



สำนักงานกองทุนสนับสนุนการวิจัย

The Thailand Research Fund

รายงานวิจัยฉบับสมบูรณ์

**โครงการ บทบาทของ NSI โปรตีนของไวรัสไข้เลือดออกกับพยาธิกำเนิดของ
ไข้เลือดออก**

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คณะผู้วิจัย

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

สาเหตุหลักที่ทำให้เกิดการเสียชีวิตในโรคไข้เลือดออกคือภาวะเลือดออกและการรั่วของพลาสมาจำนวนมากออกนอกหลอดเลือดแบบเฉียบพลัน ซึ่งในกลุ่มผู้ป่วยมีอาการดังกล่าวนี้จะพบว่าการกระตุ้นระบบคอมพลิเมนต์ที่รุนแรงเกิดขึ้นร่วมด้วย แต่พยาธิกำเนิดของโรครวมถึงสาเหตุที่ทำให้เกิดการกระตุ้นระบบคอมพลิเมนต์ยังไม่เป็นที่ทราบแน่ชัดจึงเป็นที่มาของงานวิจัยชิ้นนี้เพื่อศึกษาบทบาทของโปรตีน NS1 ต่อพยาธิกำเนิดของโรค โปรตีน NS1 เป็นโปรตีนที่มีขนาดโมเลกุลประมาณ 45 kDa ซึ่งเป็นโปรตีนที่ไม่เป็นส่วนประกอบของตัวไวรัสแต่เมื่อไวรัสเข้าสู่เซลล์จะมีการสร้างโปรตีนชนิดนี้ขึ้นภายในเซลล์ แล้วจะถูกปล่อยออกมานอกตัวเซลล์ที่ติดเชื้อเป็นจำนวนมาก จากการศึกษาพบว่า NS1 เป็นโปรตีนที่สามารถกระตุ้นระบบภูมิคุ้มกันให้สร้างแอนติบอดีได้ และยังพบว่าในพลาสมาของคนไข้ที่ติดเชื้อเด็งกีจะมีปริมาณ NS1 ที่ถูกสร้างและปล่อยออกมาจากเซลล์ที่ติดเชื้อมีความสัมพันธ์กับความรุนแรงของโรค ดังนั้น NS1 น่าจะเป็นโปรตีนที่สำคัญตัวหนึ่งที่มีบทบาทเกี่ยวข้องกับพยาธิกำเนิดของโรคไข้เลือดออก

ในงานวิจัยนี้เป็นครั้งแรกที่ศึกษา ความสามารถในการกระตุ้นระบบคอมพลิเมนต์ของมนุษย์โดยโปรตีน NS1 ของไวรัสเด็งกี ซึ่งจากการศึกษาพบว่า membrane associated NS1 บนผิวของเซลล์ที่ติดเชื้อเด็งกีมีความสามารถในการกระตุ้นระบบคอมพลิเมนต์ในสถานะที่มีแอนติบอดีที่จำเพาะต่อโปรตีน NS1 โดยผ่านทาง Classical และ Alternative pathway ซึ่งผลการทดลองได้ถูกยืนยันโดยใช้ cells stably expressing NS1 นอกจากนั้นในสถานะที่ไม่มีแอนติบอดีพบว่า purified soluble NS1 มีความสามารถกระตุ้นระบบคอมพลิเมนต์ได้ และพบว่า การกระตุ้นระบบคอมพลิเมนต์ของทั้ง membrane associated และ soluble NS1 สามารถเกิดขึ้นไปจนถึง terminal pathway คือสามารถตรวจพบ C5b-9 ที่เกิดขึ้นบนผิวเซลล์ และ SC5b-9 โดยวิธี ELISA นอกจากนั้นยังพบว่าระดับของโปรตีน NS1 และ SC5b-9 สูงในเลือดของผู้ป่วย และยังพบว่ามีความสัมพันธ์กับระดับความรุนแรงของโรค และสามารถตรวจพบทั้ง NS1, C5a และ SC5b-9 ปริมาณมากในน้ำจากช่องปอดของผู้ป่วยที่มีอาการรุนแรง ดังนั้นความสามารถในการกระตุ้นระบบคอมพลิเมนต์ของโปรตีน NS1 น่าจะมีผลต่อกระบวนการของพยาธิกำเนิดอันส่งผลทำให้เกิดภาวะช็อกและการรั่วของพลาสมาออกนอกหลอดเลือดซึ่งจะพบในคนไข้ที่มีอาการรุนแรง และ การตรวจวัดทั้งระดับ NS1 และ SC5b-9 อาจจะใช้ช่วยในการให้การวินิจฉัยและพยากรณ์ความรุนแรงของโรคได้

ABSTRACT

Severe vascular leakage and shock are the major causes of death in patients with dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS). Complement activation was proposed to be a key underlying event 30 years ago, but the cause of complement activation has remained unknown to the present day. The major nonstructural-protein NS1 of dengue virus (DV) was tested for its capacity to activate human complement in its cell-bound and soluble form. Plasma samples from 163 patients with DV-infection and from 19 patients with other febrile illnesses (OFI) were prospectively analyzed for levels of viremia, NS1, and complement activation products. Blood and pleural fluids from 9 patients with DSS were also analyzed. We discovered that soluble NS1 activated complement to completion, and activation was enhanced by polyclonal and monoclonal antibodies against NS1. Complement was also activated by cell-associated NS1 in the presence of specific antibodies. Plasma levels of NS1 and terminal SC5b-9 complexes correlated with disease severity. Large amounts of NS1, complement anaphylatoxin C5a and the terminal complement complex SC5b-9 were present in pleural fluids of DSS patients. The present study provides the link between NS1 load, complement activation and plasma leakage in severe DV-infection. High concentrations of complement activation products may be directly responsible for vascular leakage occurring in DHF/DSS patients. Massive complement activation in DV infections is triggered by NS1 both on cell surfaces and in the circulation. Measurements of NS1 and SC5b-9 in plasma may render it possible to identify patients at risk of developing vascular leakage and shock.

EXECUTIVE SUMMARY

Project Summary

This project has been supported from Thailand Research Fund (TRF) starting from July 1, 2003 to June 30, 2005 with the total period of 2 years. It aimed towards the study of the contribution of dengue nonstructural protein-1 (NS1) to the pathogenesis of severe dengue infection, dengue hemorrhagic fever or dengue shock syndrome (DHF/DSS). Accelerated complement consumption and marked reduction of plasma complement components were observed in DSS patients during shock since thirty years ago, which led to the assumption that complement activation plays an important role in disease pathogenesis. In the following decades the thrust of international research shifted towards the possible role of lymphocytes and cytokines, and the significance of complement receded to the background. Thereby, the important issue regarding the cause of complement activation has remained untouched. In a previous investigation, we observed that surfaces of dengue virus (DV)-infected cells bind DV-antibodies, which leads to complement activation and cytokine secretion. The search for the responsible viral antigen led to NS1, a 45 kD non-virion associated protein that is synthesized in the endoplasmic reticulum and exported along the cellular secretory pathway. NS1 resides in the plasma membrane of infected cells and is also released in oligomeric form to the extracellular milieu. NS1 is strongly immunogenic and anti-NS-1 antibodies play a role in protection against disease. However, protection is afforded only by type-specific antibodies. High levels of anti-NS1 antibodies are found in the circulation of DV-infected patients during the late-acute and convalescent phase. Moreover, high levels of soluble NS1 have been detected in the blood of DHF/DSS patients during the acute phase of the disease. Therefore, DV-NS1 might be one of the major dengue antigens responsible for complement activation in DV-infection.

In the present project, we discovered that soluble and cell-bound NS1 activate human complement, and that plasma levels of soluble NS1 protein and the terminal SC5b-9 complement complex correlate with disease severity. Large amounts of complement activation products and soluble NS1 were found in the pleural fluids of DSS patients, indicative of massive complement activation occurring at the sites of vascular leakage. Complement anaphylatoxins as well as the terminal SC5b-9

complement complex increase vascular permeability and SC5b-9 increases lung hydraulic conductivity. A link thus emerges between NS1 load, complement activation and the clinical manifestation of DHF/DSS.

Outputs of the research project

A manuscript entitled “A Role for Dengue Nonstructural Protein-1 and Complement in Severe Dengue Infection” is now being submitted to the Journal of Infectious Disease. Another spin off is the development of NS1 ELISA which is proven to be good and sensitive for the identification of dengue infection in clinical specimens. Other available tests in the market nowadays rely on the determination of dengue specific antibodies which have several drawbacks. Rising of dengue specific antibodies (IgG or IgM) to a detectable level in blood circulation generally occur in late acute and convalescent phase when most of the patients have been already discharged from the hospital. Additionally, more than 90% of cases in Thailand or countries in dengue endemic areas have secondary immune response so that an increase in dengue specific antibody of IgG isotype between the first and second blood specimens collected from two different time points (acute and convalescence) is necessary to differentiate acute from recent DV-infection. Therefore, antibody detection is not an ideal mean for case identification during acute period of the disease. Detection of dengue virus genome by RT-PCR, although it is a gold standard for DV identification during early febrile phase, is not applicable for general usages due to expensiveness and complexity. From the reasons described above, detection of NS1 by capture ELISA or rapid immunochromatography is an assay of choice which could possibly replace RT-PCR due to its simplicity, easy to perform, and low cost. Another major discovery from this project is that fluid phase terminal complement complexes, SC5b-9 levels are rising and coincide with those of NS1 during acute phase of the disease. Moreover, DSS patients have significantly higher levels of SC5b-9 than non-shock cases. We proposed that the combination of SC5b-9 and NS1 detection could not only identify dengue infected cases but may also predict the patients who are at risk to develop severe vascular leakage or shock. This idea has now been processing for patent in Thailand and outsides. At the same time, we are in the process of dealing with private companies to develop rapid immunochromatographic test for dengue NS1 and SC5b-9. Once the test is available, prospective study to verify its capacity to identify patients at risk to develop severe

shock and leakage will be further conducted. This is a very important issue in dengue field once a severe case can be early diagnosed and identified, with proper care and treatment, the morbidity and mortality would certainly be prevented.

Collaboration and training

The project has successfully reached their final outputs with the assistance of Prof. Sucharit Bhakdi who is the Director of Institute of Medical Microbiology and Hygiene, University of Mainz, Germany. His major contribution is the supports on funding and facilities for the measurements of complement fragments in clinical specimens. Cells stably expressing NS1 used in this project were kindly provided by Dr. Sansanee Noisakran who is the staff of BIOTEC. The project has also provided a training opportunity for Miss Nuntaya Punyadee who is a master degree student of the Department of Immunology, Faculty of Medicine Siriraj Hospital under the supervision of Dr. Panisadee Avirutnan. Miss Nuntaya's thesis entitled "The Complement Fixing Activity of Dengue Virus Nonstructural Protein-1" involved the investigation of complement fixing activity of both membrane associated and soluble NS1 *in vitro*. Clinical specimens used in the project were supplied from the serum bank established by a project funded by T-2, entitled "Establishment of dengue clinical research centers supporting basic and clinical research" by Dr. Prida Malasit as the principal investigator.

RESEARCH CONTENTS

Introduction

Dengue hemorrhagic fever is one of the major health problems in tropical and subtropical areas of the world. The disease is caused by infection with dengue virus (DV) which is an arthropod-borne flavivirus and subdivided into 4 serotypes, dengue 1-4 (1). Majority of DV infections are asymptomatic or self-limiting febrile illness known as dengue fever (DF) which is characterized by high grade or biphasic fever with myalgia, headache, and leucopenia. However, there is a risk to develop dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). DHF is a severe febrile disease characterized by abnormalities in hemostasis and increase vascular permeability leading to plasma leakage, and bleeding tendency (2). DHF is classified into 4 grades according to WHO criteria. Grade I is characterized by high grade fever, thrombocytopenia, haemoconcentration, positive tourniquet test and/or easy bruising. Characteristics of grade II patients compose of all DHF I criteria with spontaneous bleeding. The more severe form, DHF grade III, alternatively designated as DSS, are associated with massive vascular leakage leading to hypotension (if undetectable blood pressure or profound shock, DHF IV). Uniquely, plasma leakage in DHF/DSS is caused by a selective increase in capillary permeability mainly at pleural and peritoneal spaces resulting in pleural effusion and ascites. In some cases, serious bleeding with disseminated intravascular coagulopathy (DIC) may complicate and worsen patient's condition. Without proper fluid supplementation and appropriate care, DSS patients rapidly deteriorate and death may result.

The mechanism involved in the pathogenesis underlying DHF/DSS is not well understood. Three major risk components including environmental, viral, and host factors are being concerned as the elements contributing to the development of DHF/DSS (2). Genetic changes in the genome of DV during its

replication process under human host immunological pressures may render a mutated virus possessing more virulence than its original one (2). It has been reported that DV of South East Asian (SEA) isolates may relate to more severe disease outcomes and the difference in nucleotide sequences in certain areas of DV-2 genome between isolates of SEA and America origins has previously been demonstrated (3). From several epidemiological studies, DHF is commonly associated with secondary DV infection (4). Halstead proposed a model of antibody dependent enhancement (ADE) of DV infection i.e. pre-existing non-neutralizing dengue antibodies generated from previous primary infection opsonize the virus of different serotype during secondary infection and enhance its uptake via FcR into monocytes and macrophages. ADE has long been thought to play a central role in development of severe dengue infection (5, 6). Recently, Mongkolsapaya and Dejnirattisai *et al.* discovered a new phenomenon in patients with DHF/DSS, i.e. a few DV-specific CD8+ T cells were recovered during acute phase of the disease and most of them were highly activated and undergoing apoptosis. They proposed the mechanism so called T cell original antigenic sin in DV-infection which may suppress or delay viral elimination, leading to higher viral loads and increased immunopathology (7). Roles of T cell activation in DHF and DSS has been demonstrated by several authors based on the data from both *in vitro* and clinical studies that the magnitude of T cell activation and cytokine generation are evident in DHF/DSS and may be of importance in the pathogenesis of severe disease (8). However, measurements of cytokines in DV-infection showed inconclusive results. (9-11). A recent study showing that a variant in the CD 209 promoter (DC-SIGN) is associated with the protection against DF supports the idea that host genetic factors also influence the outcome of DV infection (12). However, these data do not explain the cause of vascular leakage in DHF/DSS.

Several studies since almost 30 years ago have suggested a role of complement activation in the pathogenesis of plasma leakage in DHF/DSS. Bokisch

et al. found a decrease serum level of C3, C4, and C5 and increase metabolism of radiolabelled C3 and C1q in patients with grade III and IV diseases indicating active complement consumption during acute severe DV-infection (13). Malasit detected elevated plasma levels of complement breakdown products C3a and C5a in blood circulation of patients with DHF which peaked at the day of maximum leakage or shock (14). The mechanism of complement activation remains unclear. Soluble immune complexes formed by circulating DV and DV-specific antibodies (sIC) have been suggested to trigger complement activation in DV-infection. However, very small amounts of sIC could be detected in the circulation of patients during acute phase of the disease (14, 15). Moreover, sIC is known to be a poor complement activator (16). Only very low levels of SC5b-9 complexes were detected in serum from active systemic lupus erythematosus (SLE) (17) and none of the patients with immune complex disease develops vascular leakage or shock. Therefore it is unlikely that circulating immune complexes are the major cause of complement activation in DHF/DSS.

Alternative hypothesis for complement activation in DHF/DSS has been proposed by Bhakdi *et al* in 1990. During secondary infection, antibodies against DV are generated in a large amount due to an anamnestic response which would happen at the same period as most of the infected cells express relevant quantity of DV antigens on their surfaces resulting in the formation of large immune complexes on DV-infected cell surfaces which are capable of efficiently activating complement system (18). The *in vitro* findings that surface of DV-infected cells in the presence of anti-DV antibodies efficiently activated the complement system causing C5b-9 complexes deposition support the alternative hypothesis explaining how complement activation occurs in DHF/DSS (19).

