession no. AB214879), and dengue virus type-4 (DENV-4) TVP-360 strain were used. DENV-1 01-44-1HuNIID strain was isolated from a returnee to Japan from Tahiti in 2001 (Ito et al., 2007). DENV-2 TL-30 strain and DENV-3 TL-18 strain were isolated from patients in Comoro, East Timor in 2005. DENV-2 (S16803) and DENV-4 (TVP-360) are World Health Organization reference laboratory strains and were supplied by Walter Reed Army Institute of Research, Washington, DC, USA, through National Institute for Biological Standards and Control, Hertfordshire, UK (Roehrig, 2007). Virus was propagated on Vero cells. DENV-2 TL-30 strain was harvested at the 5th culture passage, and virus from this single passage was used in all the infection enhancement experiments.

2.3. Serum specimens and antibody

Serum specimens were heat inactivated at 56 °C for 30 min before use in the experiments. All sera used had been tested for DENV antibody by IgG ELISA and IgM ELISA. Dengue serotype-cross-reactive mouse monoclonal IgG antibody 4G2 (MAbs, ATCC MAb HB-112 D1-4G2-4-15) was also used for the ADE assay.

2.4. FcγRIIA plasmid construction

Human FcγRIIA cDNA (Brooks et al., 1989, GenBank accession no. M31932) was generously provided by Dr. Jeffrey V. Ravetch, Rockefeller University, NY, USA. The cDNA was subcloned into the pcDNA 3.1/neo+ vector (Invitrogen, Calsbad, CA, USA) and full length sequence of the construct was verified by DNA sequence analysis.

2.5. Stable expression of FcγRIIA in BHK-21 cells

Transfection of BHK-21 cells with pcDNA3.1/neo+ vector carrying FcγRIIA cDNA was performed with lipofectamine LTX (Invitrogen) and nupherin-neuron reagent (Biomol Research Laboratories, Plymouth Meeting, PA), according to the manufacturers' protocols. Transfected cells were selected with 0.5 mg/ml neomycin (G418, PAA Laboratories GmbH, Austria) for 2 weeks and then further selected by the limiting dilution method. Cells stably transfected with the neomycin resistant vector were maintained in EMEM, 0.5 mg/ml neomycin supplemented with 10% FCS (Sigma) at 37 °C in 5% CO_2.

2.6. Flow cytometry

FcγRIIA transfected BHK-21 cells were examined for surface expression of FcγRIIA by flow cytometry. Cells were washed in PBS (Invitrogen) and stained with phycoerythrin (PE)-conjugated monoclonal antibody to human FcγRIIA (CD32 MAb, Clone 190723; R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Stained cells were analyzed with the Guava EasyCyte Mini cytometer (Guava Technologies, Millipore, MA, USA). More than 5000 cells were counted and the results were examined by FlowJo software (Tree Star, Ashland, OA, USA).

2.7. Preparation of virus–antibody complex

Each serum specimen was serially diluted 10-fold from $1:10^1$ to $1:10^6$ with EMEM supplemented with 10% FCS. Mouse monoclonal antibody 4G2 was serially diluted 10-fold from 2.6 mg/ml ($1:10^1$) to $1:10^6$ with EMEM supplemented with 10% FCS. DENV–antibody complex was prepared by mixing 50 μl of DENV-2 TL-30 strain at titers of 2×10^3, 2×10^2, or 2×10^1 PFU/ml with 50 μl of diluted serum samples, diluted mouse monoclonal antibody 4G2 or EMEM supplemented with 10% FCS. Virus–antibody mixture was incubated at 37 °C for 60 min before use in infection experiments.

2.8. Virus infection

One-tenth of a milliliter of virus–antibody mixture was inoculated onto BHK-21 monolayers in 12-well plates. Plates were incubated at 37 °C for 60 min in 5% CO_2. After virus absorption, cells were washed with 1 ml of EMEM and overlaid with maintenance medium containing 1% methylcellulose (Wako Pure Chemical Industry, Osaka, Japan). The plates were incubated at 37 °C in 5% CO_2 for 5 days.

2.9. Plaque visualization

After 5 days of incubation, cells were fixed with 10% formalin for 60 min at room temperature and washed with tap water. Cells were then stained with methylene blue for 60 min at room temperature and washed with tap water. Plaques were counted by naked eye and the virus infectivity titer was expressed as plaque forming units (PFU) per ml.

3. Results

3.1. Establishment of stable BHK-21 cell lines expressing FcγRIIA (BHK-FcγRIIA)

BHK-21 cell line was selected for transfection with FcγRIIA, because it forms plaques upon DENV infection. Four BHK-21 cell lines stably expressing the FcγRIIA were established (data not shown), and two of the cell lines, BHK-FcγRIIA/2 and BHK-FcγRIIA/4 were selected for further studies. The expression of FcγRIIA was verified by flow cytometry (Table 1 and Fig. 1). More than 50% of the transfected cells expressed FcγRIIA for as long as 18 continuous passages, during a span of 5 months (Table 1).

3.2. Efficiency of DENV plaque formation in BHK-FcγRIIA

Four serotypes of DENV were quantified in the absence of DENV antibody using two BHK-FcγRIIA cell lines, parent BHK-21 cells and Vero cells (Table 2). The titers of DENV were similar among these four cell lines. The results indicate that the efficiency of DENV plaque formation in BHK-FcγRIIA/2 and BHK-FcγRIIA/4 were similar to that of Vero and parent BHK-21 cell lines.

Table 1
Characteristics of stable BHK-21 cell lines expressing FcγRIIA.

Cell line	Passage number	% of FcγRIIA expressing cells[a]	Fold increase[b]
BHK-FcγRIIA/2	2	85	ND[c]
	4	74	14
	5	66	ND
	9	69	15
	18	56	15
BHK-FcγRIIA/4	2	83	ND
	4	68	13
	5	65	ND
	9	64	13
	18	71	14

[a] The percentage of BHK-21 cells expressing FcγRIIA was determined by flow cytometry as described in Section 2.
[b] Fold increase was calculated by the formula: (DENV-2 titer in the presence of mouse monoclonal antibody 4G2 at 1:1000 dilution)/(DENV-2 titer in the absence of anti-dengue antibody).
[c] Not determined.

Table 2
Comparison of DENV titers in Vero, BHK-21, and BHK-FcγRIIA cell lines.

Cell Line	Virus titers (PFU/ml)				
	DENV-1[a]	DENV-2[b]	DENV-2[c]	DENV-3[d]	DENV-4[e]
Vero	6.3×10^7	4.0×10^6	2.5×10^5	3.2×10^4	1.2×10^6
BHK-21	3.2×10^7	2.5×10^6	2.0×10^5	1.0×10^4	1.0×10^6
BHK-FcγRIIA/2	4.0×10^7	3.2×10^6	1.6×10^5	2.5×10^4	1.1×10^6
BHK-FcγRIIA/4	4.0×10^7	2.5×10^6	1.3×10^5	2.0×10^4	1.2×10^6

Virus titers are shown as the mean of two readings.
[a] Strain used was 01-44-01 HuNIID.
[b] Strain used was TL-30.
[c] Strain used was S16803.
[d] Strain used was TL-18.
[e] Strain used was TVP-360.

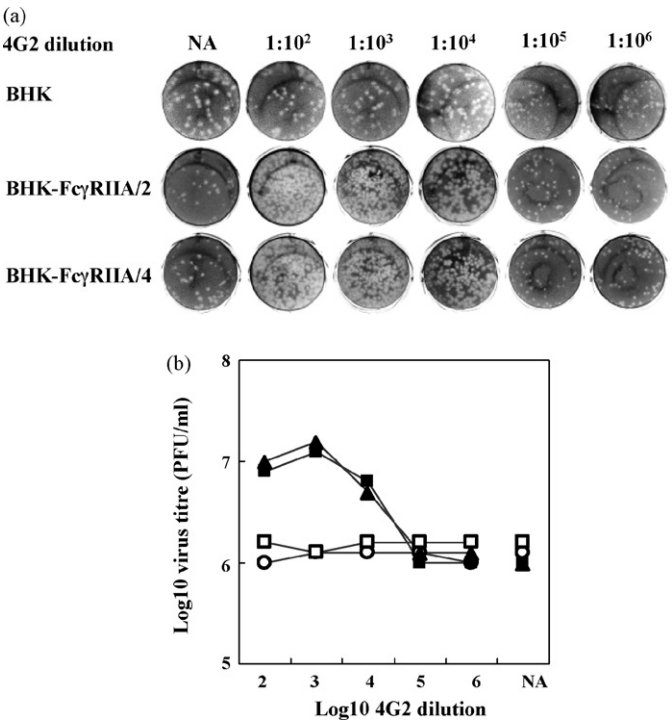

Fig. 2. Figures of plaques developed by DENV-2-4G2 antibody complex. (a) Mouse monoclonal antibody 4G2 was 10-fold serially diluted, starting from $1:10^2$. Untransfected BHK-21 cells did not demonstrate infection enhancement. In contrast, two cell lines transfected with FcγRIIA showed DENV-2 infection enhancement at antibody dilutions from $1:10^2$ to $1:10^4$. NA indicates no antibody. (b) Virus titers as determined by plaque assay. (○) Untransfected BHK-21 cells, (□) BHK-21 cells transfected with empty vector, (▲) BHK-FcγRIIA/2, and (■) BHK-FcγRIIA/4. NA indicates no human serum.

3.3. Antibody-dependent enhancement of DENV-2 infection using mouse monoclonal antibody, 4G2

Mouse monoclonal antibody 4G2, which reacts with a broad range of flaviviruses, was used to prepare virus–antibody complexes for examining ADE. The 4G2 antibody enhanced DENV-2 infection of BHK-FcγRIIA/2 and BHK-FcγRIIA/4 at final dilutions of $1:10^2$–$1:10^4$ (Fig. 2). Antibody-dependent enhancement was not detected in BHK-21 cells and BHK-21 cells transfected with empty vector (pcDNA3.1/neo+) (Fig. 2b). ADE in BHK-FcγRIIA cell lines was consistently observed until passage 18 using monoclonal antibody 4G2 at a $1:10^3$ dilution (Table 1).

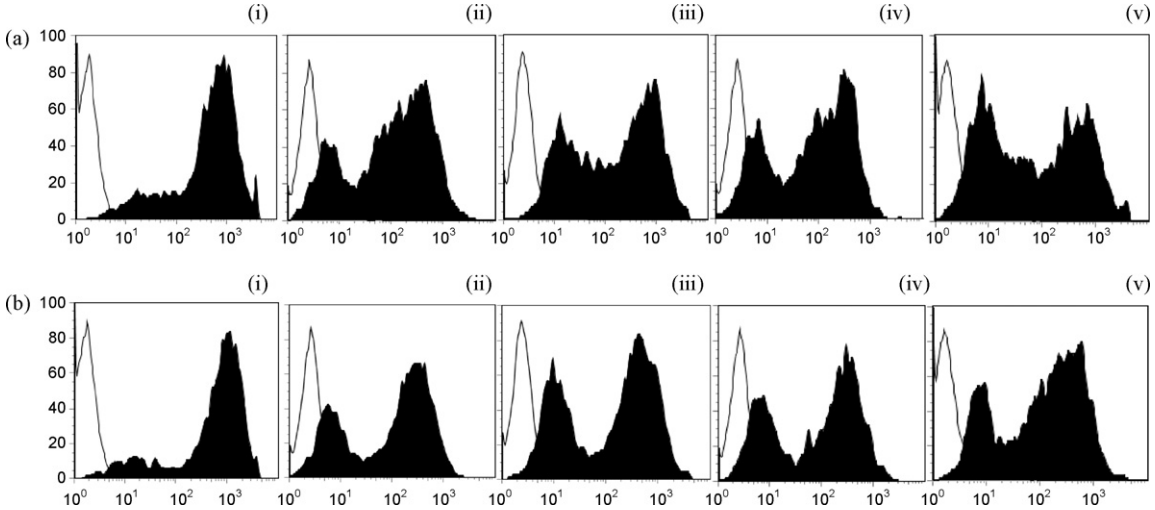

Fig. 1. Expression of FcγRIIA in BHK-21 cells as determined by flow cytometry. PE-labeled monoclonal (mAb 16320) to FcγRIIA was used to measure the percentage of BHK-21 cells expressing FcγRIIA. Solid black graph shows BHK-21 cells transfected with FcγRIIA and open graph shows untransfected parent BHK-21 cells. (a) FcγRIIA transfected BHK-21 cell line 2 (FcγRIIA/2): (i) passage 2, (ii) passage 4, (iii) passage 5, (iv) passage 9, and (v) passage 18. (b) FcγRIIA transfected BHK-21 cell line 4 (FcγRIIA/4): (i) passage 2, (ii) passage 4, (iii) passage 5, (iv) passage 9, and (v) passage 18.