However, which DV-antigens responsible for complement activation are still unknown. In the past, two types of DV-antigens, hemagglutinin (HA) and a non-hemagglutinating soluble antigen obtained from a rate zonal centrifugation of crude DV-infected culture supernatants or suckling mouse brain lysates were first described to have activities in traditional complement fixation (CF) test (20). Electron micrographs of rapidly sedimenting infectious hemagglutinin (RHA) revealed intact dengue virions, 48 to 50 nm in diameter while a non hemagglutinating soluble

complement fixing antigen (SCF) displayed debris as well as spherical particles measuring 5 nm in diameter (20). Subsequent studies suggested that SCF is a nonstructural antigen possessing at least two antigenic determinants, group- and type-specific and does not stimulate the induction of dengue neutralizing antibodies (21). The protein was demonstrated to have a molecular weight between 38,000 and 40,000 (22) and was observed its presence on the surfaces of infected cells (23). In 1985, Smith and Wright described a 46,000 kDa DV-nonstructural glycoprotein containing two N-linked glycosylation sites, so called 46-kilodalton glycoprotein (gp46) (24) and it was later renamed as nonstructural protein-1 (NS1) following the sequence analysis of yellow fever virus genome (25). Immediately after DV-protein synthesis in the cytoplasm of host cells, NS1 is associated with the membranes of endoplasmic reticulum and transported through the secretory pathway to reside in plasma membrane of infected cells (26) and also release into extracellular milieu as oligomeric complexes (27). Recently, it was found that high levels of secreted NS1 were detected in serum of patients experiencing secondary DV-infection and its level in DHF was greater than that of DF patients (28, 29). Therefore, DV-NS1 might be one of the major dengue antigens responsible for complement activation in DV-infection.

In the present study, the capability of soluble- DV-NS1 to activate human complement was demonstrated *in vitro* by using conventional hemolytic complement assay (CH 50) (30) or commercial ELISA measuring complement activation products SC5b-9 while the complement activation of membrane associated-NS1 was determined by the deposition of membrane attack complexes C5b-9 on cellular surfaces. Complement activation of two forms of NS1 (soluble and membrane associated) was tested in the presence or absence of specific antibodies.—Plasma and pleural fluids of dengue infected patients were collected and measured for viral load, NS1, and complement activation products (C3a, C5a, and SC5b-9).

We discovered that soluble and cell-bound NS1 activate human complement, and that plasma levels of soluble NS1 protein and the terminal SC5b-9 complement complex correlate with disease severity. Large amounts of complement activation products and soluble NS1 were found in the pleural fluids of DSS patients, indicative of massive complement activation occurring at the sites of vascular leakage.

Complement anaphylatoxins as well as the terminal SC5b-9 complement complex increase vascular permeability (31, 32) and SC5b-9 increases lung hydraulic conductivity (33). A link thus emerges between NS1 load, complement activation and the clinical manifestation of DHF/DSS.

Objectives

2.1. To determine the efficiency of soluble and cell associated-dengue NS1 protein in complement activation both in the absence and presence of antibodies specific to the protein.

2.2 To investigate the relationships between NS1 protein, the host complement system *in vivo* and clinical severity by measuring the levels of soluble NS1, and complement activation products generated in the circulation of dengue infected patients during acute and convalescent phase of the disease. Quantitative RT-PCR will be also performed to measure dengue viral RNA in clinical specimens.

Material and Methods

Cell culture

C6/36, a cell line from *Aedes albopictus* (ATCC CRL-1660) and Psc1oneD, a swine fibroblast cell line were cultured at 28 °C and 37 °C respectively, in L-15 medium (GIBCO BRL) containing 10% heat inactivated fetal bovine serum (FBS, GIBCO BRL), 10% tryptose phosphate broth (TPB, SIGMA, St.Louis, MO), 100 U/ml penicillin and 100 µg/ml streptomycin (SIGMA, St.Louis, MO). Human embryonic kidney epithelial cell line (HEK-293T) and HEK-293T lines stably expressing recombinant NS1 were cultured in RPMI 1640 (GIBCO BRL, Invitrogen, Grand Island, NY) containing 10% heat inactivated fetal bovine serum (FBS, Hyclone, Logan, UT), 37 µg/ml of penicillin, 60 µg/ml of streptomycin (SIGMA, St.Louis, MO) and 2 mM L-glutamine (SIGMA, St.Louis, MO) at 37 °C in 5% CO₂ incubator.

Antibodies

1. Rabbit anti-mouse Igs conjugated with HRP, P0260, DAKO
2. Swine anti-Rabbit Igs conjugated HRP, P0217, DAKO
3. Rabbit anti-mouse Igs conjugated with FITC, P0261, DAKO
4. C3c Rabbit Igs to human C3c, A062, DAKO
5. C3d (anti 2D) Rabbit Igs to human C3d, A063, DAKO
6. Goat anti-rabbit IgG conjugated Cy3, Jackson Immuno Research Laboratories, Inc., West Grove, PA
7. Goat anti-rabbit IgM conjugated Cy3, Jackson Immuno Research Laboratories, Inc., West Grove, PA
8. Rabbit anti-mouse Igs, Z0259, DAKO, Denmark
9. FITC-conjugated C3dg mAb (In house)
10. Monoclonal antibodies (mAbs)
 - 10.1 mAb 1A4 (BF2/1A4-1), specific for conformation epitope of NS1 protein of DV-2, isotype IgG2a (unpublished data)
 - 10.2 mAb 1B2 (BF2/1B2-6), specific for linear epitope of NS1 protein of DV serotype 1-4, isotype IgG1 (unpublished data)

- 10.3 mAb 1B10 (1PF 12F) specific for linear epitope of NS1 protein of DV-2, isotype IgG1 (unpublished data)
- 10.4 mAb 1F11 (2NS 3F), specific for linear epitope of NS1 protein of DV serotype 1-4, isotype IgG2a (34)
- 10.5 mAb 2C5 (NS 2S), specific for linear epitope of NS1 protein of DV serotype 1-4, isotype IgM (34)
- 10.6 mAb 2H4 (2NS 2F), specific for linear epitope of NS1 protein of DV-2, isotype IgM (unpublished data)
- 10.7 mAb 2E3 (2NS 4F), specific for linear epitope of NS1 protein of DV serotype 1-4, isotype IgG1 (34)
- 10.8 mAb 2E11 (2NS 1F), specific for linear epitope of NS1 protein of DV serotype 1-4 and JE, isotype IgM (34)
- 10.9 mAb 2G6 (1PF 6S), specific for linear epitope of NS1 protein of DV serotype 1-4, isotype IgG2a (unpublished data)
- 10.10 mAbs specific for C3dg, clone 9 (ascitic fluid), provided by Dr. P.J. Lachmann (35)
- 10.11 Pooled convalescent dengue sera (PCS); collected from a pool of at least 30 dengue patient's sera with hyper immune which HI titer against all four serotype of DV $\geq 1:10,240$. Purified Ig fraction were obtained from protein G affinity column chromatography (Pharmacia)
- 10.12 Pooled negative sera (PND); collected from a pool of healthy person individual sera which HI titer against all four serotype of DV $< 1:20$. Purified Ig fraction were obtained from protein G affinity column chromatography (Pharmacia)

Virus

Preparation of virus stock

In order to generate large stock of DV-2 strain 16681 for the whole study, DV strain 16681 was propagated in C6/36 cells. Approximately 1×10^7 C6/36 cells were grown in T75 cm² tissue culture flask (Costar, Cambridge, MA, USA) in growth medium (L-15 containing 10% FBS, 10% TPB, 100 U/ml penicillin and 100 µg/ml streptomycin (SIGMA, St.Louis, MO) and incubated at 28 °C for 2 days. The confluent cells monolayer was then incubated with the virus at a multiplicity of

infection (MOI) of 0.01 in maintenance medium (1.5% FBS 10% TPB, 100 U/ml penicillin and 100 µg/ml streptomycin) in total volume of 5 ml at 28 °C for 3 h on a rocker. After that, supernatants were replaced with maintenance medium in total volume of 15 ml/ T75 cm² flask and further incubated at 28 °C until approximately 7 days after infection. The culture supernatants were then collected and clarified by centrifugation at 1000xg for 10 min at 4°C. Clarified supernatants were aliquoted and store at -70°C.

Virus titration

Culture supernatants from DV-infected cells were titrated by focus forming assay in Psc1oneD cell lines. Briefly, approximately 4×10^4 Psc1oneD cells were plates on each well of 96 well plated in growth medium and incubated at 37 °C in humidified chamber for 24 h. The collected DV-supernatants were 10-fold serially diluted (10^1 - 10^8) in maintenance medium (L-15 containing 3% FBS, 10% TPB, 100 U/ml penicillin and 100 µg/ml streptomycin) and subsequently 50 µl of diluted samples was added into each well in duplicates. Following 2 h incubation in a humidified chamber at 37 °C, 100 µl of overlay medium (maintenance medium containing 1% gum tragacanth (SIGMA)) was added into each well, and incubated at 37 °C in a humidified chamber for 72 h. After that, culture supernatants were removed and the cells were washed with phosphate buffer saline (PBS) pH 7.4 and 100 µl of 3.7% formaldehyde (BDH, England) in PBS was used to fix the cells for 10 min. Subsequently, fixed cells were permeabilized by 100µl of 1% Triton X-100 (Fluka) in PBS for 10 min. After washing with PBS, 50 µl of mouse mAbs specific to flavivirus envelope protein (4G2) was added in each well and incubated at 37 °C in a humidified chamber for 30 min. The cells were then washed, and incubated with 50 µl of HRP-conjugated rabbit anti-mouse Igs (DAKO) in dark at 37 °C in a humidified chamber for 30 min. After washes, substrate solution containing 3, 3' diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide in Tris-HCl buffer, pH 7.4 was used for color development. Calculation of virus titer was based on the following formular.

$$\text{Virus titer (ffu/ml)} = \frac{(A+B)}{\text{Total volume of virus} \times (A+B)} \times 10^C \times 10^3$$

A= number of foci counted in the first dilution

B= number of foci counted in the final dilution

C= the first dilution that foci were counted

Establishing HEK-293T lines stably expressing recombinant NS1

Construction of plasmid cDNA clones expressing recombinant dengue NS1

Three cDNA cassettes carrying different recombinant dengue NS1 form were constructed as shown in Fig. 1. Briefly, genomic RNA of strain NGC was extracted from infected C6/36 cells and used as the template for PCR with various primer pairs listed in Table 1. To generate the plasmid construct encoding NS1 connected to the 26-amino acid region at the N-terminus of NS2A form (rNS1m), and the plasmid construct only encoding NS1 form (rNS1s) was subcloned into pDisplay vector at the Bgl II-Not I sites. The Kpn I-Not I fragment excised from this subclone was ligated into pcDNA3.1/Hygro in order to generate pcDNAhygro/soluble NS1.

After the amplification of dengue cDNA template, cloning of the amplified DNA fragment were then confirmed by nucleotide sequence analysis of the final pcDNAhygro-based plasmid clones. The NS1 encoding sequence in pcDNAhygro/soluble NS1 contained only a silent mutation at the nucleotide position 2677. The missense mutation at the nucleotide position 3455 was not incorporated into the soluble NS1 clone as the reverse primer D (Table 1) used for the construction of this clone spanned the nucleotide positions 3439 to 3477.

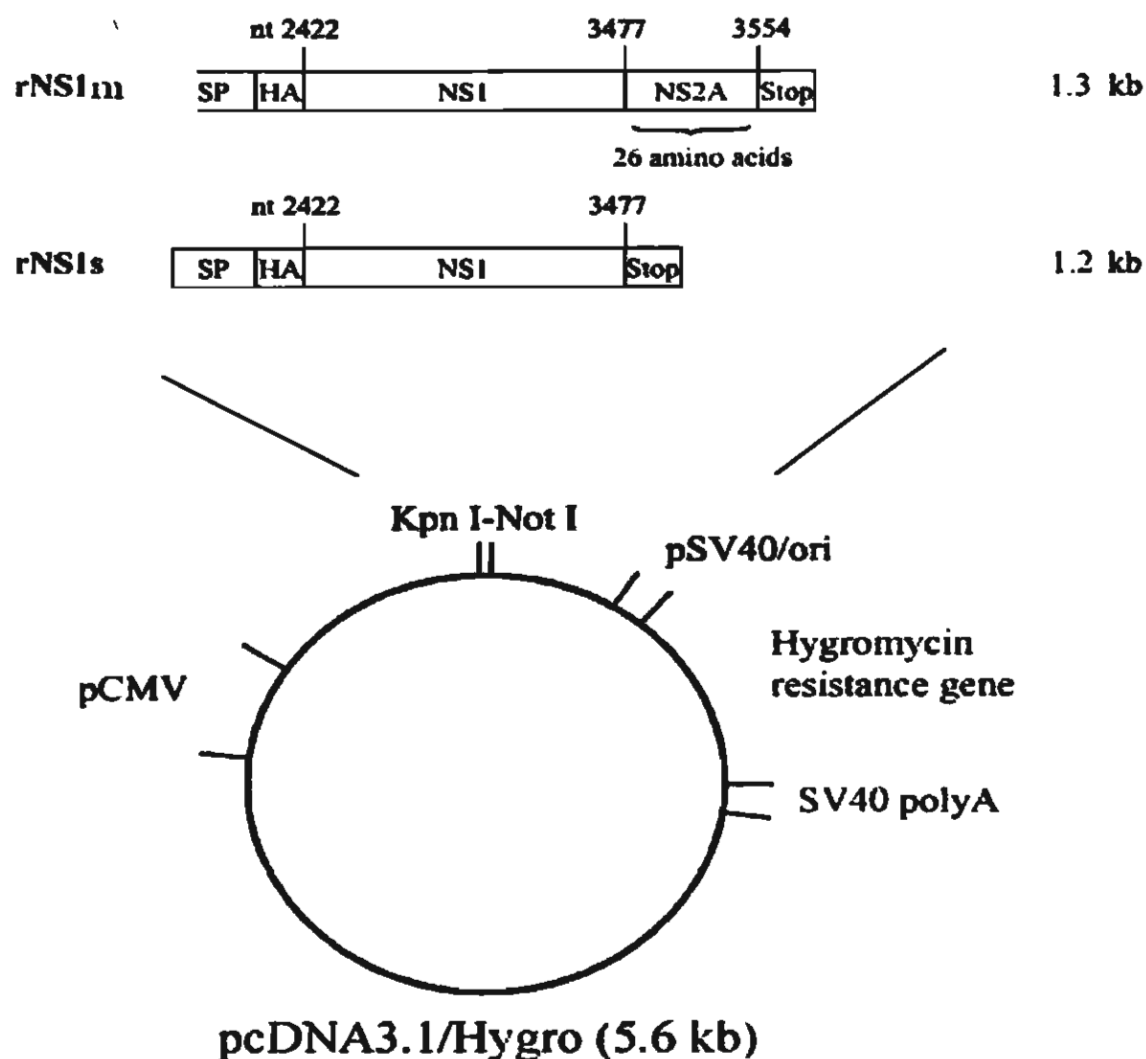


Figure 1 A schematic diagram demonstrating the construction of plasmid cDNA encoding 2 form of dengue virus NS1

cDNA of dengue2 virus (strain NGC) used as template for amplification of NS1 segments by using different sets of oligonucleotide primers and the amplified products were then inserted into plasmid display to generate NS1 subclones. The NS1 fragments flanked by the coding sequences for signal peptide and stop codon were excised from the subclones and ligated into pcDNA3.1/Hygro at the Not I and Kpn I recognition sites to generate the plasmid constructs expressing recombinant forms of dengue NS1 as follows: i) NS1 connected to the 26-amino acid region at the N-terminus of NS2A, rNS1m; (ii) only the complete sequence for dengue NS1, rNS1s.

Table 1. Oligonucleotide primers for cloning of recombinant NS1.

Product	Designation	Orientation	Sequence
NS1-NS2 (rNS1 _m)	A	Sense	5' CCGGCCAGATCT ^a GATAGTGGTTGCGTTGTGAGC 3'
	B	Antisense	5' CAAGCTTCGCGGCCGC ^b GTAAAC ^c TAA ^d TCGGGTCCT GAGCATTCTTCCAG 3'
NS1 (rNS1s)	C	Sense	5' CCGGCCAGATCT ^a GATAGTGGTTGCGTTGTGAGC 3'
	D	Antisense	5' GATCGATCGCGGCCGC ^b TAA ^d GGCTGTGACCAAG GAGTAAAC ^c CAAATTCTCTTCTTCTC 3'

^a, Bgl II restriction enzyme recognition site. ^b, Not I restriction enzyme recognition site. ^c, Hpa I restriction enzyme recognition site (a bold letter represents a silent mutation introduced to generate Hpa I site). ^d, Translation termination codon.

Transfection and selection of cells expressing recombinant NS1 forms

Approximately, 5×10^5 HEK-293T cells were grown on a 35 mm² tissue culture dish in 2 ml of RPMI 1640 containing 10% FBS and cultured for 2 days. After that, the cells (70% confluent) were washed twice with Dulbecco's modified Eagle medium (DMEM) adjusted pH to 7.5 with 1 M HEPES buffer and then added with 2 ml of DMEM supplement with 10% FBS. Consequently, the cells were transfected with pcDNA3.1/Hygro (vector control, rControl), pcDNAhygro/m NS1 (rNS1_m), pcDNA hygro/soluble NS1 (rNS1s), or with calcium phosphate-DNA precipitate as follows. One hundred μ l of 0.25 M CaCl₂ was added to 5 μ g of plasmid DNA in a 15 ml centrifuge tube. An equal volume of 2x HEPES-buffered saline (pH 7.0) was added drop wise into the DNA-CaCl₂ mixture, mixed gently, and maintained at 37 °C in the 5% CO₂ incubator for 12 h and then replaced with RPMI 1640-10%FBS. To verify NS1 protein expression on the surface of transfected cells, an indirect immunofluorescence assay (IFA) 3 days after transfection with anti-NS1 mAbs was performed. Subsequently, the transfected cells were propagated in the presence of 100-400 μ g/ml of hygromycin B (GIBCO BRL). The culture was

observed daily for the appearance of surviving colonies. Individual colonies were selected and maintained in RPMI 1640 containing 10% FBS medium and 100 µg/ml of hygromycin B. These individual colonies were screened twice by IFA for NS1 expression within 3-week period to obtain transfectant with highest percentage of expressing cells as well as the great degree of NS1 expression of NS1 expression on individual cells. In addition, pcDNA3.1/Hygro-transfected HEK-293T cells were selected with hygromycin B for use as a negative control in subsequent experiments.

Analysis of cell surface associated NS1

Approximately 1×10^6 harvested cells were washed with washing medium (RPMI 1640 containing 5% FBS and 10 mM NaN_3) and incubated with 10% normal human AB serum for 30 min on ice to block FcR. After washing, the cells were incubated with 50 µl of mouse mAbs specific to NS1 on ice for 1 h and followed by 6 µg/ml FITC-conjugated rabbit anti mouse Igs (DAKO) for 30 min on ice in dark. Propidium iodide was added to cell suspension at a final concentration 0.2 µg/ml and analyzed by flow cytometry.

Analysis of cytoplasmic DV-NS1

1×10^6 harvested cells were washed with PBS and fixed with 2% formaldehyde (BDH, England) in PBS for 1 h at RT. After that, the fixed cells were washed once with a large volume of PBS and permeabilized with 0.1% Triton X-100 in PBS. Permeabilized cells were incubated with 50 µl of mouse mAbs specific to NS1 for 1 h at RT. Subsequently, the cells were washed once with large volume of 0.1% Triton X-100 in PBS and incubated with 6 µg/ml FITC labeled rabbit anti-mouse Igs (DAKO) for 30 min at RT in dark. After a final wash with large volume of 0.1% Triton X-100 in PBS, the cells were analyzed by flow cytometry.

Detection of cell bound C3dg on the surfaces of complement attacked cells

HEK 293T cells were infected with DV strain 16681 at MOI of 10 and harvested 24 h after infection. The harvested mock and DV-infected cells were washed with RPMI 1640 and incubated with 1:5 dilution of human dengue non immune serum as a complement source in the presence or absence of dengue or NS1 specific Abs in a total of volume 200 µl. After 1 h incubation at 37° C, the cells were

washed with a large volume of cold washing medium (RPMI 1640 containing 5% FBS and 10 mM NaN_3). Complement activation on the surface of infected cells was determined by incubation of the cells with mAbs specific for C3dg, clone 9 (ascitic fluid), provided by Dr. P.J. Lachmann, at the final dilution of 1:50 50 μl for 1 h on ice. After wash once, the cells were incubated with FITC-conjugated rabbit anti-mouse Igs (DAKO) at a final concentration of 6 $\mu\text{g/ml}$ for 30 min on ice, in dark. After washing step, propidium iodide at a final concentration of 0.2 $\mu\text{g/ml}$ was added and analyzed by flow cytometry. Complement activation capability of membrane bound NS1 was also studied by using cells stably expressing NS1. The cells were cultured for 48 h and detached from culture flask by using 0.5 mM EDTA (USB, Amersham) in PBS and then washed with large volume of RPMI 1640. After complement activation, the C3dg deposition was analyzed by IFA as described above.

In some experiments, the cells were incubated with anti-dengue or NS1 specific antibodies plus heat inactivated (HI) serum or anti-dengue or NS1 specific antibodies plus serum containing 10 mM EDTA as negative controls to inhibit complement activation.

Detection of C5b-9 complex on the surfaces of complement attacked cells

Mock and DV-infected cells at MOI of 10 harvested at 24 h after infection, and cell stably expressed NS1 harvested at 48 h after culture were washed with RPMI 1640 (GIBCO) and incubated with fresh human dengue non-immune serum as a complement source at 1:5 dilution in the presence or absence PCS or mouse mAbs specific to NS1 for 1 h at 37°C. After that, the cells were washed with cold washing buffer (RPMI 1640 containing 5% FBS and 10 mM NaN_3) and stained with mAbs specific for SC5b-9 (Quidel, USA) at a concentration of 1 $\mu\text{g/ml}$ for 1 h on ice. After once wash the cells were incubated with FITC-conjugated rabbit anti-mouse Igs (DAKO) at a final concentration of 6 $\mu\text{g/ml}$ in dark for 30 min on ice. After washing step, propidium iodide at a final concentration of 0.2 $\mu\text{g/ml}$ was added and analyzed by flow cytometry.

Determination of complement activation pathway

Mock and DV-infected cells were harvested at 24 h post infection. The harvested cells were washed with RPMI 1640 (GIBCO) and incubated with PCS or a mixture mAbs specific to NS1 plus fresh non-immune serum at 1:5 dilution in the presence of 10 mM EGTA (Sigma, USA) and 10 mM $MgCl_2$ (Merk, Germany) for 1 h at 37°C to inhibit only the classical pathway of complement activation or with 10 mM EDTA (USB, Amersham) to inhibit both the classical and alternative pathway. After complement was activated, the deposition of C3dg on the surface was performed according to the protocol described above and analyzed by flow cytometry.

Double immunofluorescence staining and confocal microscopic examination

Mock and DV-infected cells or cells stably expressed NS1 (1×10^6) were incubated with fresh non-immune serum at a final dilution of 1:5 in the presence or absence of PCS or mouse mAbs for NS1 at 37°C for 1 h. After that the cells were washed once with RPMI 1640 containing 5% FBS and 10 mM NaN_3 . (Merk, Germany) and fixed with 2% formaldehyde in PBS at RT for 10 min. Subsequently, the cells were washed and incubated with 50 μ l of rabbit anti human C3c and C3d (DAKO) at a dilution of 1:800 and 1:400 respectively for 1 h at 4°C. After that the cells were washed once and incubated with 50 μ l of FITC-conjugated swine anti-rabbit Igs and Cy3-conjugated goat anti mouse-IgG, IgM at a dilution of 1:50 (Jackson Immuno research laboratory, inc., West Grove, Pennsylvania) at 4°C for 30 min. Consequently, the cells were washed once and resuspended in 20 μ l of 5% FBS/RPMI 1640 10 mM NaN_3 and then 20 μ l of anti fade was added to cell suspensions. Finally, the cells were observed under a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Germany) with a 63x oil immersion lens. FITC was excited at 488 nm and emission collected at 505-530 nm and Cy3 was excited at 543 nm and emission collected at 560-615 nm to avoid a overlap in emission spectra. All background auto fluorescence was corrected on all slides for all wavelengths. Photography was performed by using an image capture program (LSM 510 software version 3.2, Carl Zeiss).

Affinity purification of NS1

Approximately 1.4×10^7 PscloD cells were grown in 162 cm^2 tissue culture flask (Costar, Cambridge, MA, USA) in growth medium at 37°C for 48 h. Confluent cell monolayers were incubated with DV serotype 2 (strain 16681) at a MOI of 1 in maintenance medium (L-15 containing 1.5% FBS 10% TPB, 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin) in a total volume of 13 ml at 28°C for 3 h on a rocker. After that supernatants were replaced with 40 ml protein free medium (Ultradoma) and cultured for 3 days. Culture supernatants were collected and ultra centrifuged at $200,000 \times g$ for 4 h at 4°C to remove dengue virions and subjected to immunoaffinity chromatography with a column prepared with anti NS1 specific mAb 2G6. For column preparation, anti NS1 specific mAbs 2G6 were coupled with CNBr-activated sepharose bead according to the protocol supplied by the manufacturer (Pharmacia). Virion depleted-supernatants were passed through the column at the rate of 0.2 ml/min. After washing with a large volume of PBS, column bound NS1 was eluted with 20 mM Diethylamine (DEA) (Merk, Germany) pH 11.3. Concentration of NS1 was determined by ELISA and its purity and immunogenicity were assessed by SDS-PAGE and western blot analysis. For purification of soluble NS1 from cells stably expressing NS1 (rNS1s), culture supernatants were collected every 3-4 days and replaced with fresh protein free medium. Culture supernatants were filtered through $0.2 \mu\text{m}$ membrane filter (Sartorius) prior to affinity chromatography. Purified NS1 was passed through protein G column (Pharmacia) in order to remove contaminating antibodies. The absence of Ig was confirmed by ELISA.

Igs capture ELISA

Briefly, the polystyrene micro ELISA plates (NUNC) were coated with 50 μl rabbit anti-mouse Igs (DAKO) at a final dilution 1:5000 (0.35 $\mu\text{g/ml}$) in coating buffer (Carbonate-bicarbonate, pH 9.6) overnight at 4°C . After that 200 μl blocking buffer (0.5% BSA in PBS) was added to each wells and incubated for 2 h at RT. After 4 washes with washing buffer (PBS containing 0.05% Tween-20), 50 μl samples was added to each well and incubated for 37°C in a humidified chamber for 1 h. After 4 washes 100 μl of rabbit anti-mouse Igs conjugated-HRP (DAKO) at a final dilution 1:2000 (0.65 $\mu\text{g/ml}$) was then added to each well and incubated for 1 h at RT.

After 4 washes, 100 µl of 0.1 M citrate buffer, pH 5.5 containing 0.4 mg/ml o-phenylene diamine (SIGMA) and 3% H₂O₂ (Sahakarn) was added to each well and incubated at RT for 10 min. After that color development was stopped by the addition of 50 µl of 4 M H₂SO₄ to each well. The absorbance was read with an ELISA reader at 492 nm.

ELISA methods for the detection of NS1 protein

The polystyrene micro ELISA plates (NUNC) were coated with 50 µl anti NS1 mAbs 2E11 (IgM) at 50 µg/ml overnight at 4°C. After that 200 µl blocking buffer (15%FBS in PBS) was added to each well and incubated for 2 h. After 5 washes with (PBS containing 0.05% Tween-20), 100 µl of sample was then added to each well and incubated for 1 h at RT. 100 µl of anti NS1 mAbs 2E3 (IgG1) at a final concentration 50 µg/ml was added to each well and incubated for 1 h at RT. After 5 washes, 100 µl of rabbit anti-mouse IgG conjugated-HRP (DAKO) at a final dilution 1:2000 (0.65 µg/ml) was then added to each well and incubated for 1 h at RT. After 5 washes 100 µl of 0.1 M citrate buffer, pH 5.5 containing 0.6 mg/ml o-phenylene diamine (SIGMA) and 3% H₂O₂ (Sahakarn) was added to each well and incubated at RT for 10 min. After that color development was stopped by the addition of 50 µl of 4 M H₂SO₄ to each well. The absorbance was read with an ELISA reader at 492 nm.

Determination of purity and immunogenicity of purified NS1

SDS-PAGE and silver staining

Purified NS1 (1 µg/well) was mixed with 4x loading buffer (0.2M Tris pH 6.8, 8% (w/v) SDS, 40% glycerol, and 0.005% bromophenol blue) and then loaded into 12% SDS-PAGE, run under constant 100 volts for approximately 2 h and subjected to silver staining. Some samples were heated to 95°C for 10 min prior to SDS-PAGE.

Western blot analysis

Samples were separated by 12% SDS-PAGE 2 h, and transferred onto nitrocellulose membrane with Towin buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, and 0.1% SDS) for 2 h by using a SemiPhor semi-dry transphor unit (Amersham Bioscience, Uppsala, Sweden). The blotted membrane was blocked with

blocking buffer (5% skim milk in PBS) at RT for 1 h and then washed with PBS and incubated with mouse mAbs specific to NS1 (1F11) overnight at 4°C. After that the membrane was washed 3 times with PBS and incubated with HRP conjugated rabbit anti mouse Igs (DAKO) at 1:1000 dilution in blocking buffer at RT for 1 h. Finally the membrane was washed with PBS 3 times and then reacted with the substrate solution, containing 0.06 mg/ml of diaminobenzidine in PBS, 2 µl/ml of H₂O₂ and 5 µl of 8% NiCl₂ was added approximately 5 min to visualize dark-brown band on the membrane. The size of reactive protein band was determined by comparing with standard protein molecular weight marker separate on the same gel.

CH 50 assays

Complement activation by soluble NS1 and DV-infected supernatants was determined by the hemolytic complement titers (CH50) in the conventional manner (30). Briefly, various amounts of samples were incubated with or without anti dengue antibody or mouse mAbs specific for NS1 plus fresh non-immune serum at a final dilution of 1:8 in a total volume of 200 µl for 1 h 37°C. After that, the mixtures were two-fold serially diluted with complement fixing diluents (gelatin-veronal buffer with metal ions (GVB²⁺)) and each diluted sample was then incubated with 50 µl of sensitized sheep erythrocytes. 50 µl of GVB²⁺ was added into the 0% hemolysis and 50 µl of distilled water was added into 100% hemolysis tube and incubated for 30 min at 37°C respectively. After that 1.2 ml of cold 0.15 M NaCl (Merk, Germany) was added to each tube except the 100% hemolysis control tube in which 1.2 ml of distilled water was added. Subsequently, the mixtures were spun at 1250xg to remove cell pellets and supernatants were measured by a spectrophotometer at 412 nm (Shimadzu UV-160A). Distilled water or GVB²⁺ were used zero blank for spectrophotometric measurement. Percent hemolysis for each sample were determined by dividing its OD₄₁₂ by the OD₄₁₂ of the 100% lysis control tube and multiply by 100 and calculated the hemolytic complement titers (CH50) of each tube.

SC5b-9 ELISA

To determine the capability of purified NS1 in complement activation, purified NS1 was incubated with or without anti dengue antibody (PCS or mouse mAbs specific for NS1) plus 1:8 dilution of fresh non-immune serum at 37°C for 1 h,

After that the samples were subjected to a commercial SC5b-9 ELISA from Quidel (SC5b-9 Enzyme Immunoassay). Briefly, in the first step, standards, controls, and test samples were added to micro wells that precoated with an anti-SC5b-9 specific mAbs for 60 min at RT. After incubation, the plates were washed 5 times to remove unbound materials and then 50 µl HRP-conjugated antibodies to SC5b-9 was added to each test wells for 60 min at RT and followed by 5 washes to remove unbound materials. In the last step, 100 µl of a chromogenic enzyme substrate was added to each micro well for 30 min at RT and then 50 µl of stop solution were added to each well. The absorbance was read with an ELISA reader at 405 nm.

Quantitative RT-PCR of dengue viral genome

RNA was extracted from DV-infected cell supernatants or patients' plasma using QIAamp Viral RNA Mini Kit (QIAGEN), aliquoted, and stored at -70° C. Levels of dengue viral RNA were subsequently quantified by a single tube one-step real-time RT-PCR using a LightCycler instrument and software version 3.5 (Roche Molecular Biochemicals, Germany) as described by Shu *et al* (36).

Measurement of complement fragments in clinical specimens

The anaphylatoxins C3a, and C5a were quantified by flow cytometry using a commercial cytometric bead array kit (Becton Dickinson). SC5b-9 was quantified with the ELISA from Quidel.

Patient enrolment and study design

Pediatric patients admitted to the ward of Khon Khan Provincial Hospital, Thailand between November 2001 and December 2003 with the clinical diagnosis of dengue infection (DF or DHF) and the following criteria were included in the study: age 1 to 15 years, pyrexia not more than 4 days with no obvious source of infection, Tourniquet test positive, history of signs/symptoms of bleeding/hemorrhagic diathesis). At the time of enrolment, subjects and their parents were interviewed by a study nurse to collect demographic data and medical history. Blood specimens were taken daily until one day after defervescence. Plasma aliquots were collected in 5 mM EDTA containing vacuum tubes (Becton Dickinson, Cat.No.367661) and stored at -70°C. Diagnosis of dengue infection was confirmed by measuring anti-DV IgM/IgG and by virus identification by RT-PCR (37). Aspiration of pleural fluid has

been conducted as part of treatment to relieve excess fluid collected within the pleural cavity, only in patients experiencing respiratory difficulty.