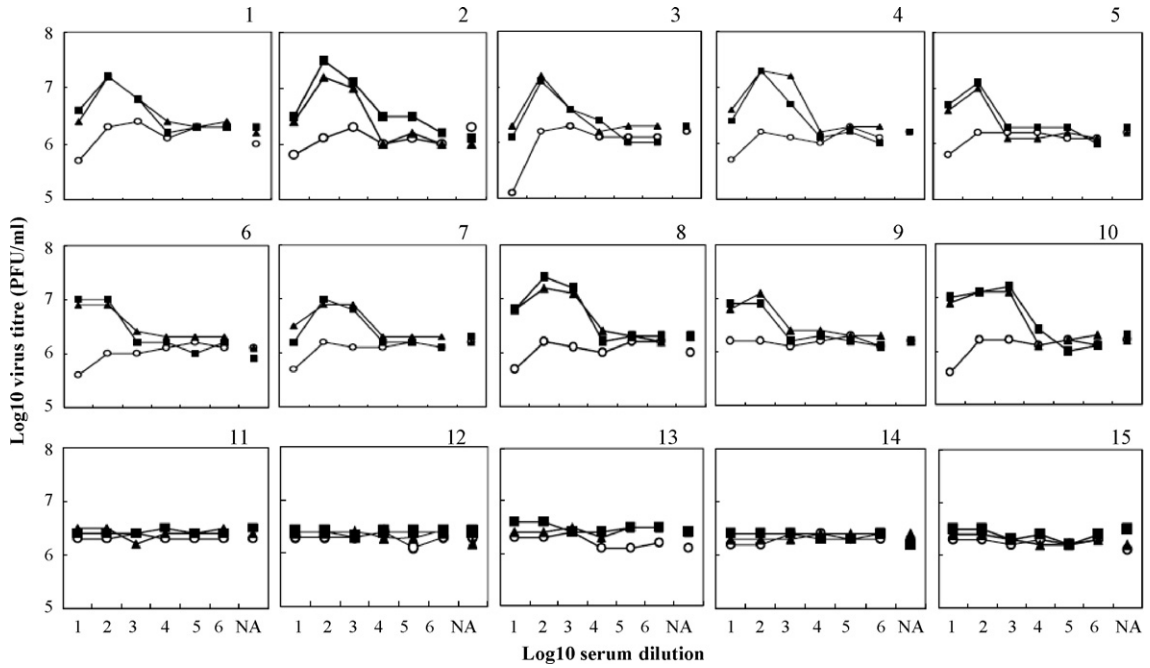

Fig. 3. Enhancement of DENV-2 infection by DENV antibody-positive human sera. DENV was incubated with human serum samples, 10-fold serially diluted from 1:10 to $1:10^6$. Figures are presented according to serum sample number. Sera 1–10 were anti-DENV IgG-positive and sera 11–15 were anti-DENV IgG negative. (○) Untransfected BHK-21 cells, (▲) BHK-FcγRIIA/2, and (■) BHK-FcγRIIA/4. NA indicates result no antibody.

3.4. Antibody-dependent enhancement of DENV-2 infection using DENV antibody-positive human sera

Ten DENV IgG-positive serum samples were tested for their capacity to enhance DENV-2 infection in BHK-FcγRIIA/2 and BHK-FcγRIIA/4. ADE activity was detected with all the dengue IgG-positive serum samples (Fig. 3, nos. 1–10). DENV IgG-negative serum samples did not enhance DENV-2 infection in either FcγRIIA expressing cell line (Fig. 3, nos. 11–15). The results indicate that BHK-FcγRIIA/2 and BHK-FcγRIIA/4 are suitable for detecting ADE activity of DENV antibody-positive human sera.

4. Discussion

An assay was established to determine and compare the in vitro ADE activity of human serum specimens. The plaque method described employs BHK-21 cells that were transfected with human FcγRIIA, an IgG Fc-receptor that is widely distributed among cells of macrophage/monocyte lineage (Daëron, 1997), and which was known to mediate ADE (Littaua et al., 1990; Mady et al., 1991). Similarly, BHK-21 cells transfected with FcγRIIA have proved useful for functional studies of FcγRIIA, such as phagocytosis and IgG complex clearance (Kwiatkowska et al., 2003; Sobota et al., 2005).

Infection enhancement of DENV-2 TL-30 strain complexed with 4G2, an IgG2a flavivirus group-reactive monoclonal mouse antibody, was reproducible in BHK-FcγRIIA cell lines and provided an internal assay control. This antibody had been reported to enhance DENV infection of FcγRIIA expressing K562 cells (Guy et al., 2004; Littaua et al., 1990; Mady et al., 1991).

Immune serum may possess two competing effects on DENV growth in the presence of FcγR expressing cells: neutralization and enhancement. Using the BHK-FcγRIIA cell lines, enhancing activity was detected for all serum specimens that were positive for anti-DENV IgG. None of the DENV-IgG-negative sera demonstrated ADE activity. In contrast, using the BHK-21 cells without FcγRIIA, DENV IgG-positive serum specimens exhibited high levels of neutralizing activity, but not enhancing activity. Thus, the newly developed assay was capable of detecting ADE activity of human sera. Assessment of neutralizing antibody titers of the serum specimens in BHK-FcγRIIA cell lines is an interesting research subject, should be done in the next series of experiments.

ADE assays that employed COS-7 and CV-1 cells expressing FcγR have been described previously (Rodrigo et al., 2006, 2009a,b). In these reports, plaques were detected by an indirect immunostaining method using anti-NS1 protein monoclonal antibody. In comparison, the DENV plaque detection method adopted in the present study is a widely used conventional titration method (Roehrig, 2007), in which cells were stained directly by vital stain, and plaques counted by naked eye.

The newly established BHK-FcγRIIA cell lines, constantly demonstrated ADE of DENV with anti-DENV antibody positive human sera. The ADE activity of serum samples detected by conventional plaque formation assay offers several advantages over ADE assays using hematopoeitic origin non-adherent cell lines. First, in the non-adherent cell lines, virus titers in the culture supernatant need to be assayed using surrogate plaque titration assays. Second, the conventional plaque assay used in the present study makes it possible to visualize plaque size and introduce flexibility into both incubation times and experimental workflow.

Recent studies have suggested that ADE infection not only facilitates the entry process, but also modifies innate and adaptive intracellular antiviral mechanisms, resulting in unrestricted DENV replication, using THP-1 cells-derived macrophages or peripheral blood mononuclear cells (PBMCs) (Chareonsirisuthigul et al., 2007; Ubol et al., 2008). Most hematopoietic cells express more than one FcγR or other myeloid-specific receptors, that might influence biological function (Daëron, 1997; Lund-Johansen et al., 1992; Ziegler et al., 1980), and thus it has been difficult to determine the exact roles of FcγR and/or other myeloid-specific receptors that may co-operatively mediate ADE in the presence of co-operative role from other FcγR and myeloid-specific receptors. The new ADE assay using non-hematopoeitic origin BHK-21 cells, which lack endogenous FcγR and other myeloid-specific receptors is suitable for determining the role of FcγRIIA in ADE including virus initiation, replication, and host innate immunity.

BHK-FcγRIIA cell lines are suitable for determining the enhancing activity of DENV in the sera of patients, and provides a valuable tool for defining the role of antibody in the pathogenesis of DENV infection.

Acknowledgements

The authors would like to thank Dr. Jeffrey V. Ravetch (Rockfeller University, NY, USA) for generously providing the FcγRIIA cDNA. The authors also would like to thank 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 Publicly Essential Drug and Medical Devices from the Japan Health Sciences Foundation (KH53333 and KHC3332), and Research on Emerging and Re-emerging Infectious Diseases (H20-shinkou-ippan-013 and H20-shinkou-ippan-015) from the Ministry of Health, Labour and Welfare, Japan.

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CVI Accepts, published online ahead of print on 30 December 2009
Clin. Vaccine Immunol. doi:10.1128/CVI.00396-09

TITLE: Discrepancy in Neutralizing Antibody Titers between Plaque Reduction Neutralizing Tests Using FcγR-negative and FcγR-expressing BHK-21 cells

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RUNNING TITLE: Neutralizing antibody titers in FcγR expressing BHK-21 cells

ABSTRACT

Protective immunity against dengue virus (DENV) is best reflected by the presence of neutralizing antibodies. A conventional plaque reduction neutralizing test (PRNT) is performed using FcγR-negative assay cells. Because FcγR plays a key role in antibody-dependent enhancement, we examined neutralizing antibody titers of mouse monoclonal antibodies and human serum samples in PRNT assays using FcγRIIA-negative and FcγRIIA-expressing BHK cells. There was a discrepancy in neutralizing antibody titers between PRNTs using FcγRIIA-negative and FcγRIIA-expressing BHK cells. Neutralizing antibody titers to DENV-1 and DENV-2 of monoclonal antibodies, and most of the tested human serum samples, were higher in assays using BHK cells than those using FcγRIIA-expressing BHK cells. The results suggest that neutralizing antibody titers determined using FcγRIIA-expressing cells may better reflect the protective capacity of anti-DENV antibodies, as the major target cells of DENV infection are FcγR-positive cells.

INTRODUCTION

Dengue virus (DENV), a member of the family Flaviviridae, represents a major health problem in tropical and sub-tropical regions of the world. There are four serotypes including dengue virus types 1–4 (DENV-1–DENV-4). DENV causes a wide range of symptoms, from mild febrile illness known as dengue fever (DF), to severe life threatening illness including dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Infection with one serotype induces life-long protection against homologous serotypes, but protection against other serotypes is short-lived. In secondary infection, cross-reactive, non-neutralizing antibodies bind to DENV. DENV-antibody complexes are taken-up more efficiently by FcγR expressing cells, and higher levels of viremia are developed (5, 7, 10, 12, 15, 16). This phenomenon, known as antibody-dependent enhancement (ADE) is considered to be a risk factor for DHF and DSS.

Protective immunity against DENV is best reflected by the presence of neutralizing antibody. High neutralizing antibody levels induced by primary infection are considered central in offering life-long protective immunity against homologous serotype. Thus, a vaccine against DENV infection is expected to induce high levels of neutralizing antibody against all four serotypes. The plaque reduction neutralizing test (PRNT) is a widely accepted approach to measure the neutralizing activity of antibodies (14). PRNTs, which employs Vero, LLC-MK2 or BHK-21 cells as assay cells (11, 14) is, however, limited to measuring neutralizing activity of viral infectivity in the absence of FcγR (1). It is possible that neutralizing antibody titers of anti-DENV antibodies induced by natural infection or by vaccines may differ when assayed in the presence of enhancing activity. The neutralizing antibody titers determined using FcγR-expressing BHK-21 cells may better reflect protective immunity, because the principal target cells of DENV are FcγR-expressing cells

1 such as monocytes (6). In the present study, we sought to determine if neutralizing antibody
2 titers were at the same or different levels when BHK-21 cells and lines expressing FcγR
3 were used as the assay cells.
4

MATERIALS AND METHODS

Cell Lines. BHK-21 cells, a hamster kidney cell line, and Vero cells (Vero 9013, Japan Health Science Research Resources Bank), which are an African green monkey kidney-derived epithelial cell line, were used. Two stable BHK-21 cell lines were established that express FcγRIIA (BHK-FcγRIIA/2 and BHK-FcγRIIA/4) previously and were used in this study (9). BHK-21 and Vero cells were cultured in Eagle's Minimum Essential Medium (EMEM), (Sigma, St. Louis, MO, USA), supplemented with heat inactivated 10% FCS (Sigma), without antibiotics at 37 ˚C in 5% CO_2. BHK-FcγRIIA/2 and BHK-FcγRIIA/4 cell lines were cultured in Eagle's Minimum Essential Medium (EMEM), (Sigma), supplemented with heat inactivated 10% FCS (Sigma) and 0.5 mg/ml neomycin (G418, PAA Laboratories GmbH, Austria) at 37 °C in 5% CO_2.