Clinical diagnosis and grading of DHF followed the WHO criteria (38). Study day 0 was defined as the calendar day during which the temperature fell and stayed below 37.8 °C. Evidence of plasma leakage included a peak of hematocrit value more than 20% above the value at the convalescent visit, a pleural effusion demonstrated on the chest radiograph, or detection of ascites on physical examination.

Thrombocytopenia was defined as a count of $\leq 100,000/\text{mm}^3$. Any subject with serological or virological evidence of acute dengue infection who did not meet the criteria for DHF was assigned to the DF group. Subjects were diagnosed as having OFI (other febrile illnesses) when there was no clinical evidence for a bacterial infection and no serological or virological evidence for DV-infection. The study protocol has been approved by the Ministry of Public Health (approval date, 7th May 2003), the Faculty of Medicine Siriraj Hospital (certificate of approval, 156/2002 and 115/2004), and the Khon Khan hospital (approval date, 31st October 2002). Informed consent was individually obtained from all subjects.

Statistical analysis

Data analysis was performed using software package StatView for Windows version 5.0 (SAS Institute Inc., NC). First, the mean and standard deviation (SD) of NS1, viral load, and SC5b-9 were presented for selected subgroups. Median and range were also displayed when the data were highly skewed. The aim of the statistical analysis was to investigate whether patients with DF, DHF grade 1, 2, and 3 differed regarding the NS1, viral load and SC5b-9. Kolmogorov Smirnov test was used to test for normality. Comparisons between DF and DHF (any group) were done by t-test if the distribution of the variables was comparable to a normal distribution; otherwise the Mann Whitney test was used. Multiple comparisons were performed using ANOVA. $P \leq 0.05$ was considered to be statistical significant. All analyzed P values were 2-sided.

Results

The study of complement fixing capability of membrane associated DV NS1 DV-infected HEK 293T cells expressed NS1 protein on their surfaces

Kinetics of NS1 protein expression on surface of DV-infected HEK 293T cells were studied using 9 clones (1A4, 1B2, 1B11, 1F11, 2C5, 2E3, 2E11, 2G6 and 2H4) of mAbs specific for NS1 and analyzed by flow cytometry. As shown in Fig. 2 dengue NS1 antigens was detected on cell surfaces at 24 h after infection and a slightly increase of NS1 expression at 48 h after infection was observed. All 9 clones of anti NS1 specific- but not isotype control antibodies (Nap2F, IgG1 and Nap7F, IgM) stained NS1 on the surface of DV-infected cells but not on mock cells. However, each clone stained with different intensity: 2G6 was able to react very strongly with surface NS1, while 1A4 and 1B2 moderately reacted and the remaining clones, 1B10, 1F11, 2C5, 2E3, 2E11 and 2H4 showed only weak positive staining. Analysis of cytoplasmic NS1 expression using the same series of mAbs was demonstrated in Fig. 3. The result showed that approximately 90% of DV-infected HEK 293T cells at 24 h and 48 h after infection at MOI of 10 expressed NS1 in their cytoplasm as detected by positive staining of 1A4, 1B2, 1B10 and 2G6 all these clones equally react to cytoplasmic NS1 as indicated by mean fluorescence intensity (data not shown) and 2E3 and 2E11 weakly reacted to cytoplasmic NS1 in (about 10%) while staining with 2C5 and 2H4 gave absolutely negative results (Fig. 3 A-B).

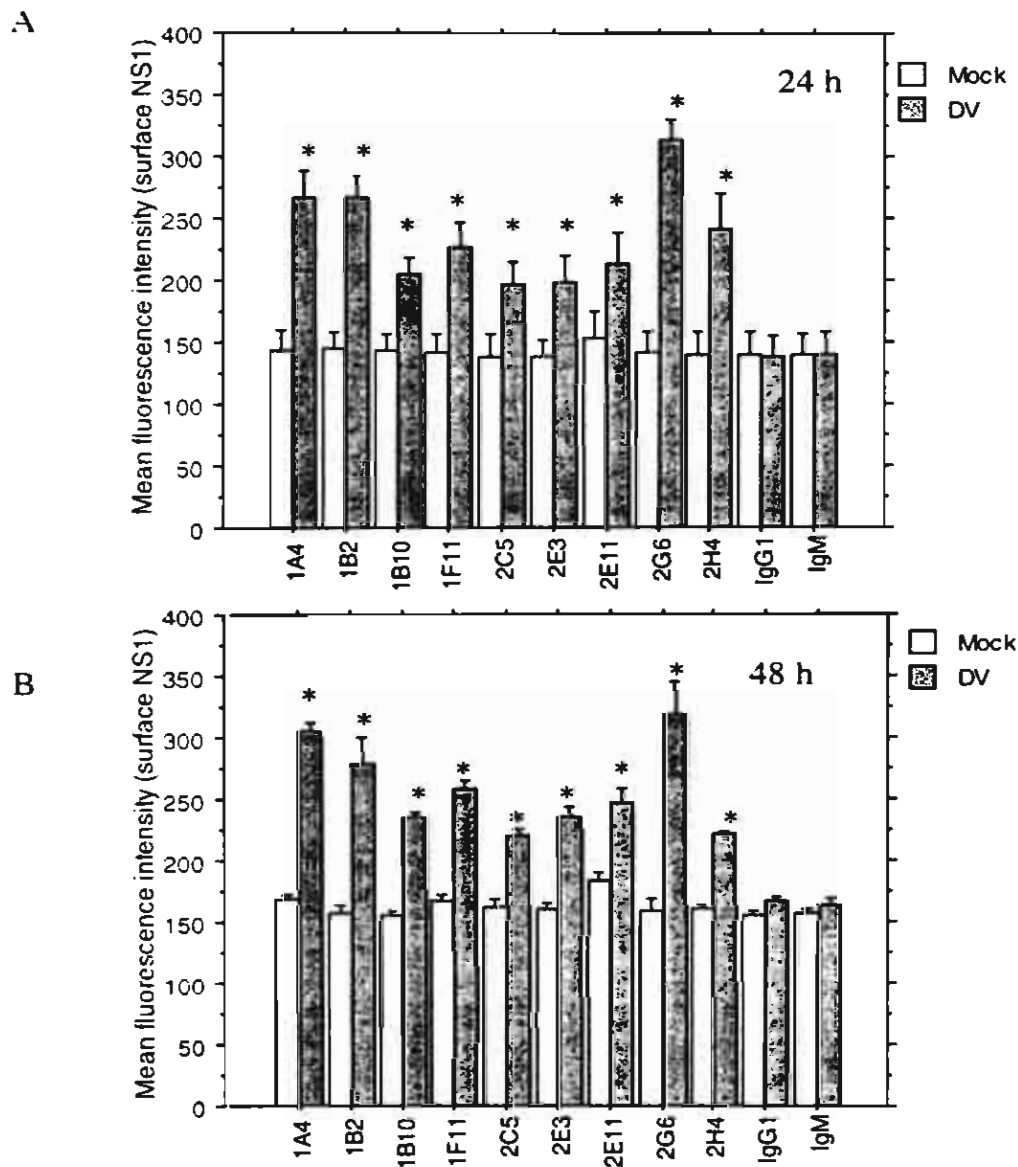


Figure 2 Kinetics of NS1 proteins expressed on the surface of DV-infected HEK 293T cells

HEK 293T cells were infected with DV at MOI of 10. DV-infected cells were harvested at 24 and 48 h after infection and Fc receptor were blocked by incubation with 10% human serum in RPMI for 30 min on ice. After that the cells were stained with mouse mAbs specific to dengue NS1 mAbs or isotype control antibodies and followed by with FITC-conjugated rabbit anti mouse Igs. Propidium iodide (0.2 μ g/ml) was added to cell suspension prior to flow cytometry analysis. Each bar represents the mean fluorescence intensity of surface NS1 of propidium iodide negative population from 3 independent experiments $\pm$ SD. * indicated p value < 0.001 (mock vs DV).

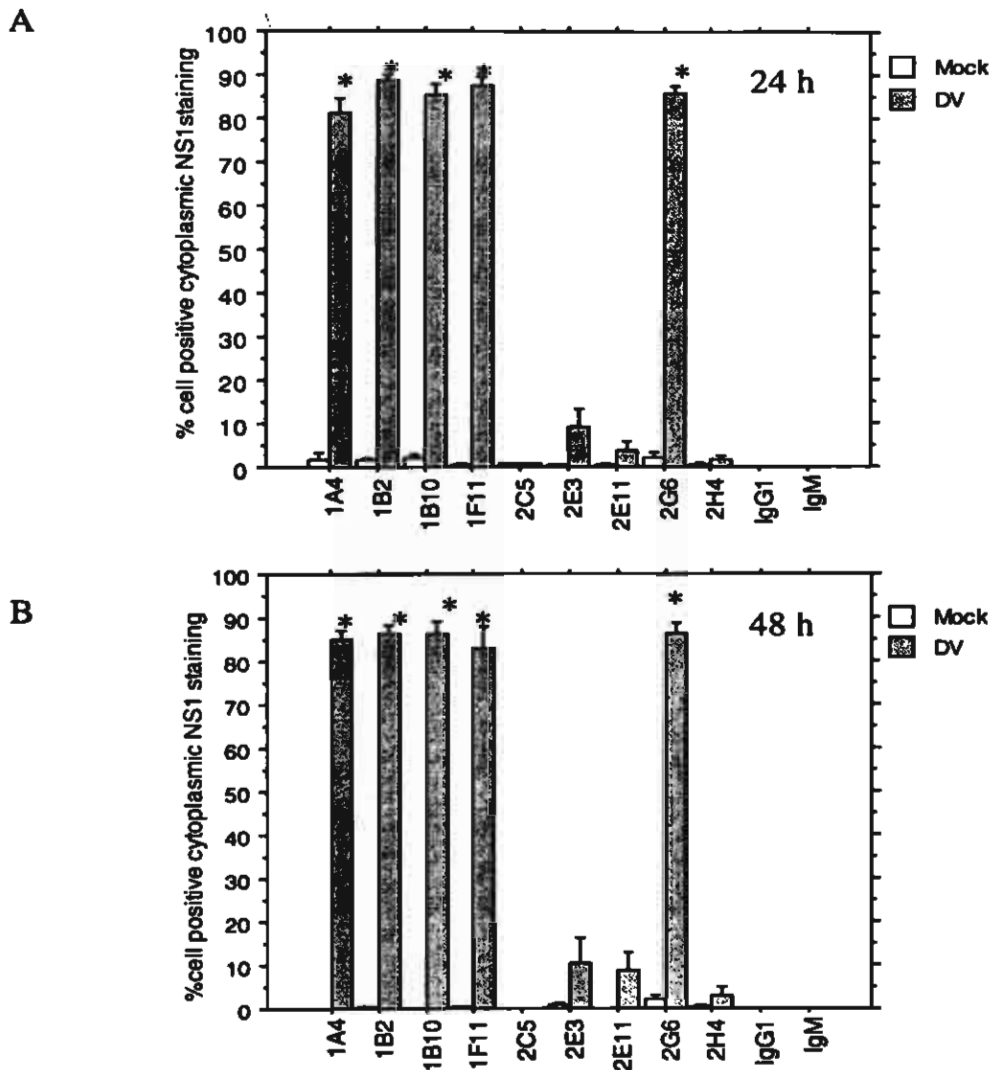


Figure 3 Kinetics of intracellular NS1 expressed in DV-infected HEK 293T cells

HEK 293T cells were infected with DV at MOI of 10. DV-infected cells were harvested at 24 and 48 h after infection and then fixed, permeabilized and stained with mouse mAbs specific to dengue NS1 or isotype control antibodies and followed by FITC-conjugated rabbit anti mouse Igs. The stained cells were analyzed by flow cytometry. Each bar represents the average percentage of cells positive for intracellular NS1 staining from 3 independent experiments $\pm$ SD. * indicated p value < 0.001 (mock vs DV).

DV-infected HEK-293T cells were capable of activating the complement system in the presence of anti-dengue antibodies

DV or mock-infected HEK-293T cells were incubated with 25% fresh dengue non immune serum as complement source in the presence or absence of anti dengue antibodies for 1 h at 37°C. Complement activation on cell surfaces was determined by using mAbs specific for a neo-antigen that exposed on C3dg fragments (35). As shown in Fig. 4, the deposition of C3dg fragments was seen when DV-infected HEK-293T cells were incubated with human complement in the presence of purified PCS (A) but not found on cell incubated with human complement in the absence of anti dengue-antibodies (B) or in the presence of 10 mM EDTA (C) or with heat inactivated serum (D). Fig. 5 demonstrated dose-dependent effect of anti dengue-antibodies on complement activation of DV-infected cells. DV-infected and mock HEK-293T cells were incubated with various concentrations of PCS in fresh DV-non immune human serum and the deposition of C3dg fragments on cells surfaces was analyzed by flow cytometry. The degree of C3dg deposition on the surface of DV-infected HEK 293T cells was dependent on the dosage of anti-dengue antibodies. Whereas, no difference in the amount of C3dg detected when various concentrations of purified dengue negative antibodies (PND) were added to DV-infected cells.

Complement activation by DV-infected HEK 293T cells is induced by anti-NS1 antibodies

The fact that surface of DV-infected cells in the presence of dengue antibodies could serve as the site for complement activation and that DV-infected cells expressed NS1 on their surfaces prompted us to investigate whether NS1 on surface of infected cells could activate the complement system in the presence of NS1 specific antibodies. DV-infected HEK 293T cells were incubated with each clone of anti-NS1 mAbs or mixtures of all clones plus 25% fresh dengue non-immune serum as complement source. Consequently, the deposition of C3dg fragments was analyzed by flow cytometry. As shown in Fig. 6, complement activation on the surface of DV-infected HEK 293T cells in the presence of 1F11 and 2G6 showed significant C3dg deposition while complement activation induced by 1A4 and 1B2 resulted in a slight increase of C3dg deposition compared with the condition without anti-NS1 mAbs or

with isotype control antibodies. Additionally, in the presence of mixture anti-NS1 mAbs (1A4, 1B2, 1F11 and 1F11), DV-infected cells efficiently activated complement resulting in the highest amounts of C3dg deposition on complement-attacked-surfaces.

Complement activation by cells stably expressing NS1 is antibody dependent

Complement activation of membrane associated NS1 on the surface of DV-infected cells was confirmed by using cells stably expressing NS1. Three types of transfected cells, rNS1m (cells transfected with plasmid containing natural dengue NS1 plus 26 amino acid of membrane anchoring domain of NS2A), rNS1s (cells containing plasmid of natural NS1 without membrane anchoring domain of NS2A) and rControl (cells transfected with empty vector) were used in the experiments. Surface NS1 expression of transfected cells was determined by mouse mAbs specific for NS1 protein (1A4). More than 70% of rNS1m cells were positive for surface NS1 expression (Fig. 7) whereas rNS1s and rControl cells were negative for surface NS1 detection as well as mock-infected cells. DV-infected HEK 293T cells served as positive controls for surface NS1 staining in Fig. 7. Fig. 8 demonstrated C3dg deposition after rNS1m was treated with 25% fresh non-immune serum plus PCS at 400 μ g/ml but not with PND or without anti-dengue antibodies. C3dg deposition on surface of DV-infected cells was observed when the cells were incubated with fresh non-immune serum plus lower concentration of PCS (25 μ g/ml). These results were in line with the previous experiments that higher concentration of PCS (400 μ g/ml) was needed to positively stain surface NS1 of rNS1m while only 25 μ g/ml of PCS was able to give positive staining on the surfaces of DV-infected cells (data not shown). The deposition of C3dg on rNS1m was also seen after complement treatment in the presence of 25 μ g/ml of a mixture of anti-NS1 mAb (1A4, 1B2, 1F11 and 1F11) similar to that of DV-infected cells (Fig. 8B). The deposition of C3dg was not detected on the surfaces of rNS1s and rControl after complement treatment in the presence of PCS, PND or mixed isotype control antibodies (Fig 8A, B). Complement activation of membrane associated NS1 in the presence of a mixture of anti-NS1 antibodies was also confirmed by co-localization of NS1 and C3 fragments on the surfaces of both DV-infected cells (Fig. 9A) and cells stably expressing NS1 (Fig.

9B). Co-localization of membrane associated NS1 and C3dg was also observed after complement treatment in the presence of PCS (data not shown).

Complement activation by DV-infected HEK 293T and cells stably expressing NS1 reached the terminal pathway

The insertion of complement terminal complexes (C5b-9) on plasma membrane of rNS1m and DV-infected cells after complement treatment in the presence of PCS at 400 µg/ml and 25 µg/ml respectively was shown in Fig. 10. C5b-9 formation was not seen when incubating the cells in the presence of PND or without DV-specific antibodies.

Antibody-dependent complement activation of membrane associated NS1 occurred via both classical and alternative pathway

Next pathway of complement activation by membrane associated NS1 was clarified using buffer containing 10 mM EDTA or 10 mM EGTA plus 10 mM MgCl₂ to inhibit both classical and alternative or only classical pathway respectively. Complement activation by DV-infected cells in the presence of PCS (Fig. 11A) or NS1 specific antibodies (Fig 11B) occurred through both classical and alternative pathway since the addition of 10 mM EGTA and 10 mM MgCl₂ only partially inhibited the deposition of C3dg on the surface of cells while the C3dg deposition was not seen when using heat inactivated serum or serum containing 10 mM EDTA.

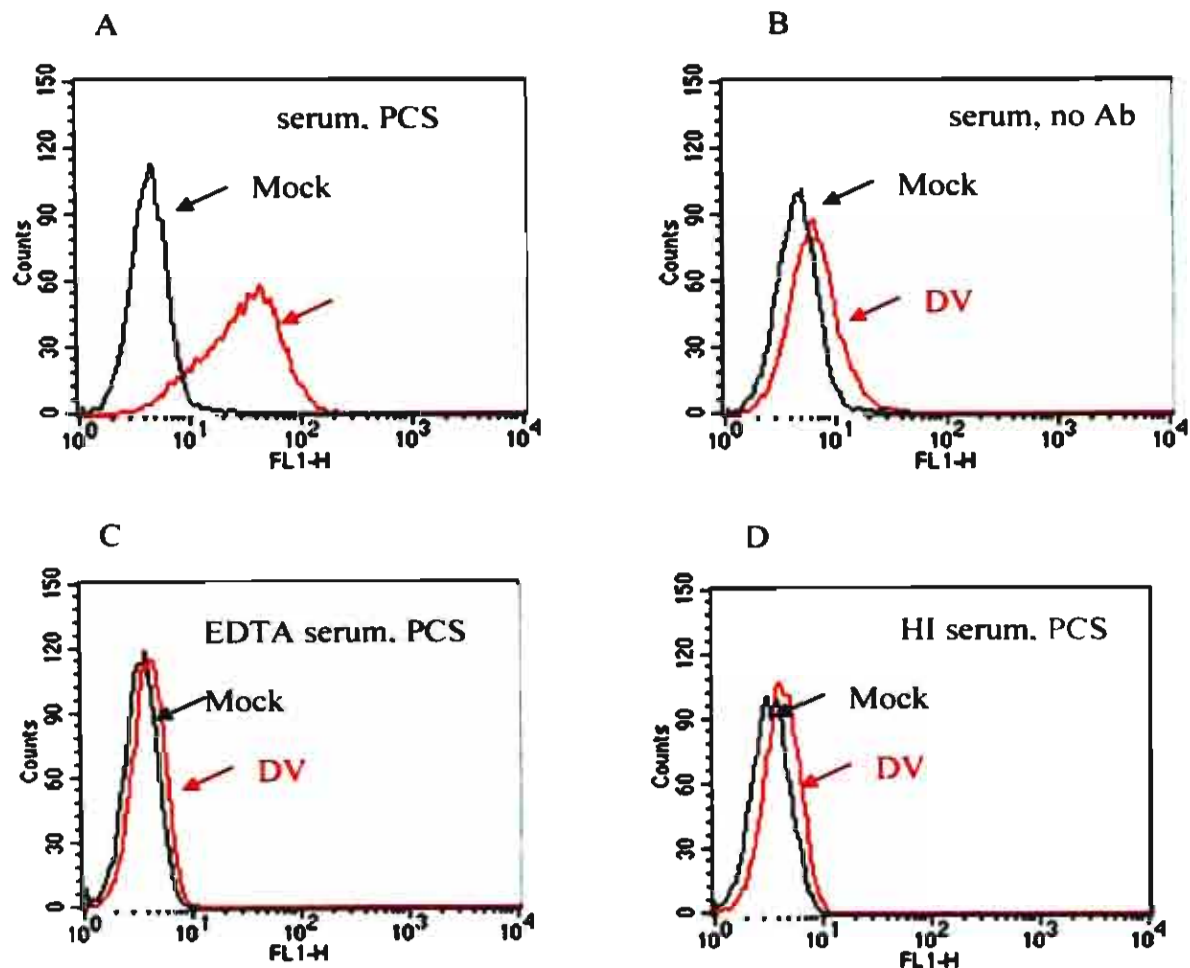


Figure 4 Histograms of fluorescence intensity showing the deposition of C3dg on the surface of DV-infected HEK-293T cells

Mock and DV-infected HEK 293T cells at 24 h after infection were incubated with 25% dengue-non immune serum in the presence (A) or absence (B) of PCS at 37°C for 1 h. Inhibition of complement activation by 10 mM EDTA (C) or heat inactivated (HI) serum (D) served as negative controls. After that, the deposition of C3dg was detected by using its specific mAbs, clone 9 and followed by FITC-conjugated rabbit anti-mouse Igs. Propidium iodide (0.2 µg/ml) was added to cell suspension prior to flow cytometry analysis. The histograms of green fluorescence intensity of viable propidium iodide-negative populations of infected (red line) and mock (black line) cells were depicted. The results shown here was the representative of 3 independent experiments.

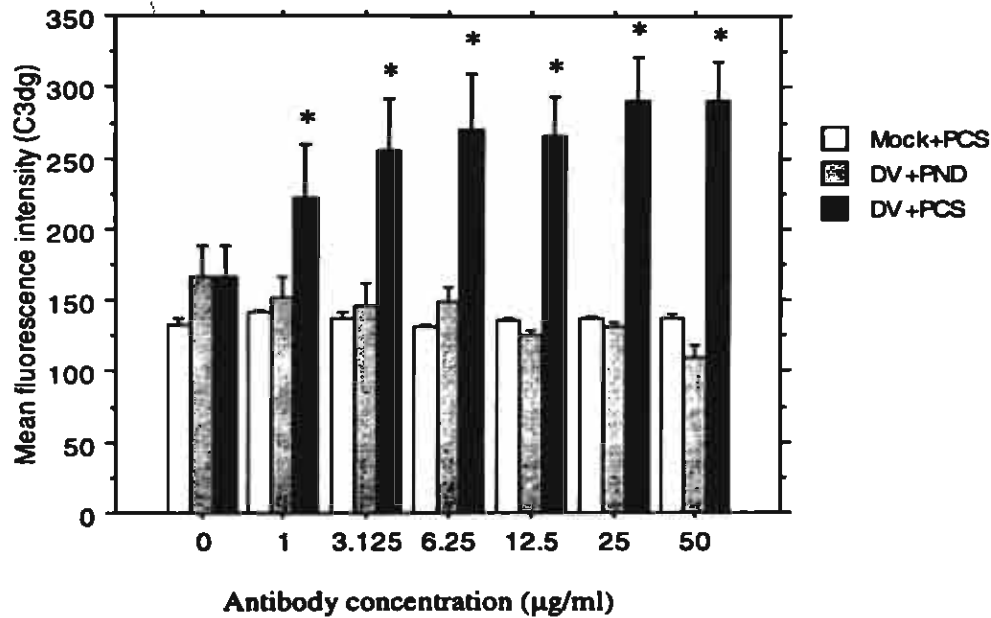


Figure 5 Dose-dependent enhancement of complement activation on DV-infected HEK-293T cells by anti-DV-antibodies

Mock and DV-infected HEK 293T cells at 24 h after infection were incubated with 25% fresh dengue-non immune serum in the presence of various concentrations of PCS and PND (1, 3.125, 6.25, 12.5, 25 and 50 µg/ml) at 37°C 1 h. The deposition of C3dg was consequently detected by using mAb specific for a neoantigen, clone 9 and followed by with FITC-conjugated rabbit anti-mouse Igs. Propidium iodide (0.2 µg/ml) was added to cell suspensions prior to flow cytometry analysis. Each bar represents the population of propidium iodide-negative cells that are positive for surface C3dg staining. The data represents the mean fluorescence intensity of surface C3dg deposition from of 3 independent experiments $\pm$ SD. * indicated p value < 0.05 (mock vs DV).

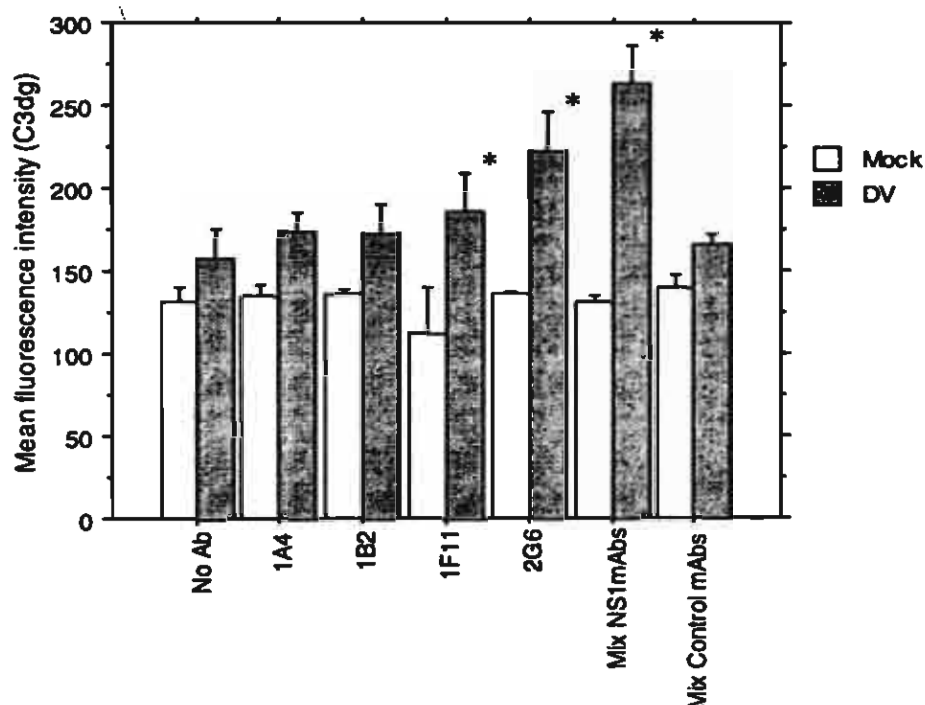


Figure 6 NS1-specific mAbs enhanced complement activation by DV-infected HEK-293T cells

Mock and DV-infected HEK 293T cells at 24 h after infection were incubated with 25% fresh dengue non immune serum in the presence of 25 μ g/ml of NS1-specific mAbs (1A4, 1B2, 1F11, 2G6, or a mixture of all clones (mix NS1mAbs) and a mixture of isotype control antibodies (mix Control mAbs) at 37°C 1 h. The deposition of C3dg was consequently detected by using mAb specific for a neoantigen, clone 9 and followed by FITC-conjugated rabbit anti mouse Igs. Propidium iodide (0.2 μ g/ml) was added to cell suspensions prior to flow cytometry analysis. Each bar represents the population of propidium iodide negative cells that are positive for surface C3dg staining. The data represents the mean fluorescence intensity of surface C3dg deposition from 3 independent experiments $\pm$ SD. * indicated p value < 0.05 (mock vs DV).

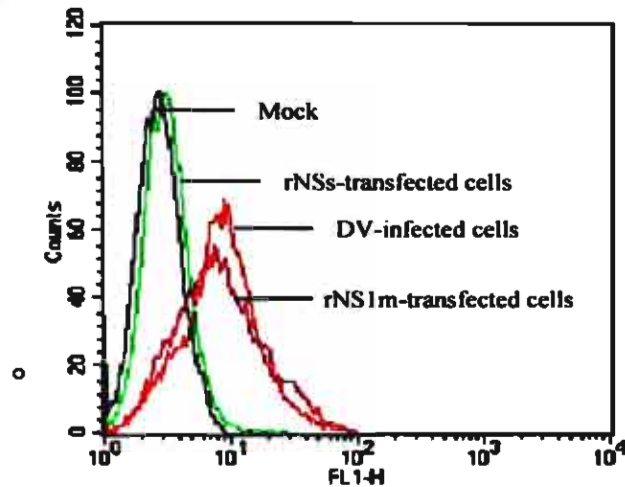


Figure 7 NS1 expression on the surface of cells stably expressing NS1

Cells stably expressing NS1, rNSm were grown in T25 cm² flasks for 48 h, after that the cells were harvested, washed and stained with anti-NS1 specific mAb (1A4) and followed by FITC-conjugated rabbit anti-mouse Igs. Propidium iodide (0.2 µg/ml) was added to cell suspensions prior to flow cytometry analysis. The histograms of green fluorescence harbored propidium iodide negative populations were demonstrated.

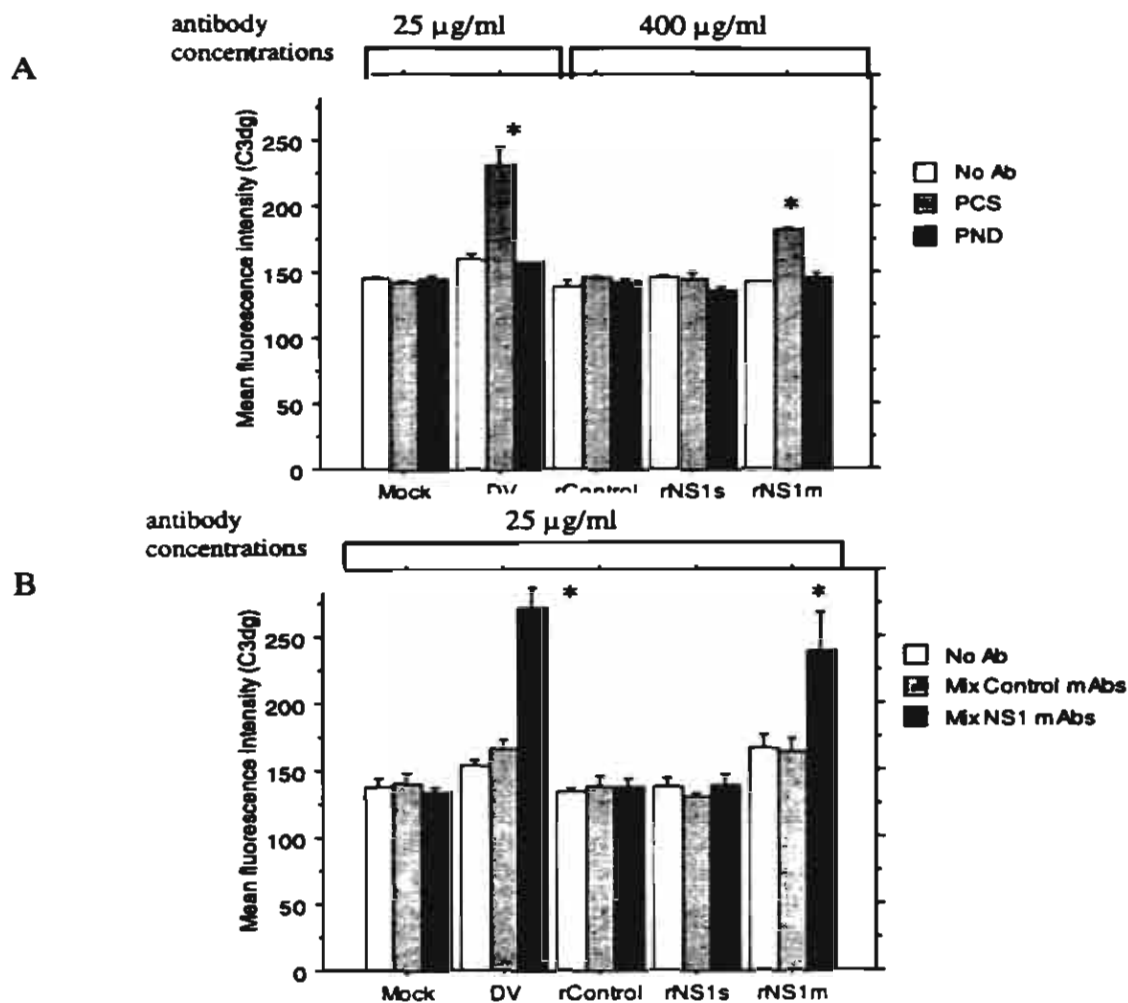


Figure 8 Complement activation by cells stably expressing NS1 is antibody dependent

rNS1m, rNS1s and rControl cells were incubated with 25% of fresh dengue non-immune serum in the presence of 400 µg/ml of PCS or PND (A) or 25 µg/ml of a mixture of NS1-specific mAbs (MixNS1mAbs) or a mixture of isotype control antibodies (Mix ControlmAbs) (B). DV-and mock-infected cells were incubated with 25% of fresh dengue non-immune serum plus 25 µg/ml of PCS or PND (A) or equivalent concentrations of MixNS1mAbs and Mix ControlmAbs (B). The deposition of C3dg was consequently detected by using mAb specific for a neoantigen, clone 9 and followed with FITC-conjugated rabbit anti-mouse Igs. Propidium iodide (0.2 µg/ml) was added to cell suspensions prior to flow cytometry analysis. Each bar represents the population of propidium iodide negative cells that are positive for surface C3dg staining. The data represents the mean fluorescence intensity surface C3dg position from 3 independent experiments $\pm$ SD. * indicated p value < 0.05 (PCS vs PND or MixNS1mAbs vs Mix ControlmAbs).