Virus. Dengue virus type-1 (DENV-1) 01-44-1HuNIID strain (GenBank accession number AB111070), dengue virus type-2 (DENV-2) D2/Hu/OPD030NIID/2005 strain (GenBank accession number AB219135), dengue virus type-3 (DENV-3) CH53962 strain, and dengue virus type-4 (DENV-4) TVP-360 strain were used (Table 1) (4, 9, 14). DENV-1 (01-44-1HuNIID) and DENV-2 (D2/Hu/OPD030NIID/2005) were isolated from imported dengue patients and established as laboratory strain for plaque reduction neutralizing assay at Department of Virology I, National Institute of Infectious Disease (4, 9). DENV-3 (CH53962 strain) and DENV-4 (TVP-360 strain) were World Health Organization laboratory strains and were supplied by Walter Reed Army Institute of Research, Washington, DC, USA (9, 14).

Monoclonal antibodies. Flavivirus-cross-reactive mouse monoclonal IgG2a antibody (ATCC MAb HB-112 D1-4G2-4-15) and DENV-2 serotype-specific mouse monoclonal IgG1 antibody, ATCC MAb HB-46 3H5-1 (3), used in the assays were purchased from the

American Type Culture Collection (Manassas, VA, USA).

Serum specimens. The serum samples were collected for laboratory diagnostic purpose from dengue patients from 2004 to 2009. Dengue virus infection was confirmed by positive type-specific real time reverse transcriptase polymerase chain reaction (RT-PCR) and, anti-DENV antibody by IgG ELISA and IgM ELISA in our laboratory (4). Serum specimens, #1—18, were heat inactivated at 56 °C for 30 minutes and used in the experiments. Serum samples #1 and #3, and #2 and #4, were paired serum samples, respectively, obtained from two cases of primary DENV infections. Serum samples designated as early samples (#1, #2, #7, #8, #9, and #10) were collected 1—3 days after the onset of the disease. Serum samples designated as late phase samples (#3, #4, #5, #6, #11, #12, and #13) were obtained 6—14 days after the onset of the disease. The virus was isolated from primary cases #1 and #2, and secondary cases #7, #8, #9 and #10, previously (Tables 2 and 3). The serum samples #15—18 were obtained from non-DEN fever patients.

Plaque reduction neutralizing assays. Mouse monoclonal antibody 4G2 (ATCC HB-112 D1-4G2-4-15; 1.3 mg/ml) and mouse monoclonal antibody 3H5 (ATCC HB-46 3H5-1; 2.5 mg/ml) were serially diluted 2 fold from 1:10 to 1:5120 with EMEM supplemented with 10% FCS. Human serum samples were serially diluted 2 fold from 1:5 to 1:2560 with EMEM supplemented with 10% FCS. The virus-antibody mixture was prepared by mixing 25 µl of DENV-1 or DENV-2 at titers of 2.5 x 10^3 PFU/ml with 25 µl of serially diluted antibodies or serum samples. Control virus samples were prepared by mixing 25 µl of DENV-1 and DENV-2 at titers of 2.5 x 10^3 PFU/ml with 25 µl of EMEM supplemented with 10% FCS. The virus-antibody mixture was incubated at 37 °C for 1 hour. Fifty microliters of virus-antibody mixture were inoculated onto BHK-21 monolayers in 12-well plates. The plates were incubated for 1 hour at 37 °C in 5% CO_2. After virus

absorption, the cells were washed with 1 ml of EMEM and overlaid with maintenance medium containing 1% methylcellulose (Wako Pure Chemical Industry, Osaka, Japan). The plates were incubated at 37 °C in 5% CO_2 for 5 days. After 5 days of incubation, the cells were fixed with 3.7% (v/v) formaldehyde for 1 hour at room temperature and washed with water. The cells were then stained with methylene blue solution for 1 hour at room temperature and washed with water. Plaques were counted by naked eye. The neutralization titer was defined as the highest serum dilution that reduced the number of plaques by 50% (17). In the case of no plaque reduction, or an increase in the number of plaques, the percentage plaque reduction was expressed as “0%”.

RESULTS

Neutralizing titers of mouse monoclonal antibodies, 4G2 and 3H5, determined by assays using parent BHK-21 and FcγRIIA cell lines. Neutralizing titers of mAb 4G2 and 3H5 were examined against DENV-1, DENV-2, DENV-3 and DENV-4, using BHK-21 cells, and two stable BHK-21 cell lines expressing FcγRIIA, BHK-FcγRIIA/2 and BHK-FcγRIIA/4 (Fig 1, Fig 2). The mAb 4G2 demonstrated neutralizing titers of 1:320 to 1:40 to all 4 serotypes of DENV, using parent BHK-21 cells. However, when BHK-FcγRIIA/2 and BHK-FcγRIIA/4 were employed, no neutralizing activity was detected in 3 of the 4 serotypes, and neutralizing titers as low as 10 were detected in DENV-3 (Table 1).

The DENV-2 serotype-specific mAb 3H5 demonstrated a neutralizing titer of 1:40 in DENV-2, only when BHK-21 cells were used. Neutralizing activity to DENV-2 was not detected when FcγRIIA/2 and FcγRIIA/4 were used as assay cells (Table 1). The results indicate that there is a discrepancy in the neutralizing activities of the two mAbs between assays using Fcγ-negative BHK-21 cells and those using FcγRIIA-positive cells.

Neutralizing antibody titers to DENV-1 of human serum samples determined by assays using BHK-21 and BHK-FcγRIIA cells. Eighteen human serum samples were tested for their neutralizing titers to DENV-1, using BHK-21, BHK-FcγRIIA/2 and BHK-FcγRIIA/4 cell lines (Fig 3). Serum samples # 6, # 10, # 11, # 12, #13 and # 14 demonstrated neutralizing antibody titers of 1:160—1:640 when Fcγ-negative BHK-21 cells were used as assay cells. However, they demonstrated neutralizing antibody titers of <1:5—1:40, when FcγR-positive cells were used (Table 2). Interestingly, serum samples # 3 and # 10 demonstrated similar levels of neutralizing antibody titers in assays using FcγR-negative and FcγR-positive BHK-21 cells. DENV-antibody negative samples (#

15—18) did not show any neutralizing activity in BHK-21, BHK-FcγRIIA/2 and BHK-FcγRIIA/4 cells. The results indicate that neutralizing antibody titers of human serum samples from dengue patients were different between assays using FcγR-negative and FcγR-positive BHK-21 cells.

Neutralizing antibody titers to DENV-2 of human serum samples determined by assays using BHK-21 and BHK-FcγRIIA cells. Eighteen human serum samples were also tested for their neutralizing titers to DENV-2, using BHK-21, BHK-FcγRIIA/2 and BHK-FcγRIIA/4 cells (Fig 4). Serum samples # 5, #6, # 9, # 10, # 13 and # 14 demonstrated neutralizing titers of 1:20—1:320 when BHK-21 cells were used. However, they did not demonstrate detectable levels of neutralizing activity using BHK-FcγRIIA cells (Table 3). Serum samples # 4 and # 12 demonstrated neutralizing titers of 40 and 80 respectively in BHK-21 cells, and 10 and 5, respectively, in BHK-FcγRIIA cells. These results were consistent with those shown in Tables 1 and 2, and indicate that neutralizing titers were higher, when determined by assays using FcγR-negative cells than when using BHK-FcγRIIA cells.

DISCUSSION

The plaque reduction neutralizing test (PRNT) is a widely accepted method of measuring neutralizing capacity of antibodies against DENV. Conventional PRNT assays employ Vero, LLC-MK2 or BHK-21 cells that do not express FcγR (1, 11, 14). Thus, assays using these cell lines measure the effects of viral infectivity in the absence of FcγR, and activity measurements are excluded. In the present study, we compared DENV neutralizing titers between stable BHK-21 cells lines expressing FcγRIIA, BHK-FcγRIIA/2 and BHK-FcγRIIA/4, and parent FcγR-negative BHK-21 cells to examine the influence of FcγR on DENV neutralization. The assay using BHK-FcγRIIA/2 and BHK-FcγRIIA/4 cell lines in this study was developed to examine neutralizing antibody titers of anti-DENV antibody present in serum samples obtained from DENV patients from various stages of the disease. The assay had been proved useful in studies in the role on antibodies in ADE of DENV infection using human serum samples. The ability to enhance DENV using the flavivirus group reactive monoclonal mouse antibody, 4G2, by BHK-FcγRIIA/2 and BHK-FcγRIIA/4 were obtained at similar levels (9).

In this study, the monoclonal antibody, 4G2, neutralized all 4 DENV-serotypes in BHK-21 cells. In contrast, when BHK-FcγRIIA cells were used, 4G2 did not neutralize 3 of the 4 serotypes and neutralized DENV-3 at antibody dilutions as low as 1:10. The 3H5 antibody neutralized only DENV-2 and the neutralizing activity was not detected using BHK-FcγRIIA cells. The absence of neutralization using BHK-FcγRIIA cells suggests that the presence of ADE lowers the neutralizing activity of the monoclonal antibody. Human serum samples from dengue patients demonstrated similar results. The neutralizing antibody titers of most of the tested samples were higher when determined using FcγR-negative BHK-21 cells than when determined by the FcγR-expressing BHK-21 cell

lines, BHK-FcγRIIA/2 and BHK-FcγRIIA/4.

Antibodies have two competing effects on DENV growth in the presence of FcγR: neutralization and infection enhancement (10). In the presence of FcγR, infection enhancement effect may hamper neutralization (9). DENV immune complexes formed with heterologous antibodies were less susceptible to neutralization in the presence of FcγRIIA-expressing BHK-21 cells, which is consistent with earlier findings by other investigators using FcγRIIA-expressing CV-1 cells (13, 16). In contrast, DENV-1 immune complexes formed with DENV-1 antibodies, or DENV-2 immune complexes formed with DENV-2 antibodies (homologous DENV-immune complexes), were susceptible to neutralization in both BHK-FcγRIIA cells and parent BHK-21 cells. Primary infection with one DENV serotype usually induces long-term protective immunity against homologous serotypes (2). Neutralization of heterologous DENV in assays using FcγR-expressing cells, thus strongly reflects the effect of ADE activity. Some serum samples from primary infection also demonstrated higher neutralizing antibody titers when determined using BHK-21 cells than when determined using BHK-FcγRIIA cells. It is possible that some ADE activity exists in the neutralizing assay, even against homologous serotypes.