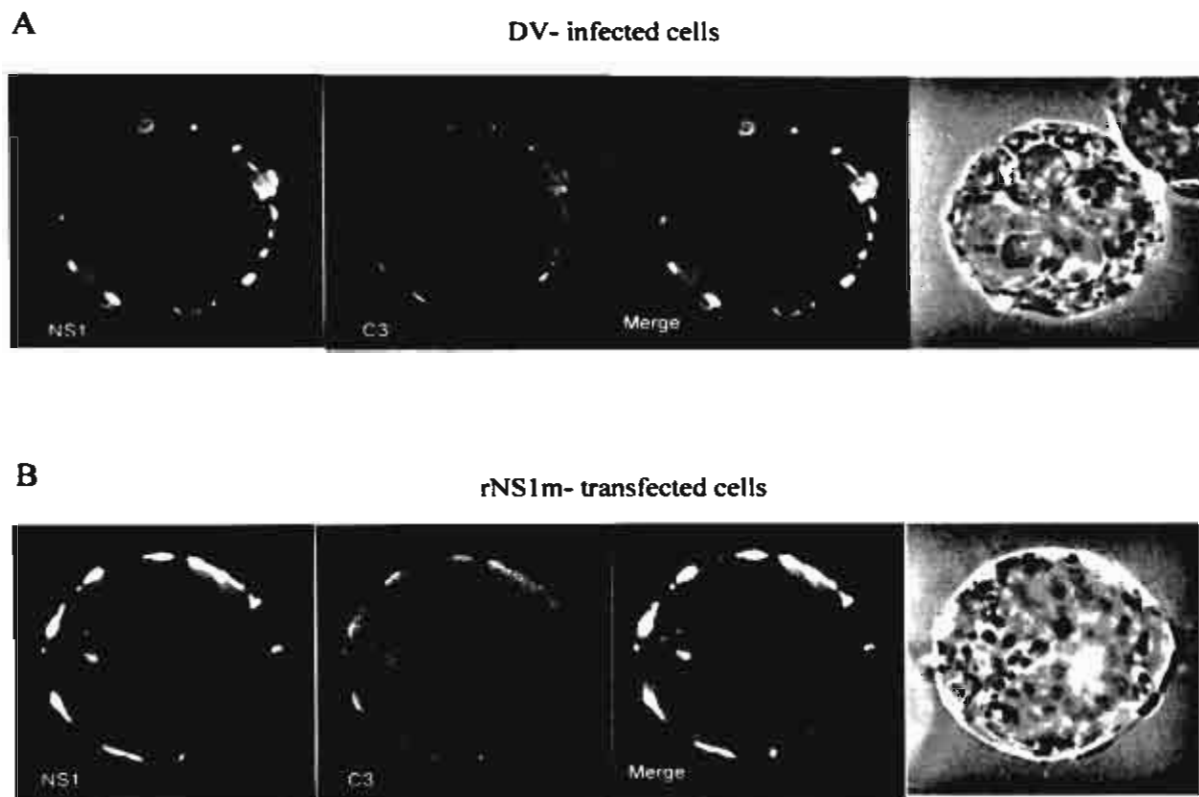


Figure 9 Co-localization of NS1 and complement C3 fragments on the surface of complement attacked cells

DV-infected HEK 293T (A) or cells stably expressing NS1 (B) were incubated with 25% of dengue-nonimmune serum in the presence of a mixture of NS1 mAbs (25 μ g/ml) at 37°C for 1 h. After washing, the cells were stained NS1 (red, Cy3) and complement C3 fragments (green, FITC) with fluorescent-conjugated antibodies and observed under a zeiss LSM 510 META confocal microscope.

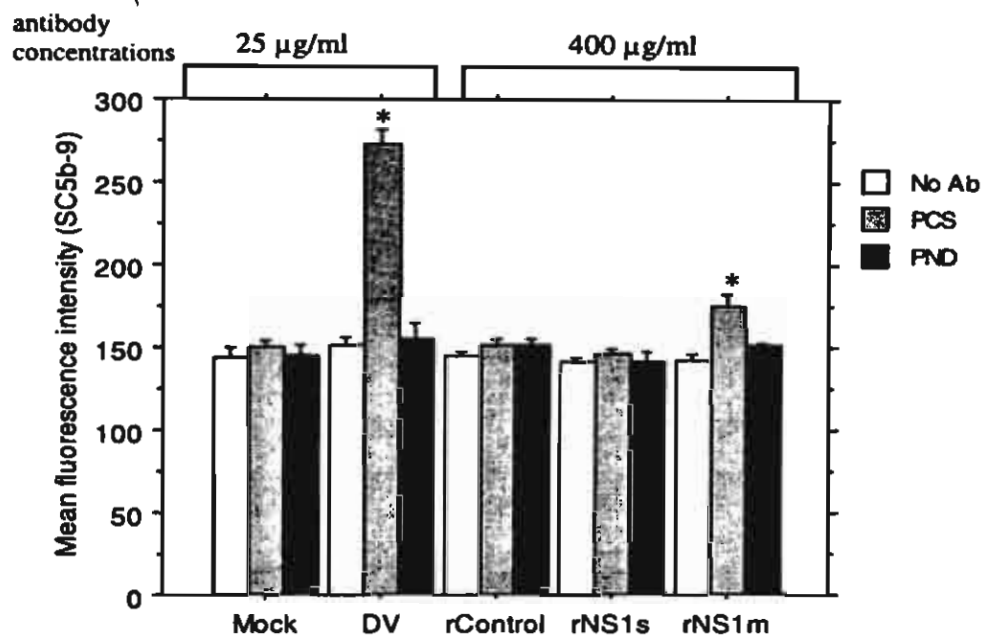


Figure 10 Membrane associated NS1 activated complement to completion

DV-and mock-infected cells were incubated with 25% of fresh dengue non-immune serum plus 25 µg/ml of PCS or PND for 37°C 1 h. rNS1m, rNS1s and rControl cells were incubated with 25% of fresh dengue non-immune serum in the presence of 400 µg/ml of PCS or PND for 37°C 1 h. The formation of C5b-9 complexes were subsequently detected by mAbs specific for C5b-9 and followed by FITC-conjugated rabbit anti-mouse Igs. Propidium iodide (0.2 µg/ml) was added to cell suspensions prior to flow cytometry analysis. Each bar represents the population of propidium iodide negative cells that are positive for surface C5b-9 complex formation. The data represents the mean fluorescence intensity of surface C5b-9 from 3 independent experiments $\pm$ SD. * indicated p value < 0.05 (mock vs DV).

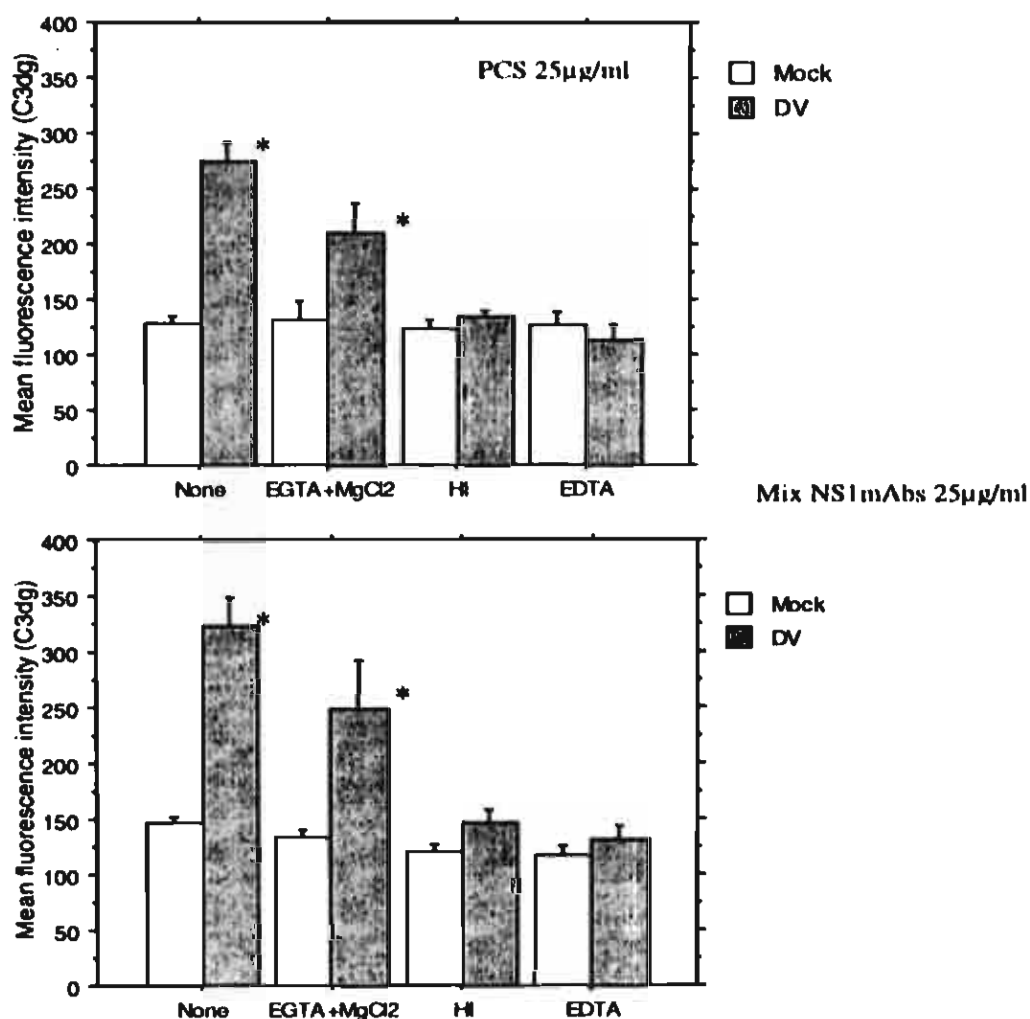
B

Figure 11 Antibody dependent-complement activation of membrane associates NS1 activated occurred via classical and alternative pathway

Mock and DV-infected HEK 293T cells at 24 h post infection were harvested and incubated with 25% of fresh dengue non immune serum in the presence of 25 µg/ml of PCS or PND for 1 h 37°C. In some experiments, 10 mM EDTA or 10 mM EGTA plus 10 mM MgCl₂ was added to determine pathway of complement activation. Subsequently, the deposition of C3dg was determined by C3dg specific mAbs and followed by FITC-conjugated rabbit anti-mouse Igs. Propidium iodide (0.2 µg/ml) was added to cell suspensions prior to flow cytometry analysis. Heat inactivated (HI) serum to destroy functions of complement was used as negative control. Each bar represents the population of propidium iodide negative cells that are positive for surface C3dg deposition. The data represents the mean channel fluorescence intensity of surface C3dg deposition from 3 independent experiments $\pm$ SD. * indicated p value <0.05 (mock vs DV).

Study of complement fixing capability of soluble DV-NS1

Culture supernatants from DV-infected PscloneD activated human complement system

As shown in Fig. 12, culture supernatants taken from DV-infected PscloneD cells but not mock-infected cells were capable of activating complement system independent of dengue specific antibodies in a dose dependent manner. However, the activation was enhanced by anti-dengue antibodies (B).

Complement activation by DV-infected supernatants was enhanced by NS1 specific antibodies

Only NS1 specific mAbs clone 2G6 and 2E11 significantly enhanced complement activation by DV-infected supernatants, while 1A4, 1B2, 1F11 did not (Fig. 13). The activation was much enhanced when a mixture of mAbs specific for NS1 was used. Isotype control antibodies could give not this effect in culture supernatants from mock and DV-infected cells (Fig. 13A). Fig. 12B demonstrated dose-dependent effect of a mixture of mAbs specific to NS1 in enhancing complement activation by DV-infected supernatants. At 12.5 µg/ml of a mixture of anti-NS1 antibodies, complement activation by DV-infected supernatants reached its highest activity.

Purified DV-NS1 was capable of activating human complement system and reached the terminal pathway

Purified DV-NS1 obtained from affinity chromatography was checked for its purity and immunogenicity by SDS-PAGE and western blotting analysis (Fig. 14). Purity and immunogenicity of purified NS1 was demonstrated by a single band on the SDS-PAGE by silver staining (Fig. 14A) and by western blot analysis (Fig. 14B) using NS1 specific monoclonal antibodies (1F11). A band of approximately 45 kDa was observed after heat treatment representing monomeric NS1 while under unheated condition, NS1 was retained in its dimeric form of 90 kDa (Fig. 14A, B). Purified NS1 also activated human complement system in a similar manner of DV-infected culture supernatants. Complement activation independent occurred of NS1 specific antibodies and also reached the terminal pathway generating fluid phase SC5b-9

complexes as detected by ELISA. A dose dependent manner of purified NS1 from DV-infected cells and cells stably expressed NS1; rNS1s in complement fixation was demonstrated in Fig. 15. The activation was also enhanced in the presence of PCS and antibodies specific to NS1 but not by PND or isotype control antibodies (Fig. 15 A-B). The generation of SC5b-9 complexes was not observed in the presence of 10 mM EDTA.