In the present study, a conventional PRNT assays was used and the serum samples used in this study was obtained from primary or secondary DENV 1 to 4 patients, at both early phase and late phase of the disease, and thus, offers insights into individual immunological respose during various stages of the disease (Table 2 and 3). The PRNT assays using BHK-FcγRIIA cells satisfy the criteria for an acceptable alternative to conventional neutralization assays: the assay detects the sum of neutralizing and enhancing activities as neutralizing titers in the presence of FcγRIIA. At the same time, the simplicity and ease of performance using the cell lines in the present study meet or exceed those of

previous studies (8, 13, 14, 16). The results suggest that PRNTs using BHK-FcγRIIA cells could be a feasible alternative to the detection of neutralizing titers of DENV. In addition, the assay holds potential in assessing protective capacity against heterologous DENV challenge using *in vivo* animal models. However, subsequent studies will be needed to determine whether PRNTs using FcγRIIA-expressing BHK-21 cells will better demonstrate the correlation between the PRNT using human serum samples and protective capacity against DENV in vivo.

ACKNOWLEGEMENTS

We thank Dr Jeffrey V. Ravetch, Rockfeller University, NY, USA, for generously providing the FcγRIIA cDNA. We also thank Dr Susheela Tridandapani, Ohio State University College of Medicine, Columbus, OH, USA, for assistance in obtaining the FcγRIIA cDNA for this work. This study was supported by grants KH 53333 and KHC 3332, from Research on Publicly Essential Drug and Medical Devices from the Japan Health Sciences Foundation and, grants (H20-shinkou-ippan-13 and H20-shinkou-ippan-15) from Research on Emerging and Re-emerging Infectious Diseases by the Ministry of Health, Labour and Welfare, Japan.

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Text to Figures:

FIG. 1. Plaque reduction neutralizing assays using BHK-21 cells and BHK-FcγRIIA cells. DENV-1 and DENV-2 were reacted with serially diluted mouse monoclonal 3H5 antibody in PRNT assays using BHK-21 and BHK-FcγRIIA cell lines in 12-well plates.

FIG. 2. Patterns of plaque reduction against DENV in neutralization assays. (**A**) 4G2 and (**B**) 3H5. (○) Untransfected BHK-21, (▲) BHK-FcγRIIA/2, and (■) BHK-FcγRIIA/4 cell lines. Each curve is the mean of duplicate experiments.

FIG. 3. Patterns of plaque reduction against DENV-1 in neutralization assays of human serum samples. DENV-1 was reacted with human serum samples, 2-fold serially diluted from 1:5 to 1:2560. Figures are presented according to serum sample number. The characterization of samples is described in Tables 2 and 3. (○) Untransfected BHK-21, (▲) BHK-FcγRIIA/2, and (■) BHK-FcγRIIA/4 cell lines. Each curve is the mean of duplicate experiments.

FIG. 4. Patterns of plaque reduction against DENV-2 in neutralization assays of human serum samples. DENV-2 was reacted with human serum samples, 2-fold serially diluted from 1:5 to 1:2560. Figures are presented according to serum sample number. The characterization of samples is described in Tables 2 and 3. (○) Untransfected BHK-21, (▲) BHK-FcγRIIA/2, and (■) BHK-FcγRIIA/4 cell lines. Each curve is the mean of duplicate determinants.

TABLE 1. Neutralizing titers of anti-DENV monoclonal antibodies determined by assays using BHK-21, BHK- FcγRIIA/2 and BHK- FcγRIIA/4 cell lines.

Monoclonal antibodies	Challenge virus[a]	Neutralizing titers[b]		
		BHK-21	BHK-FcγRIIA/2	BHK-FcγRIIA/4
4G2	DENV-1	320	<10	<10
	DENV-2	160	<10	<10
	DENV-3	80	10	10
	DENV-4	40	<10	<10
3H5	DENV-1	<10	<10	<10
	DENV-2	40	<10	<10
	DENV-3	<10	<10	<10
	DENV-4	<10	<10	<10

Text to Table 1:

[a]DENV-1 01-44-1HuNIID strain, DENV-2 D2/Hu/OPD030NIID/2005 strain, DENV-3 CH53962 strain, and DENV-4 TVP-360 strain were used as challenged virus.

[b]$PRNT_{50}$ was determined as described in Materials and Methods.

TABLE 2. Neutralizing antibody titers of human serum samples against DENV-1 as determined using BHK, BHK- FcγRIIA/2 and BHK- FcγRIIA/4.

	Disease Phase	Serum ID#	DENV type[a]	Neutralizing titers to DENV-1		
				BHK-21	BHK-FcγRIIA/2[b]	BHK-FcγRIIA/4[c]
Primary DENV infection	Early phase[d]	1[f]	1	<5	<5	<5
		2[g]	2	<5	<5	<5
	Late phase[e]	3[f]	1	160	160	160
		4[g]	2	<20[j]	<20[j]	<20[j]
		5[h]	3	10	<5	<5
		6[h]	4	320	10	10
Secondary DENV infection	Early phase[d]	7[h]	1[i]	10	<5	<5
		8[h]	1[i]	<5	<5	<5
		9[h]	2[i]	10	<5	<5
		10[h]	3[i]	320	160	160
	Late phase[e]	11[h]	1[i]	320	40	40
		12[h]	2[i]	320	40	40
		13[h]	3[i]	160	<5	<5
		14[h]	4[i]	640	10	10
Non-DENV patient	DENV-IgG negative	15		<5	<5	<5
		16		<5	<5	<5
		17		<5	<5	<5
		18		<5	<5	<5

Text to Table 2:

[a] DENV types that infected the patients. The types of dengue virus were determined by type-specific real time RT-PCR.

[b] FcγRIIA-expressing BHK cell line-2.

[c] FcγRIIA-expressing BHK cell line-4.

[d] Days 1—3 after onset of the disease.

[e] Days 6—14 after onset of the disease.

[f] Samples #1 and #3 were obtained from the same patient infected with DENV-1.

[g] Samples #2 and #4 were obtained from the same patient infected with DENV-2.

[h] Serum samples #5—14 were obtained from patients #5—14, respectively.

[i] Dengue types of primary infection of patients #7—14 were not determined. Dengue types in secondary infection are included in the table.

[j] Serum was serially diluted 2-folds starting from 1:20.

TABLE 3. Neutralizing titers of human serum samples against DENV-2 as determined using BHK-21, BHK-FcγRIIA/2, and BHK-FcγRIIA/4.

	Disease Phase	Serum ID#	DENV type[a]	Neutralizing titers to DENV-1		
				BHK-21	BHK-FcγRIIA/2[b]	BHK-FcγRIIA/4[c]
Primary DENV infection	Early phase[d]	1[f]	1	<5	<5	<5
		2[g]	2	<5	<5	<5
	Late phase[e]	3[f]	1	160	10	10
		4[g]	2	40	10	10
		5[h]	3	20	<5	<5
		6[h]	4	40	<5	<5
Secondary DENV infection	Early phase[d]	7[h]	1[i]	5	<5	<5
		8[h]	1[i]	10	<5	<5
		9[h]	2[i]	40	<5	<5
		10[h]	3[i]	20	<5	<5
	Late phase[e]	11[h]	1[i]	<5	<5	<5
		12[h]	2[i]	80	5	5
		13[h]	3[i]	40	<5	<5
		14[h]	4[i]	320	<5	<5
Non-DENV patient	DENV-IgG negative	15		<5	<5	<5
		16		<5	<5	<5
		17		<5	<5	<5
		18		<5	<5	<5

Text to Table 3:

[a] DENV types that infected the patients. The types of dengue virus were determined by type-specific real time RT-PCR.

[b] FcγRIIA-expressing BHK cell line-2.

[c] FcγRIIA-expressing BHK cell line-4.

[d] Days 1—3 after onset of the disease

[e] Days 6—14 after onset of the disease

[f] Samples #1 and #3 were obtained from the same patient infected with DENV-1.

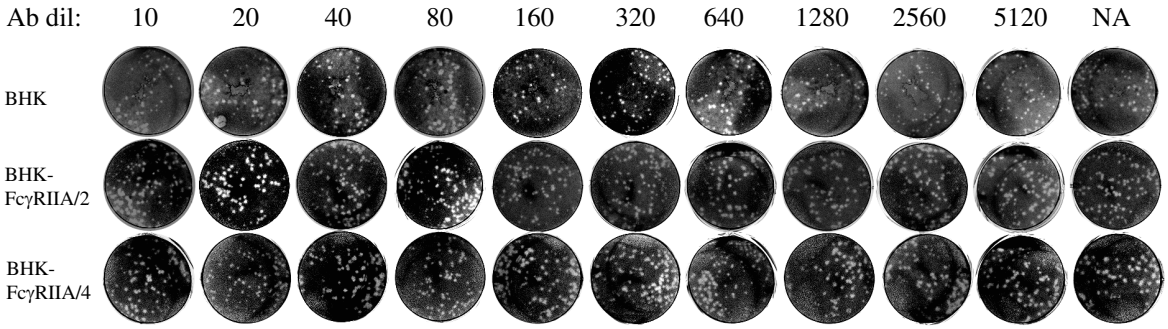
[g] Samples #2 and #4 were obtained from the same patient infected with DENV-2.

[h] Serum samples #5—14 were obtained from patients #5—14, respectively.

[i] Dengue types of primary infection of patients #7—14 were not determined. Dengue types in secondary infection are included in the table.

Figure 1

A. DENV-1



B. DENV-2

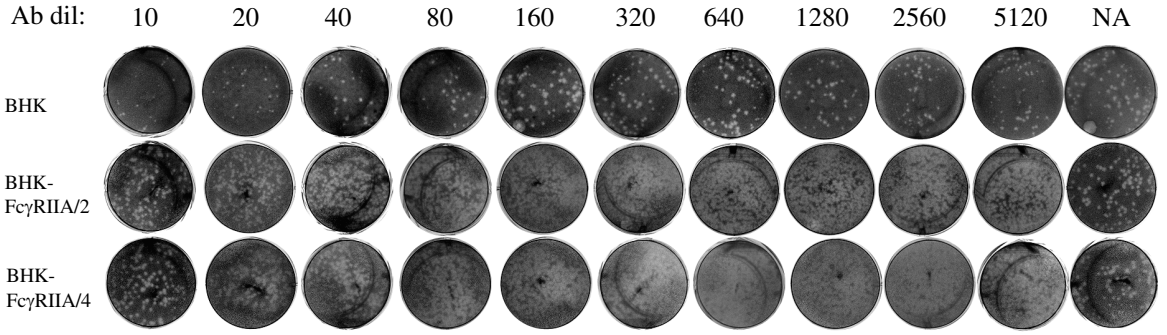
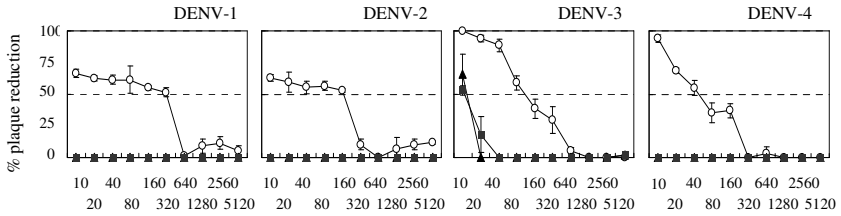


Figure 2

A. mAb 4G2



B. mAb 3H5

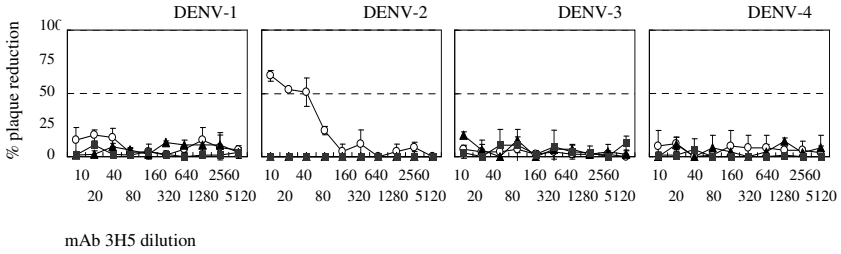


Figure 3

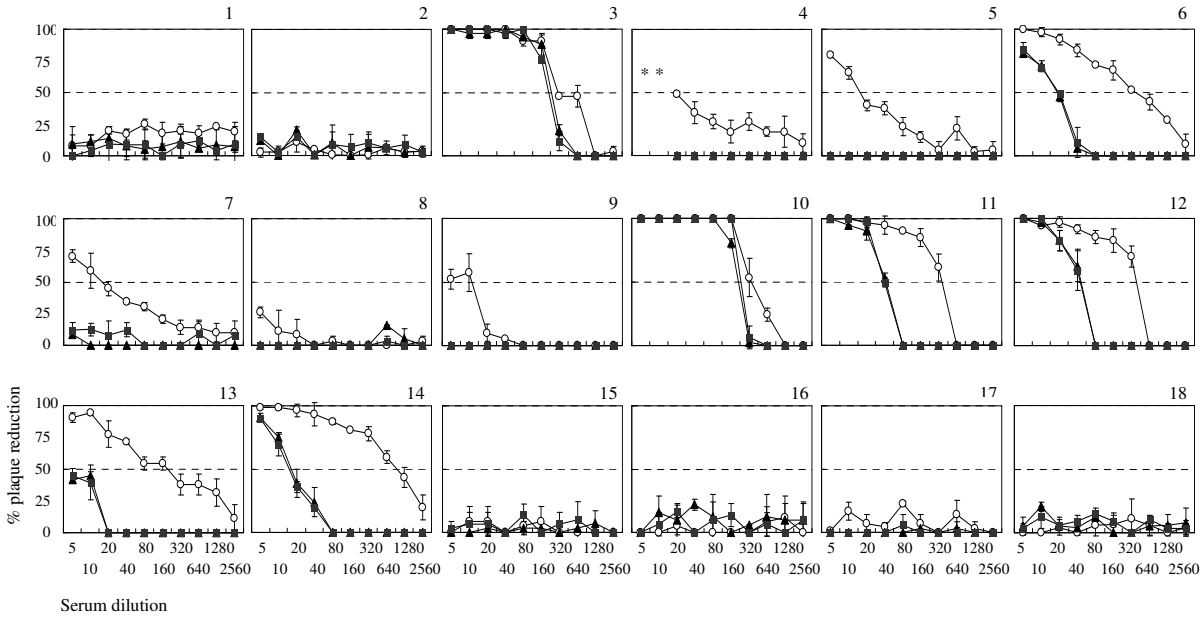
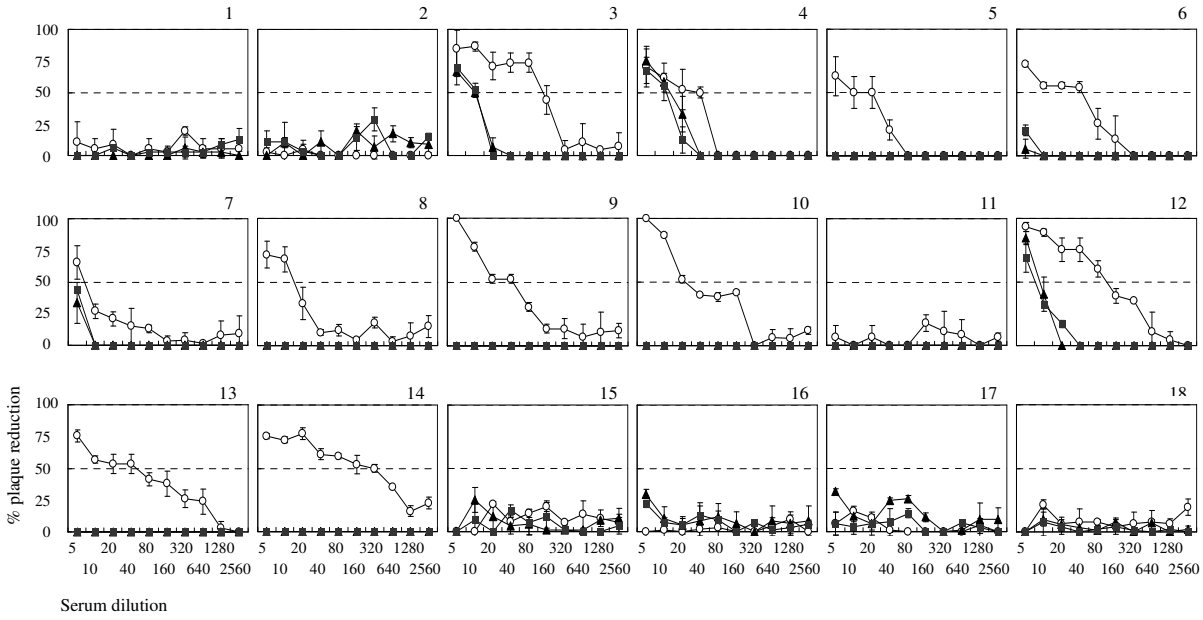


Figure 4



特集 輸入感染症の可能性がある希少感染症

1．デング熱・デング出血熱

モイ メンリン*1) 倉根 一郎*2)

デング熱・デング出血熱は，患者数，世界的な分布，重篤度等から，特に熱帯・亜熱帯地域においてはもっとも重要なウイルス感染症といえる。ワクチン開発は進んでいるものの，実用化には至っていない。近年，流行地域の拡大とともに，致死的な病態であるデング出血熱患者数の増加が大きな問題となっている。デングウイルスの日本国内への侵入は起こっていない。しかし，輸入感染症としてのデング熱・デング出血熱は年間50例以上が報告されている。デング熱・デング出血熱は輸入感染症としての対策とともに，ウイルスのわが国への侵入についても十分な対策をすべき感染症である。

Key Words：デングウイルス／デング熱／デング出血熱／ヒトスジシマカ／ネッタイシマカ

Ⅰ はじめに

節足動物媒介性ウイルスは多くのウイルスがヒトに感染し重篤な感染症を起こす。おもなウイルスとして，日本脳炎ウイルス，黄熱ウイルス，ウエストナイルウイルス，デングウイルスがあげられるが，特にデングウイルスによる感染症であるデング熱・デング出血熱は，患者数，世界的な分布，重篤度等からもっとも重要なものといえる。

Ⅱ デングウイルスと感染

デングウイルスは蚊－ヒト－蚊の感染環で維持される。ヒトはデングウイルスにもっとも感受性の高い宿主である。ネッタイシマカ（*Aedes aegypti*）およびヒトスジシマカ（*Aedes albopictus*）が主たる媒介蚊である。デングウイルスには1型～4型の4つの異なる血清型が存在する。ひとつの地域において複数の血清型のデングウイルスが存在することが多い。いずれの血清型のデングウイルスによっても同様の病態を示し，病態から感染した血清型は分からない。ある血清型のデングウイルスに感染すると，同型のデングウイルスに対する防衛免疫は終生持続するが，異なる血清型のデングウイルスに対する防衛免疫は短期間（数カ月）に消失する。他の血清型のデングウイルスによる2度目の感染が起きると，以下に述べるデング出血熱が発症する率が初感染に比べ高いことが特徴的である。

Ⅲ デング熱・デング出血熱

デングウイルスに感染した場合，多くは，不顕性感染に終わる。しかし，不顕性感染の率は明らかにされていない。単に発熱のみを症状として終わる場合もあるが，典型的な症状を示す場合，デング熱・デング出血熱と呼ばれる2つの病態を示す。世界保健機関（WHO）によりデング熱・デング出血熱の定義が示されている[1]（表1）。

1．デング熱（Dengue fever）

デングウイルス感染後5～7日の潜伏期間を経て，通常，悪寒戦慄をともなった高熱で発症する。発熱はときに二峰性を示す。また発熱前後から激しい頭痛，眼窩痛，全身の関節痛や筋肉痛，前駆

Dengue fever and dengue hemorrhagic fever
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表1　デング熱・デング出血熱の定義（文献1より改変）

デング熱
急性の熱性疾患で以下の症状のうち2つ以上存在すること。
頭痛，眼窩痛，筋肉痛，関節痛，発疹，出血傾向，白血球減少
確定診断されたデング熱患者と同時期に同じ場所で発症。
実験室診断として
デングウイルスに対するIgG抗体価がペア血清で4倍以上の上昇
デングウイルス特異的IgM抗体の存在
デングウイルス遺伝子のPCR法による検出
デングウイルスの分離
デング出血熱
発熱により発症し2～7日持続，ときに2峰性のパターンをとる。
出血傾向
Tourniquetテスト陽性
点状出血，斑状出血，紫斑
粘膜，消化管，注射部位や他の部位からの出血
血便
血小板減少（100,000/mm^{3}以下）
血管透過性亢進による血漿漏出
ヘマトリックス（Ht）の上昇（同性，同年代の人に比べ20%以上の上昇）
胸水，腹水
血清タンパク質の低下
デングショック症候群（デング出血熱でショックをともなう例）
上記デング出血熱の症状の存在に加えて
速く弱い脈拍
脈圧の低下（20mmHg未満）
低血圧
冷たく湿った皮膚，興奮状態

疹，眼結膜の充血，咽頭の発赤と痛み，白血球減少などが現れる。悪心，食欲不振，嘔気，嘔吐，全身倦怠感，腹痛をともなうこともある。第3～5病日には麻疹様発疹が躯幹から顔面や四肢に出現する場合がある。症状は1週～10日で消失し，通常後遺症なく回復する。

2．デング出血熱 (Dengue hemorrhagic fever)

デング熱と同様に発症し経過した患者において，血漿漏出と出血傾向を主症状とする病態が出現する。これらの症状は解熱時に起こることが特徴的である。血漿漏出によりヘマトクリットの上昇，胸水，腹水の貯留がみられる。肝臓の腫脹，GOT（グルタミン酸オキザロ酢酸トランスアミナーゼ），GPT（グルタミン酸ピルビン酸トランスアミナーゼ）の軽度上昇，補体の活性化によるC3減少，血小板減少（100,000/mm^{3}以下），血液凝固時間延長も特徴的である。血漿漏出が進行すると循環血液量が不足になりショックを起こすことがある。この病態はデングショック症候群（Den-

WHO（世界保健機関）
GOT（グルタミン酸オキザロ酢酸トランスアミナーゼ）
GPT（グルタミン酸ピルビン酸トランスアミナーゼ）

gue shock syndrome：DSS）と呼ばれる。デングショック症候群においては，速く弱い脈拍および脈圧の低下（20mmHg未満），低血圧を示す。デングショック症候群は適切な治療を行わないと死に至る病態である。

Ⅳ　デング熱・デング出血熱の診断

現在，日本においてはデングウイルスの国内感染はないことから，熱帯・亜熱帯から２週間以内に帰国したという情報が診断には重要である。確定診断には血清，病原体診断が必須である。①デングウイルスが分離されること，②デングウイルス遺伝子が検出されること，③特異的IgM（免疫グロブリンM）抗体が検出され，回復期において急性期に比べ上昇していること，④特異的IgG（免疫グロブリンG）抗体が検出され，急性期と回復期で４倍以上の上昇が認められることのいずれかで確定診断しうる。デングウイルス感染症では初感染，再感染いずれにおいても，有熱期にはウイルス血症が存在すると考えてよい。したがって有熱期にはウイルス分離やRT-PCR（逆転写ポリメラーゼ連鎖反応）法による遺伝子の検出が陽性となる。

血清診断としてはIgM捕捉ELISA（Enzyme-linked immunosorbent assay）法によるウイルス特異的IgM抗体の検出が広く行われている。IgM抗体の検出によって，１検体においてもデングウイルス感染を確定できるとする記述もある。しかし，デングウイルス特異的IgMは数カ月，時に半年程度持続するので，IgM抗体の検出においてもIgMレベルが急性期に比べ回復期に上昇していることを確認する必要がある。IgG抗体の検出も種々の方法によって行われている。中和法はデングウイルス特異性が高いが時間がかかるという欠点がある。赤血球凝集阻止反応（HI）法も広く行われているが，デングウイルス特異性において劣る。日本人の場合，日本脳炎ワクチンの接種等によりすでに日本脳炎ウイルスに対する免疫を有している例が多い。このような例では，デングウイルス初感染ではあってもフラビウイルス再感染としての抗体反応を示し，特にHI法，IgG-ELISA法ではデングウイルスに対する反応とともに日本脳炎ウイルスに対する抗体反応が強くみられるので，これらの検査法を用いる場合には注意が必要である。このような例においても中和抗体はデングウイルス特異性が高く，日本脳炎ウイルスに対する中和抗体価の上昇は低い。