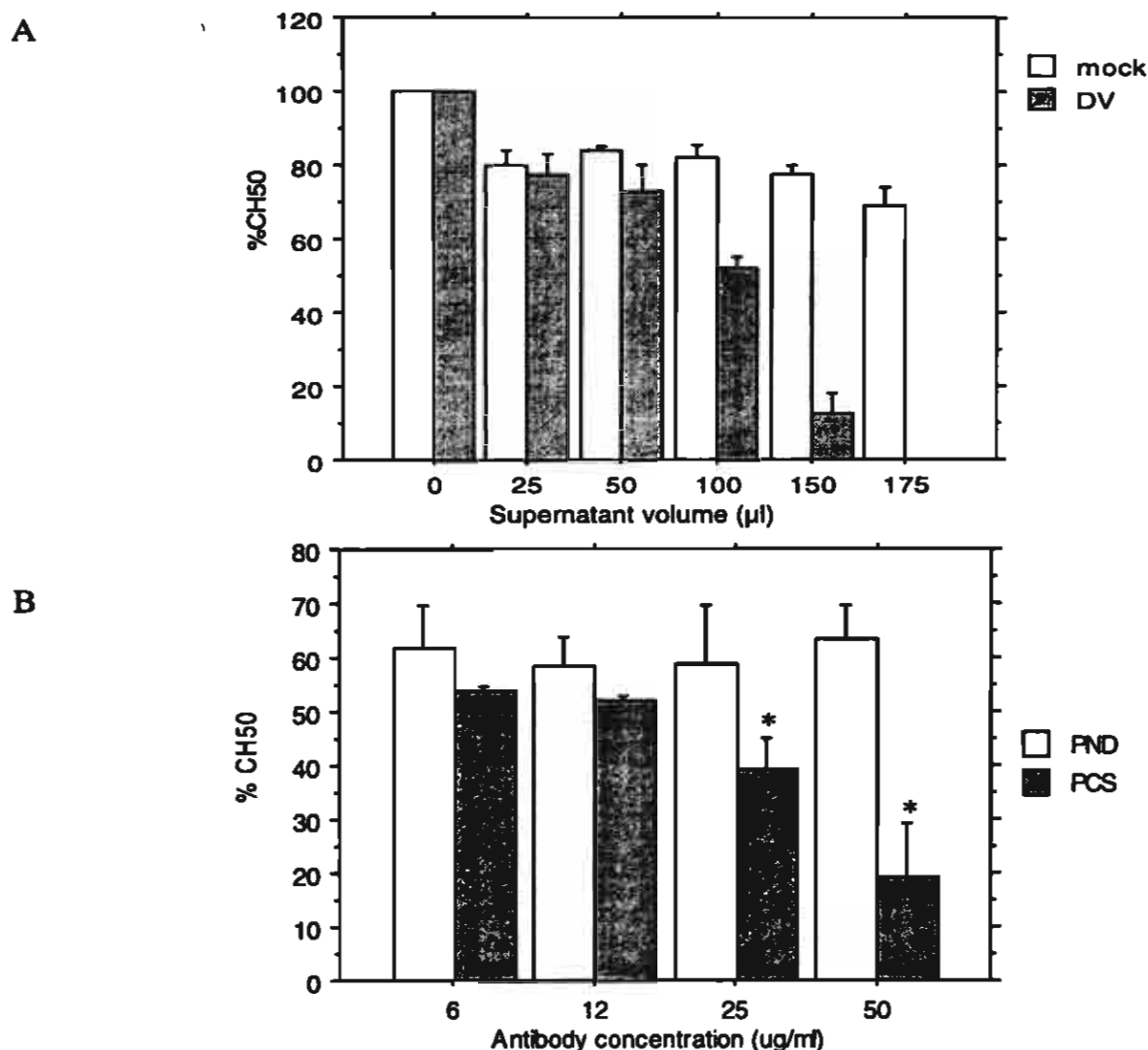
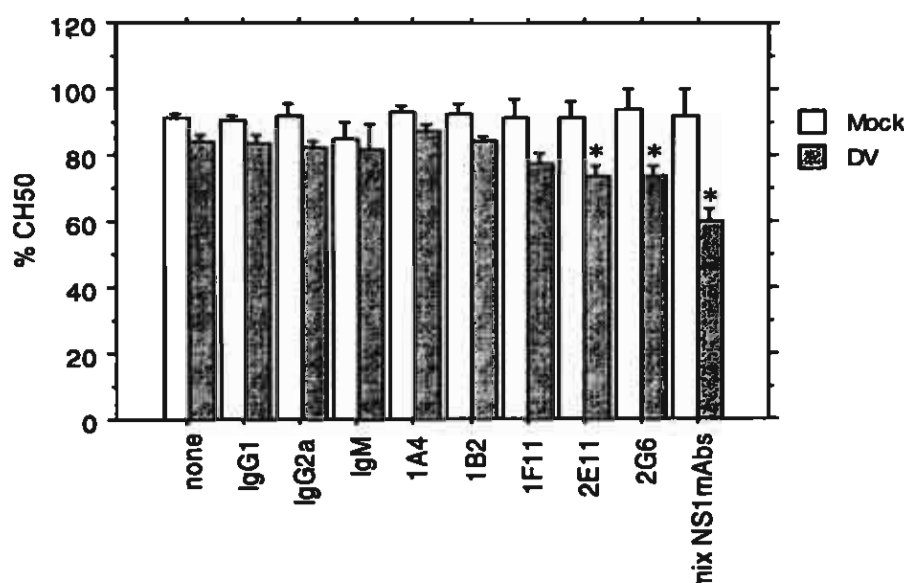


Figure 12 DV-infected supernatants spontaneously activated human complement system and the activation was enhanced by dengue-specific antibodies

Various amounts of culture supernatants (25, 50, 100, 150 and 175 μl) from DV and mock-infected cells (A) and 100 μl of DV-infected supernatants in the presence of PCS or PND (6, 12, 25 and 50 μg/ml) (B) were incubated in the total volume of 200 μl with 1:8 dilution (12.5%) of human dengue non immune serum as complement source at 37°C for 1 h. After that, samples were two-fold serially diluted and determined hemolytic complement assay (CH50). The results were demonstrated as %CH50 of each sample compared with serum control. The data was derived from 3 independent experiments $\pm$ SD. * indicated p value < 0.05 (mock vs DV and PCS vs PND).

A



B

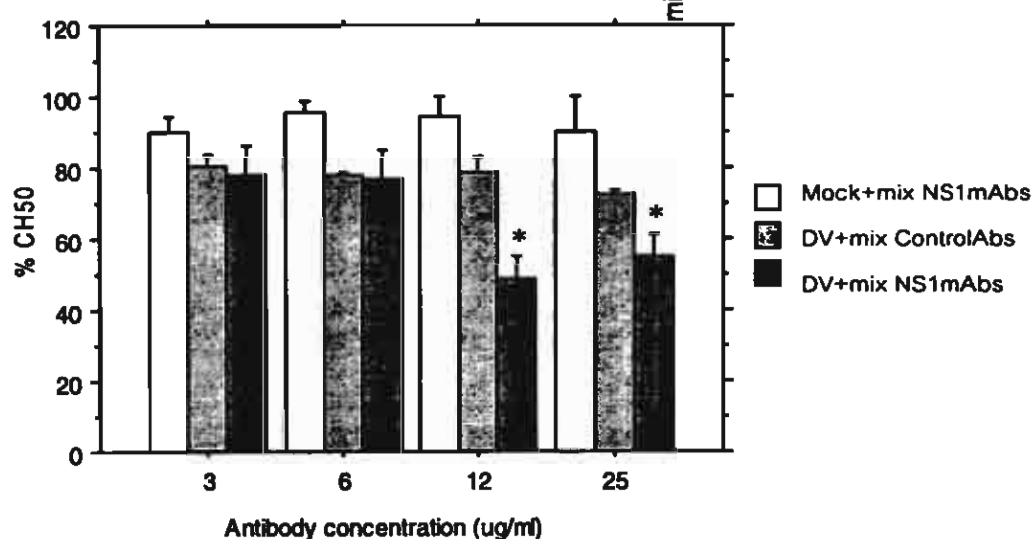


Figure 13 NS1 specific antibodies enhanced complement activation by DV-infected culture supernatants

100 µl of culture supernatants from mock and DV-infected cells were incubated in the total volume 200 µl with 1:8 dilution (12.5%) of human dengue non immune serum as complement source in the presence of 12.5 µg/ml NS1 mAbs or isotype control antibodies (A) and various concentration (3, 6, 12, and 25 µg/ml) of a mixture anti-NS1 antibodies or a mixture of isotype control antibodies (B) at 37°C for 1 h. After that, samples were two-fold serially diluted and determined hemolytic complement assay (CH50). The results were demonstrated as %CH50 of each sample compared with serum control. The data were derived from 3 independent experiments $\pm$ SD. * indicated p value < 0.05 (mock vs DV or mix control mAbs vs mix NS1mAbs).

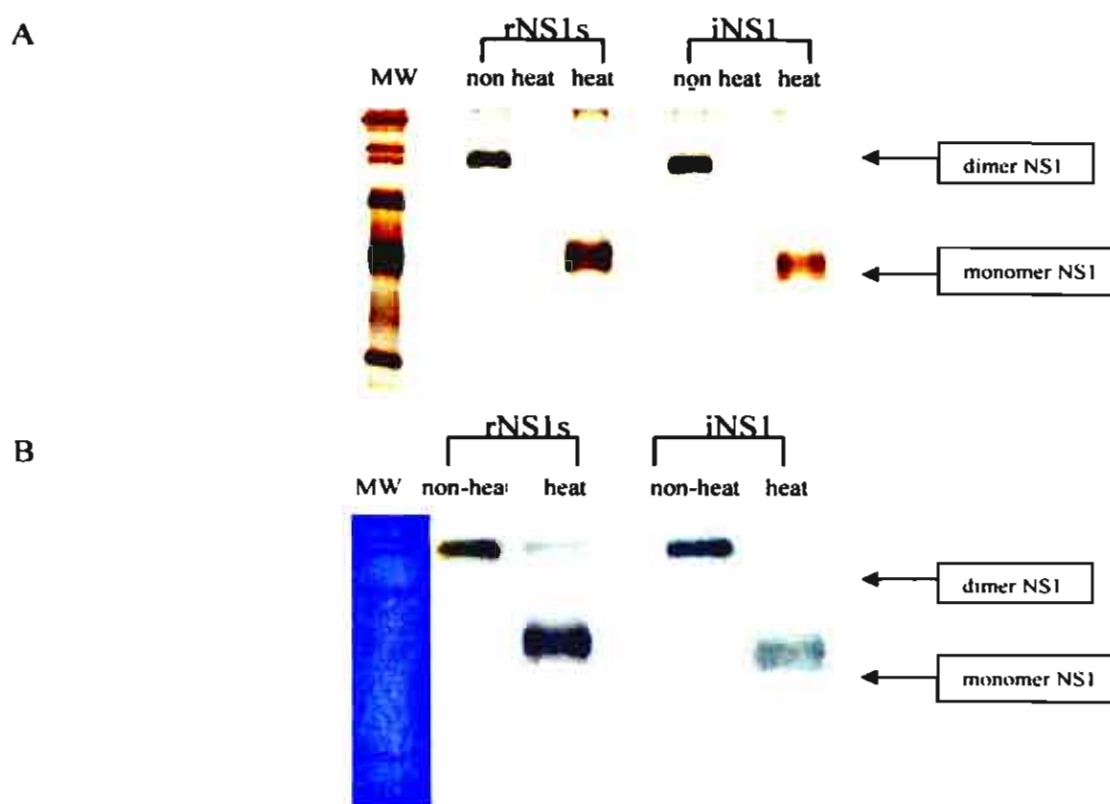


Figure 14 Purity and immunogenicity of purified DV-NS1 obtained from affinity chromatography

1 μ g/well of NS1 purified from DV-infected (iNS1) and cells stably expressed NS1 (rNS1s) in heat (96°C, 10 min) and non-heat treatment was loaded in 12% SDS-PAGE and subjected to silver staining (A) and western blotting using with anti NS1 specific antibody (B).

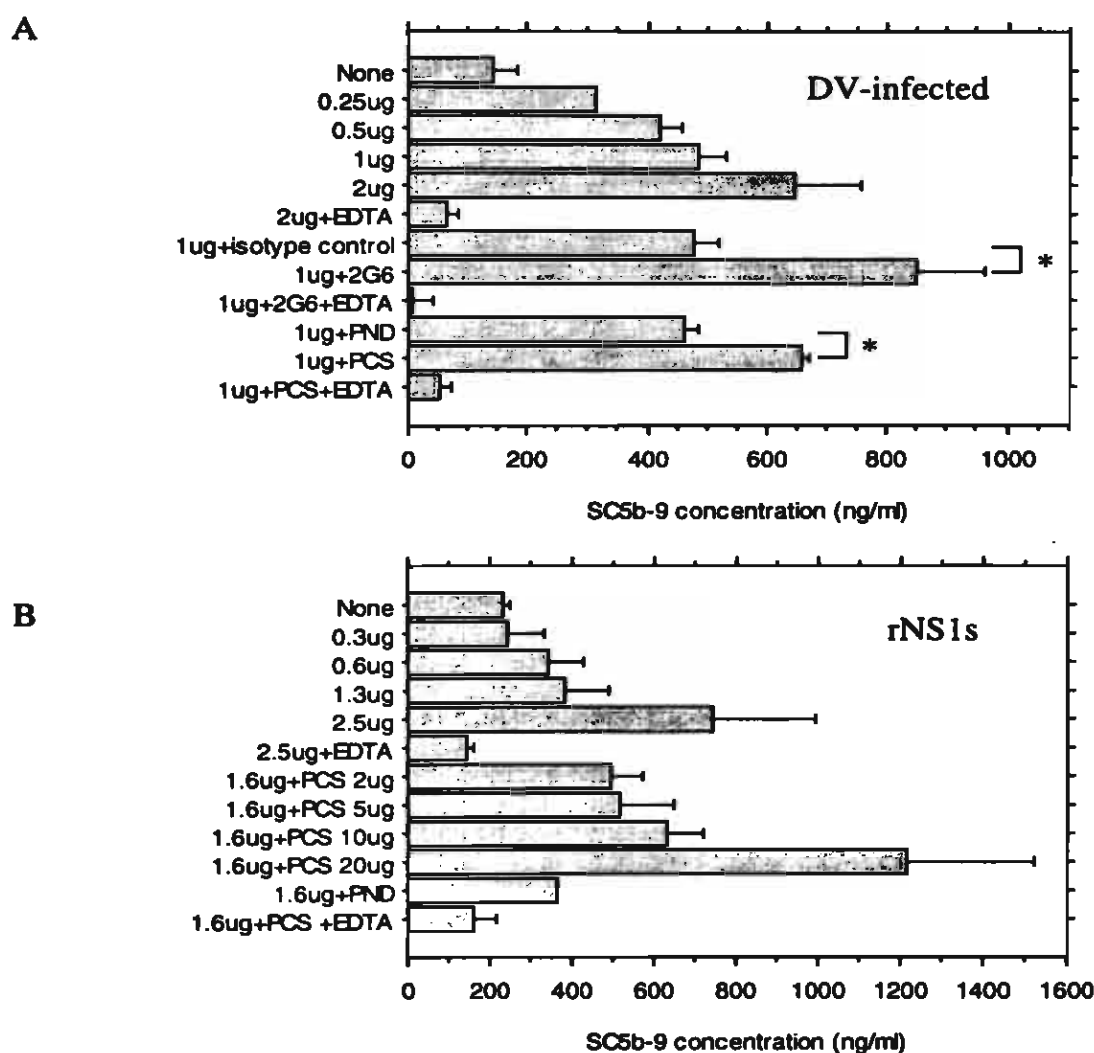


Figure 15 Purify DV-NS1 activated complement to completion

Various amounts of purified NS1 from culture supernatants of DV-infected cells (A) and cells stably expressed NS1; rNS1s (B) were incubated in the total volume 200 μ l with 1:8 dilution (12.5%) of human dengue non-immune serum in the presence or absence of PCS (20 μ g/ml) or anti-NS1 specific mAb 2G6 (10 μ g/ml) at 37°C for 1 h. Generation of fluid phase SC5b-9 was determined by ELISA. Equivalent concentrations of PND or isotype control antibodies were used as negative controls. The data represents the mean of SC5b-9 concentration $\pm$ SD from three independent experiments. * indicated p value < 0.05.

DV-RNA, soluble NS1, and complement activation products in clinical specimens

A total of 182 patients admitted to the pediatric ward of Khon Khan Provincial Hospital between November 2001 and December 2003 were enrolled into this prospective study. The overall male-to-female ratio was 1:1, and the overall mean age was 9.6 ± 3 (range 2-15, median 9) years. There were no major differences in male-to-female ratios and mean ages of patients in each group. According to the WHO criteria, the final diagnosis was DF for 49 patients and DHF for 114, including 44 of grade 1, 44 of grade 2 and 26 cases of grade 3 or dengue shock syndrome (Table 2). The residual 19 cases were diagnosed as OFI.

Dengue RT-PCR was positive for 151 out of 163 patients (92.6%). Virus types identified were DEN-1 ($n = 87$), DEN-2 ($n = 52$), DEN-3 ($n = 6$), and DEN-4 ($n = 6$). A total of 148 patients (90.8%) were diagnosed as secondary infection, while 15 patients (9.2%) had a primary dengue infection.

Viremia levels during acute phase of illness were compared over time. Highest viremia levels were detected early in clinical illness for all groups of dengue-patients and gradually declined to undetectable levels on day + 1 in DF or on day + 2 in DHF (Fig. 16A). Similar delayed virus removal from the circulation of DHF compared with DF patients has been observed earlier (39, 40). Viral clearance in patients with shock was significantly slower than in non-shock patients; mean viremia levels at day +1 of shock and non-shock cases were 62.9 ± 206 (range, 0-545 PFU/ml) and 8.9 ± 61 PFU/ml (range, 0-869 PFU/ml) respectively ($p < .05$). In confirmation of a previous report (41), mean levels of dengue viral RNA were higher in DHF grade 3 patients than in patients with DF or DHF grade 1 and 2 and reached statistical significance at day -1 ($p < .001$).