デングウイルス再感染例でもデングウイルス特異的IgMを検出することにより診断できる。しかし，再感染の場合特異的IgMが認められない例があるので注意を要する。一方，再感染時においては感染初期からデングウイルスに対するIgG抗体が高レベルであり，IgG抗体の４倍以上の上昇によって確定診断できる。検体として血清，血漿あるいは全血が用いられ，確定診断には急性期と回復期２点の採血が必要である。但し，急性期においてウイルス分離あるいは遺伝子検出がなされた場合は１検体によっても確定診断となる。

Ⅴ　治療法・予防法

デング熱は対症療法が主体である。解熱鎮痛剤としてアセトアミノフェンが勧められる。アスピリンは出血傾向の増悪やライ症候群発症の可能性があるので禁忌である。デング出血熱では補液が治療の主体である。

デングワクチンは開発が進められているが，まだ実用化されていない。デングウイルスの流行地域に滞在する場合には，蚊に刺されることを避けることが重要である。肌の露出をさけること，忌避剤を適切に使用することが重要である。幼児のベビーカーやベビーキャリアに防蚊ネットを使用し，蚊に刺されることを避けるのも重要である。また，ネッタイシマカは夜明けから数時間，夕方の数時間がもっとも活動性が高いことも考慮し行

DSS（Dengue shock syndrome；デングショック症候群）
IgM（免疫グロブリンM）
IgG（免疫グロブリンG）
RT-PCR（逆転写ポリメラーゼ連鎖反応）
ELISA（Enzyme-linked immunosorbent assay）

動する必要がある。

Ⅵ　デング熱・デング出血熱の流行状況

世界的な報告数としては年間100万～150万人であるが，実際には数千万人がデング熱を，また約50万人がデング出血熱を発症していると推察されている。デング出血熱患者の致死率が約1～5％であることからも，デング熱・デング出血熱は世界的にもっとも重要なウイルス感染症のひとつといえる[2]。1950年代にはデング熱の流行地域は数カ国に限られていたが，今日70カ国以上がデング流行国となっている（**図1**，**図2**）。現在，日本において国内感染はないが，流行地域への海外渡航者の増加にともない，帰国後発症する例も増加傾向にある（**図3**）。

近年アジアを始めとし，世界各地においてデング熱の大きな流行が起こっている。シンガポールでは2004～2005年にかけてデングウイルス1型による大きな流行があった。2005年には13,000人の患者と19人の死亡が報告された。2007年にはデングウイルス2型ウイルスによる流行が始まり，約4,000人の患者，8人の死亡が

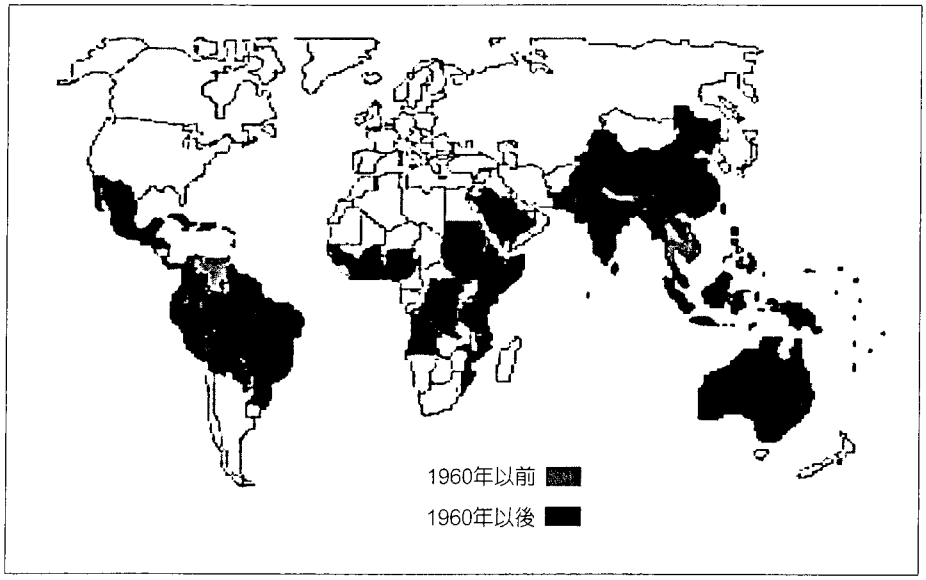

図1　世界におけるデングウイルス分布拡大（世界保健機関より改変）
1960年以降，デングウイルスは世界の熱帯・亜熱帯地域に分布を拡大している。

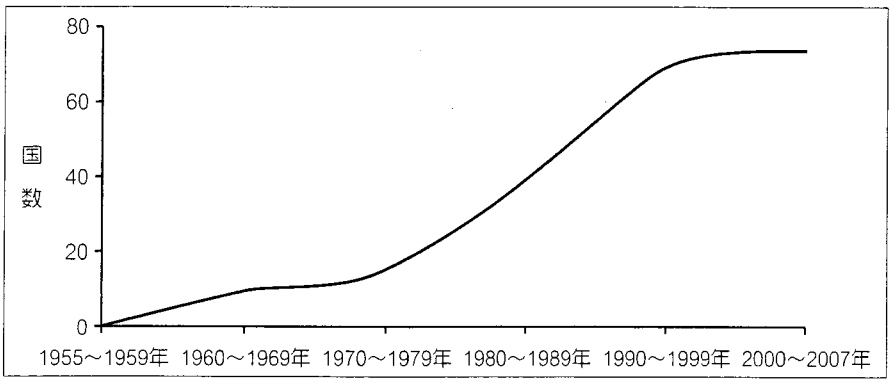

図2　デング患者の国内発生を報告している国数
デングウイルスの国内感染が報告される国数もデングウイルスの分布地域拡大にともない増加している。

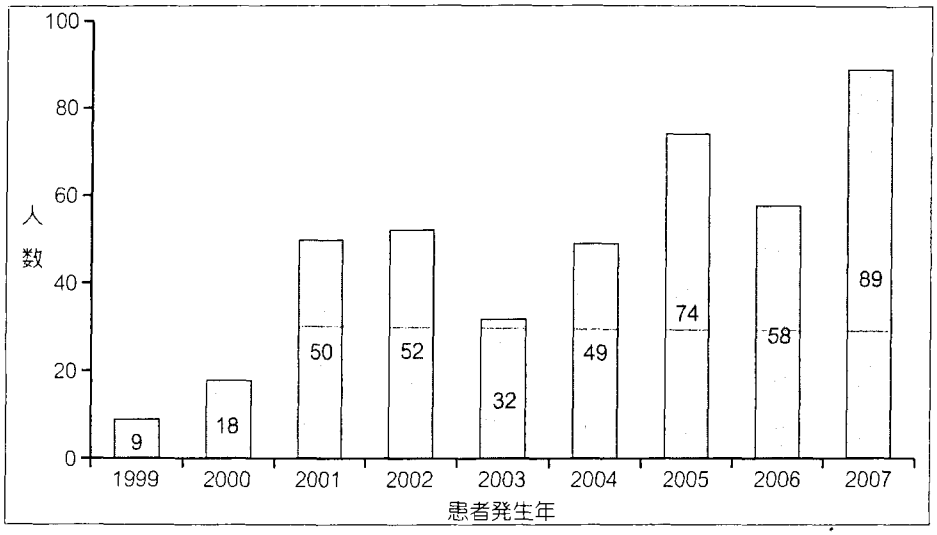

図3　日本におけるデング熱報告患者数推移（1999～2007年）
わが国で近年報告されたデング熱・デング出血熱患者はすべて輸入感染例であるが，数は増加傾向にある。

報告された。2008年の前半までにすでに2,087人の患者が報告されている。

タイのバンコクにおいては2008年の前半ですでに1,966人の患者が報告された。タイ全土におけるデング熱の患者数は前年と比べて72%増加し，2008年7月までにデング熱が22,639人，デング出血熱が30,102人，デングショック症候群が814人と報告された。うち65人が死亡している。

インドネシアにおいては2006年の報告患者数114,656人で死亡1,196人であった。カンボジアでも2007年に大きな流行が発生し，約4万人の患者が報告され，うち407人が死亡した。ベトナムでも2007年，8万人を超える患者が確認された。ハノイではデング患者数が前年同時期に比べて患者数で70%増加した。

台湾においては2002年に15,221人の患者報告があり，うち242人はデング出血熱であった。流行は南部の高雄市を中心に発生した。この大流行後，徹底的な媒介蚊の駆除対策が実施され，2003年にはデング熱の報告数は1,583人（デング出血熱は2人）に減少した。しかし，デングウイルスの活動を完全に押さえ込むことは困難であり，2004年の報告数は1,421人，2005年は1,083人，2006年は2,465人であった。2008年7月時点で台湾におけるデング熱患者は輸入例が60人，国内感染が24人であった。

一方，南米においても大きな流行が起こっている。ブラジルでは2007年におけるデング熱患者報告数は約54万人であり，2002年と比べておよそ2倍となった。2008年前半，デングウイルスの大流行があり，地域により前年の約12倍にものぼるデング熱症例が報告された。2008年の1月から4月までのデング熱の報告数はブラジル全土で約12万，このうち647人はデング出血熱であり48人が死亡した。深刻なデングウイルスの拡大のため，ブラジル国内50地域で非常事態が宣言された。

Ⅶ　日本におけるデング熱患者

上述のように，現在わが国においては国内にデングウイルスは存在せず，国内感染はない。しかし，海外において感染した，いわゆる輸入症例は毎年50～100人が報告されており，近年増加傾向にある（**図3**）。輸入例の訪問先はアジアが多いが，南米，オセアニア，アフリカからの帰国例もみられる。したがって，デング熱・デング出血熱は熱帯・亜熱帯地域から帰国した有熱患者の鑑別

疾患として重要なものといえる。

Ⅷ　デング出血熱の病態形成

デングウイルスの感染により，なぜある患者はデング熱で終わり，ある患者はデング出血熱を発症するかは明らかにされていない。現在，デング出血熱は強毒性のデングウイルスの感染によるとする説と，再感染時における免疫応答による感染の増強によるとする説が考えられている。過去この2説は相対立するものとして捕らえられてきたが，近年はむしろそれぞれ病態形成の一面を捉えていると考えられている。

1．強毒性のデングウイルスの感染によるとする説

デングウイルス株にはいわゆる毒性の強いものと弱いものがあり，強毒株はデング出血熱を，弱毒株はデング熱を起こすというものである。この説を支持するデータとして，1歳以上の小児の初感染においてもデング出血熱が発生すること，すなわち以下に述べる感染増強抗体，免疫の関与がない状況でもデング出血熱となりうることがある。分子疫学的にこの説を支持するデータとして以下のようなものがある。従来，南米にはデングウイルス2型は存在したが，デング出血熱の発生はみられなかった。しかし，南米においてもデング出血熱の発生が報告されるようになり，デング出血熱を起こしているウイルスを解析してみると東南アジア由来のデングウイルス2型であった。さらに，タイにおいてはデングウイルス2型と4型によるデング出血熱は再感染であるが，デングウイルス1型と3型によるデング出血熱のかなりの割合は初感染によって起こっている。したがって，明らかにウイルス株による病態の重篤度の違いが生じている。いわゆる強毒性という言葉がウイルスのどのような性質を反影しているかについては現在でもまだ明らかではないが，強毒性はヒトにおけるウイルスの高い増殖性によるという理解が一般的である。

2．再感染時における免疫応答による感染の増強によるとする説

特にタイでの疫学的研究に基づく。タイにおいて1964年デング出血熱患者の人，1,000人当たりの発生率を調べるとその曲線は二峰性となる。第1のピークは生後8カ月，第2のピークは4歳であった。2つのカーブの谷は生後約12カ月であった。興味深いことに，1歳以上のデング出血熱患者のほとんどは再感染による患者，1歳未満の患者のほとんどは初感染の患者であった。さらに，この1歳未満の患者のほとんどはデングウイルス抗体を有する母親から生まれていた。1歳以上でデング出血熱患者の多くが再感染であるというデータは他の調査においても報告されている。例えば，キューバでも大人において発生したデング出血熱患者の多くが再感染であったことが報告されている。以上のようなデータに基づき，デング出血熱は母子移行抗体あるいは初感染時に誘導された中和能を有しない交叉抗体が，感染時（1歳未満では初感染，1歳以上では再感染），特に単球・マクロファージ等IgG-Fcレセプターを有する細胞のデングウイルス感染を増強させることがデング出血熱の基盤であると解釈された。この感染増強抗体については，特に1歳未満の小児において母親の血中デングウイルス抗体価が高いほどデング出血熱を発症する月数が遅いというデータがあり，これは母子移行中和抗体のレベルが減少し，中和能を失いデングウイルス感染を増強させうる状態になるまでに，より長い時間を必要とすると解釈されている。

上述の2つの説として示されているように，デングウイルス自体が持つ増殖性の高さ，あるいはさらに感染増強抗体によるウイルス価の上昇が病態形成の基礎となっているとしても，ウイルスの増殖性の高さ，ウイルス価の上昇がどのような機序によって血漿漏出や出血傾向というデング出血熱に特徴的な病態形成に結びつくかは解明されておらず，今後の重要な研究対象となっている。

Ⅸ　おわりに

デング熱・デング出血熱は熱帯・亜熱帯地域においてもっとも重要な感染症のひとつといえる。今後，ワクチンが実用化され，世界各地で使用されるようになれば，デング熱・デング出血熱の状

況は大きく様変わりすると思われる。しかし，現在の状況ではワクチンの実用化には5年以上が必要であると考えられることから，ベクター対策，啓発による個人の防御を一層進める必要がある。尚，デング熱・デング出血熱は「感染症の予防及び感染症の患者に対する医療に関する法律（感染症法）」において，四類感染症に指定されており，本感染症を診断した医師はただちに保健所長を経由し都道府県知事に届け出なければならない。

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Review:

Arbovirus Infections: the Challenges of Controlling an Ever-Present Enemy

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[Received July 8, 2009; accepted July 30, 2009]

Arthropod-borne infections carried by mosquitoes and ticks are difficult to eradicate, once rooted, and have frequently caused wide-area epidemics such as dengue fever, West Nile fever, chikungunya fever, yellow fever, Japanese encephalitis and Rift Valley fever. Factors such as global warming and overpopulation have aggravated urban epidemics caused by dengue and chikungunya viruses. Measures against arthropods have their limitations, however, so nonepidemic areas must be protected against invasion by vector-borne diseases through quarantine, education and effective vaccination.

Keywords: arbovirus, vector, vaccine, flavivirus, alphavirus

1. Introduction

Infectious diseases transmitted by mosquitoes and ticks may appear less dangerous than those spread from human to human, but once a vector-borne disease enters a nonepidemic area, the pathogen becomes difficult to eradicate. Viruses transmitted by arthropod are generically called arthropod-borne viruses, or arboviruses. This is not a virological classification, but refers to the common feature of these diseases being transmitted by arthropod to vertebrates. Representative diseases caused since the beginning of this century include dengue fever, West Nile fever, chikungunya fever, yellow fever, Japanese encephalitis and Rift Valley fever, many of which are occurring more often in wider areas.

2. Arboviral Febrile Diseases

Febrile diseases caused by arboviruses include dengue and chikungunya fever, both being transmitted by the *Aedes aegypti* and *Aedes albopictus* mosquito and forming a human-mosquito-human transmission cycle. Rift Valley fever is closely associated with weather conditions such as rainfall.

2.1. Dengue Fever

Dengue virus found in most tropical and subtropical areas has caused repeated outbreaks in Southeast Asia, South Asia, and Latin America. The latest, in Brazil, affected 410,000 persons in 2001 and 780,000 in 2002. In Southeast Asia, outbreaks infecting over 100,000 have occurred annually in Thailand, Indonesia, Vietnam, and the Philippines. Singapore, one of Southeast Asia's most modern cities, has taken all-out measures against the mosquito vector, but has not eradicated *Aedes aegypti*, with the dengue outbreaks since 2004 annually infecting 100 to 400 patients [1]. Areas of outbreak are expanding, with dengue in southern Taiwan infecting 15,000 persons in 2002. Dengue outbreaks have also occurred in Nepal [2]. Tropical malaria is decreasing in some epidemic areas because of economic growth enabling people living in epidemic areas to move into urban areas.

2.1.1. Infectious Agents

(1) Viral structures and features

Dengue virus, yellow fever virus, and Japanese encephalitis virus belong to genus *flavivirus* having an envelope 40 to 60 nm in diameter and a core 30 nm in diameter. The genome is a single-strand ribonucleic acid (RNA) [3]. It causes relatively higher viremia than Japanese encephalitis and West Nile viruses during the acute phase [4]. Dengue viruses are classified type 1 to 4 having a partially common antigenicities showing serological cross-reactions but less protective against other type infections. A person infected with the type 1 virus will have permanent immunity to homo type, for example, but immunity to cross-protections against other types will disappear in several months, meaning the person may soon be infected by another type.

(2) Vector mosquito and transmission cycle

The mosquito-human-mosquito transmission cycle forms in human outbreak. The virus may cause limited viremia in an infected monkey but the simian rarely develops the disease, which human beings are much more sensitive to. The only amplifier in dengue transmission is the

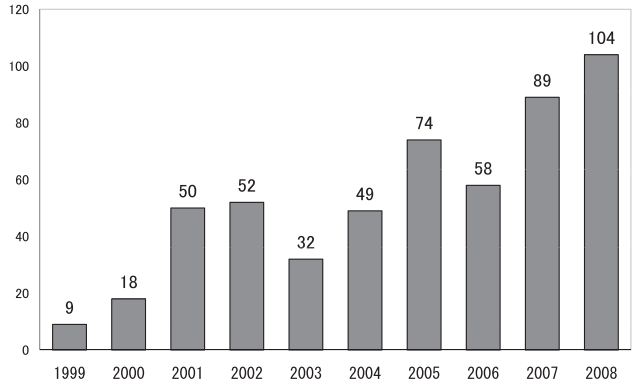

Fig. 1. Imported dengue trends in Japan. The reported number of patients has increased since 1999. The decreased number in 2003 was due to SARS decreasing the number of overseas tourists.

human being, unlike in Japanese encephalitis transmission, in which swine operate as the amplifier. Although *Aedes aegypti* is the major vector mosquito, *Aedes albopictus*, now widespread in Japan, can transmit the virus and now has a northern range extending to Akita and Aomori Prefectures because of global warming [5].

2.1.2. Dengue as an Imported Infection

No dengue onset has yet been reported in Japan after World War II, but imported cases numbered 32 in 2003, 49 in 2004, 74 in 2005, and 104 in 2008 (**Fig. 1**) [6] because of the increase in Japanese tourists traveling to endemic tropical and subtropical areas. According to Japan Association of Travel Agents statistics, Japanese overseas tourists numbered 1,740,000 in 2005, of whom 1,100,000 traveled to Asian areas having dengue epidemics [7]. Dengue fever is an especially high infection risk because tourists may be infected in urban and resort areas, unlike malaria, whose endemic areas are relatively limited.

2.1.3. Clinical Symptoms of Dengue Fever and Dengue Hemorrhagic Fever

1) Dengue fever

Most dengue patients show nonfatal, acute febrile symptoms called dengue fever, whose main symptoms are fever, skin rash, muscular and arthritic pain. The patients have sudden fever onset after 3 to 14 days incubation period, which is often accompanied by headache, postorbital (eye socket) pain, and muscular and arthritic pain and sometimes by digestive organ disorders such as appetite loss, abdominal pain, astriction, and diarrhea. Maculopapular rashes appears after 3 to 6 days after onset, spreading from the chest and trunk to the extremities and face. These acute symptoms disappear in 7 to 10 days, normally without after effects.

2) Dengue hemorrhagic fever

Dengue virus, which may cause dengue hemorrhagic fever in 0.3 to 0.5% of dengue fever patients, features blood plasma leakage and hemorrhage, typically while the fever is subsided. Patients suffer extreme anxiety, excitation, heavy sweating, cold extremities, and pleural effusion and ascites. Other symptoms and signs include liver bloating, activated complements, blood platelet often decreases to 100,000/mm^3 or less, and prolonged blood clotting time. Petechia is observed in many cases, with nasal bleeding and throat hemorrhage observed in 10 to 20% of cases involving plasma leakage that may develop into hypovolemic shock resulting from a blood shortage, also called dengue shock syndrome. Dengue hemorrhagic fever may fatal unless adequately treated. The hemorrhagic fever occurs more often in reinfection with another dengue virus type than in initial infection.

2.1.4. Dengue Fever and Environments

Northeastern Brazil was hit by dengue fever in the first half of 2008, with patients numbering over 12 times that of the previous year. The epidemic expanded due to increased breeding sites for vector mosquitoes due to abnormal rainfall and global warming. Of 120,000 dengue fever patients in Brazil, 647 patients were diagnosed with hemorrhage killing 48. Dengue fever epidemics are not always related to weather. Catch basins for rainwater are less contaminated with daily life water because of improved infrastructures for urban functions and sewage systems. Those catch basins provide breeding sites for *Aedes albopictus* and *Aedes aegypti* vector mosquitoes (**Fig. 2**). Dense urban overpopulation due economic growth also provides breeding sites the mosquito-human-mosquito transmission cycle.

2.1.5. Difficulty in Dengue Vaccine Development

Live attenuated dengue vaccines have been developed for their inexpensiveness because of economic conditions in the epidemic developing countries There are four dengue virus types, and vaccine developed against one type works only poorly against another type, with inadequate vaccine use increasing the risk of hemorrhage and shock syndrome when the infection reemerges. A vaccine must therefore induce antibodies effective against all four types, which has greatly retarded vaccine development. Pediatric vaccination must provide at least antibodies effective against all 4 types to get consent from guardians. There are problems in where the clinical trials can be conducted. Laboratory diagnosis and surveillance systems should be established there. Another problem is whether the vaccine targets dengue fever or dengue hemorrhagic fever.

2.2. Chikungunya Fever

An arbovirus attracting much attention since 2005 causes chikungunya fever, which was epidemic as of 2009 in Singapore, Malaysia, Thailand, Indonesia, India, Sri

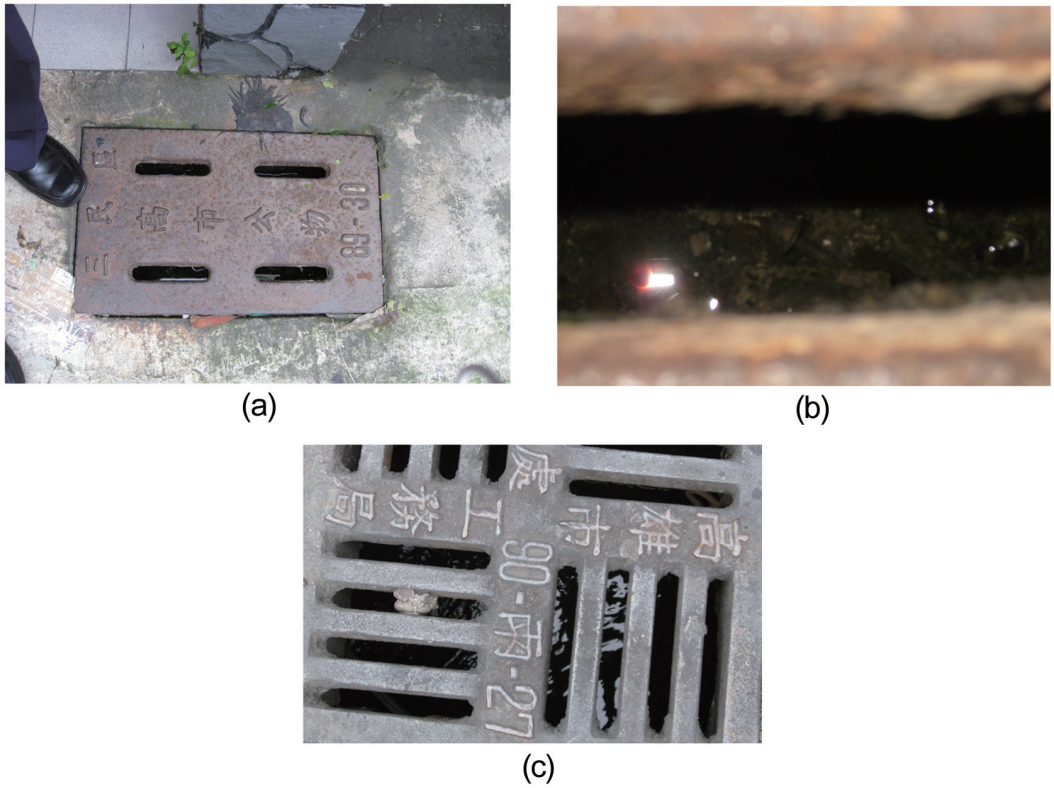

Fig. 2. Street near around dengue endemic area in Kaohsiung City. Catch basins for rainwater have been less contaminated with daily life water in Taiwan as water and sewage systems have developed, providing Aedes aegypti and Aedes albopictus breeding sites. (a), (b) Rain water is not flowing: good breeding place for *Aedes mosquitoes*. (c) Rain water is flowing, *Aedes mosquitoes* can not breed.

Lanka, the Maldives, and Pakistan. In Malaysia, epidemics in 2008 spread from Johor Province, near Singapore to nationwide, with victims numbering 4,271 in 2008, and 2,687 in the first 23 weeks of 2009. The Singapore Ministry of Health announced 299 chikungunya fever patients in just over the first 4 months of 2009. The epidemic apparently originated in the Union of the Comoros at the start of 2005 [8]. The epidemic dates back to 2004 when it first was observed on Lamu Island, Kenya [9], later spreading to other Indian Ocean islands, including Mauritius, Reunion, the Seychelles, and Mayotte. Reunion patients numbered 240,000 patients from March 2005 to February 2006, resulting in encephalopathy and hepatitis and 237 deaths. Some 490,000 cases were reported an epidemic in western Indian Ocean countries in 2004 to 2006. Another epidemic also occurred in western India and Sri Lanka in 2006, and imported cases were reported in Hong Kong, Taiwan, the US, Singapore, Australia, and Japan [10].

2.2.1. Chikungunya Fever Epidemic in Italy

Two areas of northern Italy hit by a chikungunya fever epidemic in July 2007, with patients rapidly increasing in August, was probably imported from India by tourists, and the vector mosquito was *Aedes albopictus*, which also live in Japan. Laboratory diagnoses were implemented for 284 of 334 suspected cases, and 204 were confirmed to be chikungunya fever virus, with one death occurring [11]. This was the first chikungunya fever epidemic in the Temperature Zone, and confirmed that chikungunya fever epidemics can occur in any area inhabited by *Aedes albopictus*.

2.2.2. Infectious Agents

(1) Viral structures and features

Alphavirus can be grouped into 7 antigen groups, of which chikungunya virus belongs to the Semliki Forest antigenic complex. The spherical virus is 70 nm in diameter, consisting of a spiked envelope enclosing a nucleocapsid. The virus is stable at pH 7 to 8, and readily inactivated in an acidic condition. The virus is divided to 3 genotypes – Central/East African, Asian, and West African with major epidemics caused by the Central/East African genotype. The epidemic strain reportedly caused 237 deaths in Reunion, and is more pathogenic than previous ones. An alanine residue at residue 226 in the E1 envelope glycoprotein shifted to a valine residue (A226V), leading to increased virus transmission with more severe nonconventional symptoms [11, 12]. A226V was observed in over 90% of subsequent viral sequences in Reunion [12, 13].

(2) Transmission cycles

The human-mosquito-human urban transmission cycle involves *Aedes aegypti* or *Aedes albopictus* as the vector, and is observed in Africa and maintained among Aedes mosquitoes, wild primates, rodents, and chiroptera (bats) [14]. Viremia in human is at least equivalent to that caused by dengue virus, with very similar clinical symptoms, clearly manifesting arthritis with arthritic pain and swelling. Arthritic pain may last several months after acute symptoms are subsided. Blood platelets generally decrease less than in dengue fever.

2.3. Rift Valley Fever

Rift Valley fever in sub-Saharan countries, which spread to Egypt and the Arabian Peninsula [15], is transmitted by mosquitoes, and outbreaks are aggravated by cud-chewers such as sheep, goats, cattle, and camels. Humans are infected both by mosquitoes and domestic animals through the skin or respiratory passages, e.g., during herding. Vector mosquitoes are Aedes family *mcintoshi, vexans, palpalis, and circumluteolus*. Rift Valley fever virus remains dormant in the infected mosquito eggs and female mosquitoes become vectors when hatched in rain, making epidemics closely related to rainfall [16].

2.3.1. Clinical Symptoms

The incubation period is 2 to 6 days. Patients get high fever with chill and headache suddenly. Other symptoms are muscle pain, nausea, and dizziness. The fever is subsided in 2 to 6 days but frequently causes ocular symptoms leading to decreased vision or evanescent or irreversible blindness. It may cause meningitis, encephalitis, and hemorrhagic fever.

2.3.2. Infectious Agents

The virus belongs to genus *Phlebovirus*, *Bunyaviridae.* The virions are spherical or polymorphic. It has a 3-segmented negative-strand RNA genome and is 80 to 120 nm in diameter. Live attenuated-vaccines are used for domestic animals in Africa.

3. Arbovirus Encephalitis

Arboviral encephalitis shows similar symptoms. Image-aided diagnosis and or examination show peculiarities in some cases, and laboratory diagnosis is essential for differential diagnosis. No specific treatment exists for arboviral encephalitis, with vaccination and development/production essential. West Nile virus and Japanese encephalitis virus belong to genus *Flavivirus, Flaviviridae*. Those are virologically similar to dengue and yellow fever viruses.

3.1. West Nile Fever/Encephalitis

West Nile virus is a member of the Japanese encephalitis antigen complex of genus *Flavivirus* together with Japanese, St. Louis, and Murray Valley encephalitis virus [3]. West Nile virus usually causes West Nile fever as a nonfatal febrile disorder in Africa, Europe, western Asia, and Middle East since 1937, when it was discovered in Uganda. It may sometimes cause encephalitis, and has spread since 1994, when Algeria was hit by a fever causing 50 cases and one death, including Rumania (1996 to 1998), the Czech Republic (1997), the Republic of Congo (1998), Tunisia (1997 and 2003), France (2000), Israel (1997 to 2000), Georgia (1998), Russia (1999 to 2004), the US (after 1999), Canada (after 2002) and Mexico (2003) [17]. In 1999, the epidemic occurred the first time in the Americas in New York. The virus is now rooted in North America, causing epidemics of several thousand patients annually. The virus has already invaded South America, although most victims are in North America, and shows no signs of disappearing. West Nile virus is transmitted by mosquitoes and amplified in birds. The bird-mosquito-bird transmission cycle may be transmitted to humans and horses, developing into encephalitis. The virus is noted for being transmitted by a wide variety of mosquitoes, so a major measure for reducing risk of infection with the virus is protection of persons and animals against mosquitoes. Measures taken by individuals, e.g., wearing long sleeves and pants and being sprayed with repellents, are important, as is the spread of awareness, e.g., for reducing breeding sites for mosquito. Inactivated vaccines have been produced commercially for horses to reduce the number of cases, but those for human beings remain yet to be commercialized.

3.2. Japanese Encephalitis

Active region of Japanese encephalitis virus is also expanding. An epidemic in 1995 in the Torres Strait Islands of Australia was confirmed to have invaded Cape York Peninsula, Queensland, in northern Australia [18, 19]. Unlike West Nile virus invading the US, Japanese encephalitis virus has shown no sign of spread nationwide in Australia. Only one strain was isolated from 1998 to 2004 by *Culex sitiens*, a major Australian vector mosquito, although 66 virus strains were isolated on Badu Island in the Torres Strait Islands [20, 21, 22]. While it has caused no serious health-related problems in Australia as such, arbovirus distribution is not well known, although it is considered to be affected by weather, arthropod vectors, and amplifiers.

4. Yellow Fever

Yellow fever virus, also a member of the mosquito-borne genus *Flavivirus*, takes 4 to 10 days to grow in infected mosquitoes, not being infectious before that. Infected mosquitoes remain infectious as long as they live. "Flavi" comes from "flavor," meaning yellow in Latin, and yellow fever virus is representative of the family. Endemic areas include the tropical zone in Africa between the northern and southern 15th parallels. In the American

Fig. 3. Endemic yellow fever areas in South America. Endemic areas spread to Uruguay and Paraguay and part of Argentina between these countries.

tropical zone, endemic areas range from Panama to the southern 15th parallel, and epidemics occur often in the rainy season. The virus has been active in South America since 2008 – endemics occurring both in Brazil and Paraguay and in northern Argentina – confirming that the virus has invaded into Argentina, as shown in **Fig. 3**. Clinical symptoms are sudden fever and headache after 3 to 6 days of incubation, followed by nausea, vomiting, conjunctival congestion, and protein urea, usually lasting 1 to 3 days. The clinical course of severe yellow fever is clearly divided into 3 stage; infection, symptom relief for several hours only, and intoxication. Onset starts with sudden headache, dizziness, and fever. Bradycardia with a low pulse of 48 to 52 per minute and high temperature is an early symptom known as Faget's sign occurring by the second disease day. Other symptoms include vomiting, conjunctival congestion, facial flushing, and delirium.

5. Conclusions

Epidemic areas of mosquito-borne viral infection have been expanding, and global warming provides breeding sites for vector mosquitoes. Not all vector-borne diseases are, however, such as tropical malaria, which is no longer epidemic in urban areas in Southeast Asia because of improved economic conditions. Infectious diseases such as dengue and chikungunya fever, however, involve urban breeding sites where vector mosquitoes form human-mosquito-human transmission cycles. West Nile virus spreads easily, transmitted by a variety of mosquitoes. Insect-borne infectious diseases are difficult to eradicate once they invade nonepidemic areas. Measures against vector insects are effective but have limitations, and nonepidemic areas must be protected against invasion by vector-borne diseases.

Acknowledgements

This work was partly supported by grants for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health and Walfare of Japan (H20-Shinko-ippan-015) and the Global Environment Research Fund (S-4) of the Ministry of the Environment, Japan.

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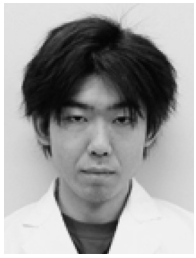

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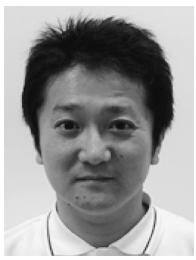

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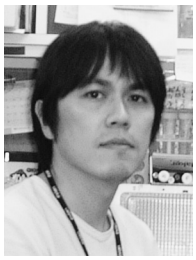

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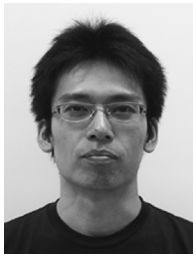

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