Unlike these kinetics of DV RNA levels, plasma NS1 levels were relatively lower in the early febrile days and peaked at day -2 (Fig. 16B). In three patients, plasma NS1 levels were extremely high at day -2 (3911, 3974, and 4474 ng/ml) and these values were not included in the statistical analysis. Mean levels of soluble NS1 in DHF (383.9 ± 620 ng/ml; range, 60-4151 ng/ml; median, 166.3 ng/ml) were higher than those of DF patients (181.6 ± 120 ng/ml; range, 78-895 ng/ml; median, 137.7 ng/ml) during acute illness (disease day ≤ 0 , $p = .003$). At day -3, -2, and -1, mean NS1 levels were 162.7 ± 61 (range, 95-267 ng/ml; median, 148.7 ng/ml), 211.4 ± 96 (range, 91-384 ng/ml; median, 218.3 ng/ml), 203.1 ± 164 ng/ml (range, 80-896 ng/ml; median,

140 ng/ml) for DF and 433.4 ± 306 (range, 141-1028 ng/ml; median, 357.4 ng/ml), 500 ± 400 (range, 99-1616 ng/ml; median, 361 ng/ml), and 438.1 ± 640 ng/ml (range, 88-3181 ng/ml; median, 183.9 ng/ml) for DHF respectively ($p=.01$, $.01$, and $.05$). At the time of maximum leakage or shock (day 0), mean levels of NS1 in DHF3 (666.2 ± 1274 ng/ml; range, 77-4152 ng/ml; median, 134.5 ng/ml) were significantly higher than in the non-shock cases (DHF2 [mean, 305.9 ± 593 ng/ml; range, 60-3785 ng/ml; median, 157.9 ng/ml; $p=.01$], DHF1 [mean, 172.2 ± 160 ng/ml; range, 83-1004 ng/ml; median, 124.4 ng/ml; $p=.001$], and DF [mean, 163.6 ± 98 ng/ml; range, 78-538 ng/ml; median, 131.9 ng/ml; $p<.001$]). Unlike viral load, enhanced levels of NS1 in DHF3 patients at day-1 were at the borderline of significance compared with patients of other groups. At day +1, soluble NS1 was cleared from the circulation in almost all cases (Fig. 16B).

Levels of the terminal complement complex SC5b-9 were measured in the same blood samples (Fig. 16C). SC5b-9 plasma concentrations were significantly higher in DHF patients (mean, 306.9 ± 174 ng/ml) as compared to DF (mean, 225.3 ± 97 ng/ml; $p<.001$) and OFI (mean, 170.3 ± 57 ng/ml; $p<.001$) patients during acute illnesses (disease day < 2). There was also a significant difference between SC5b-9 levels in DF compared to patients with OFI ($p=.02$). At day -1, there was a correlation trend between SC5b-9 levels and disease severity: mean values were highest for DHF3 and lowest for DF (Fig. 16C). The difference in mean levels of SC5b-9 in shock and non-shock cases was statistically significance ($p<.05$).

Table 2. Age and sex of children enrolled from November 2001 to December 2003

Diagnosis	Number	Mean age (years)	Male to female ratio
DF	49	9.12	1.04:1
DHF I	44	10.07	1.44:1
DHF II	44	10.11	1.2:1
DHF III	26	9.31	0.63:1
OFI	19	9	0.46:1
Total	182		

OFI, other febrile illness; DF, dengue fever; DHF I, II, and III, dengue hemorrhagic fever grade 1, 2, and 3.

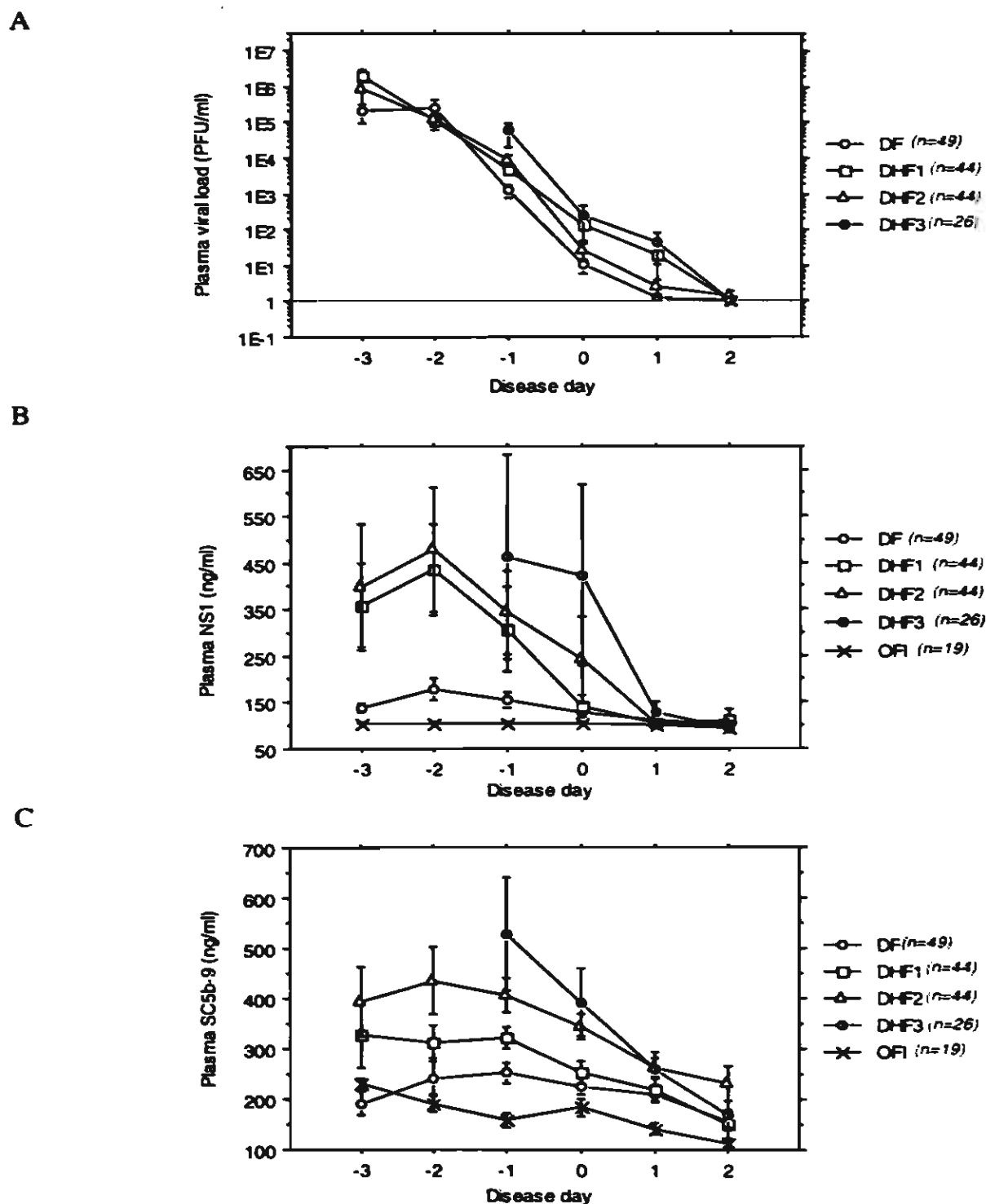


FIGURE 16 Viral load, levels of NS1, and terminal SC5b-9 complexes in the circulation of patients with DF and DHF/DSS. Plasma samples were assayed for dengue viral RNA levels using quantitative real time RT-PCR, and soluble NS1 and SC5b-9 complexes were quantified by ELISA. Disease day 0 was defined as the calendar day during which the temperature fell and stayed below 37.8 °C. Plasma samples from patients with acute febrile diseases other than dengue (OFI-other febrile illness) were also used as controls.

Soluble NS1 and complement activation products in pleural fluids of DSS patients

NS1, complement anaphylatoxins C3a and C5a, and SC5b-9 were measured in pleural fluids and in plasma of 9 patients with DSS. Samples were collected at the day of shock or 1-2 days later. Identification of viral RNA was also performed using nested RT-PCR. The results are depicted in Fig. 17. Soluble NS1 was detected in 6 pleural specimens, while only 4 of these were positive for DV. In 3 cases with undetectable NS1 in both plasma and pleural fluids, the specimens were collected after the day of shock (7-9, Fig. 17). In eight cases (1-8, Fig. 17), the quotients between albumin concentrations in pleural fluids versus plasma were 0.7- 1.5, typical of exudates. In one case, the quotient was approximately 0.28, indicative of considerable transudation (9, Fig. 17). The specimens in this case were obtained several days after shock.

NS1 concentrations displayed large variations. In four cases, (1-4, Fig. 17), concentrations ranging from 116-120 ng/ml and 122-337 ng/ml were found in plasma and pleural fluids respectively. In all cases, levels in pleural fluids were equivalent (1, Fig. 17) or higher than in plasma (2-4, Fig. 17). In one case (5, Fig. 17), the NS1 level was relatively low in plasma and 20-fold higher in the pleural fluid. In case 6 (Fig. 17), concentrations were very high (about 2000 ng/ml) in both plasma and pleural fluid. NS1 was not detectable in case 7-9 (Fig. 17).

Pleural fluid concentrations of SC5b-9 were markedly higher than the plasma concentrations in all but one case where the levels were equivalent (4, Fig. 17). Mean SC5b-9 levels in pleural fluids were 2575.9 ± 1121 ng/ml; range, 627-4865 ng/ml; median, 2312.5 ng/ml, and were significantly higher than plasma concentrations (1546.3 ± 943 ng/ml, range, 394-2935 ng/ml; median, 1722 ng/ml; $p=.04$). A similar trend was found for C5a: levels of this anaphylatoxin in pleural fluids were 47.4 ± 61.1 ng/ml; range, 7-227 ng/ml; median, 23 ng/ml, and were also greater than in plasma (25.6 ± 33.9 ng/ml; range, 5-114 ng/ml; median, 15 ng/ml; $p=.34$).

When quotients obtained for NS1, SC5b-9 and C5a shown above were plotted against the respective quotients for albumin in the individual patients, almost all plotted values came to lie above the diagonal, which indicated relative accumulation of the analytes, probably due to their local generation at the site of leakage (Fig. 18).

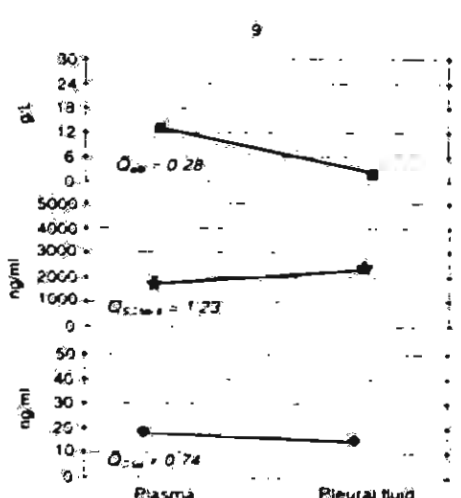
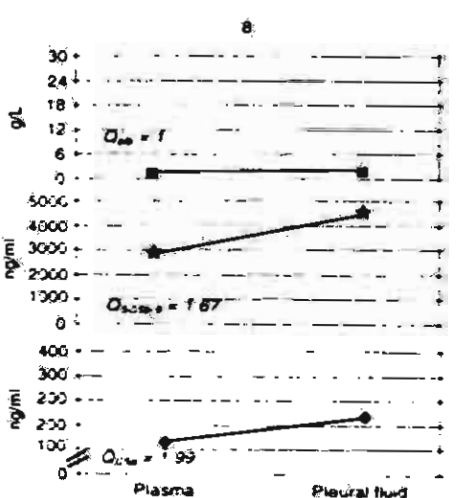
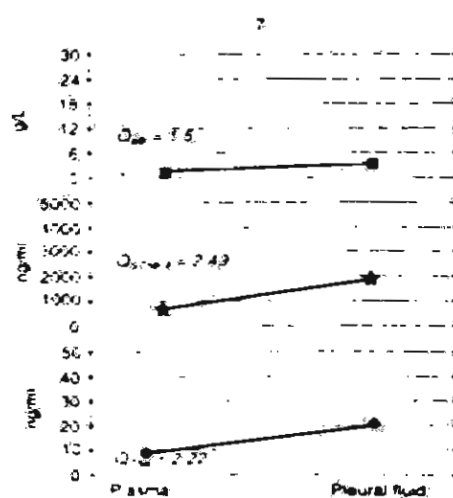
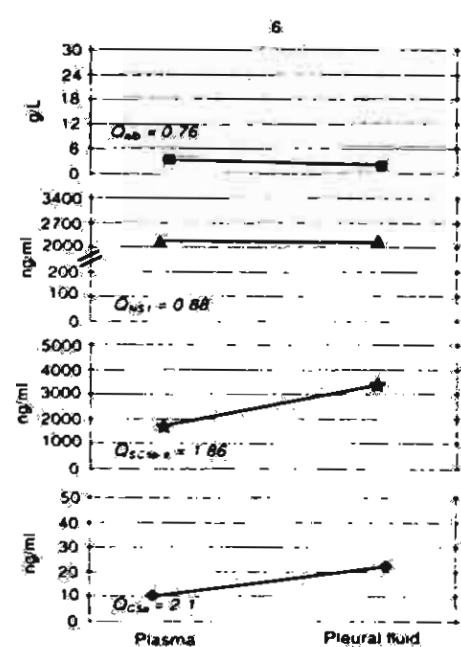
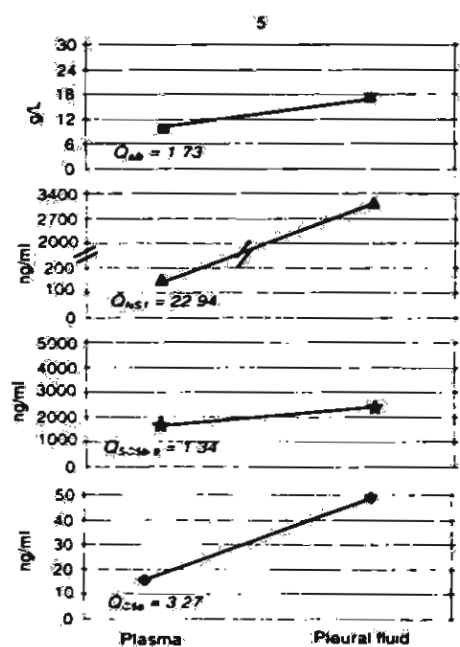
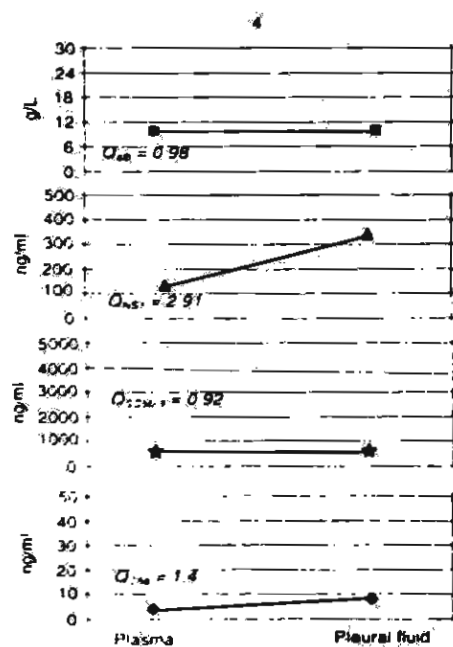
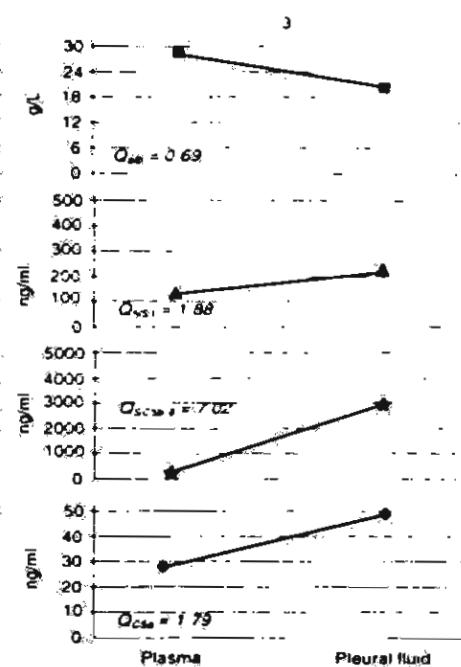
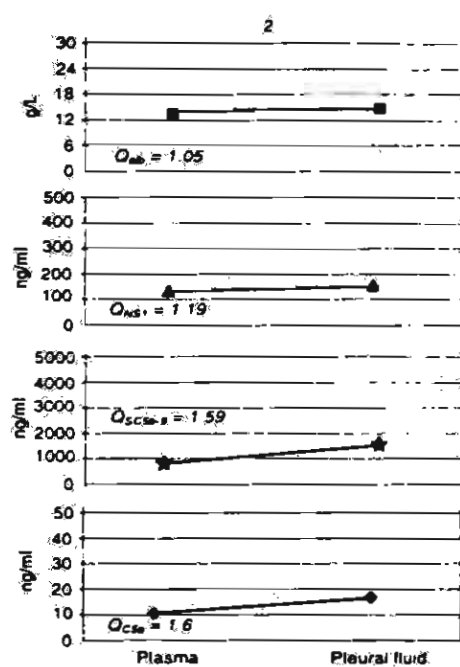
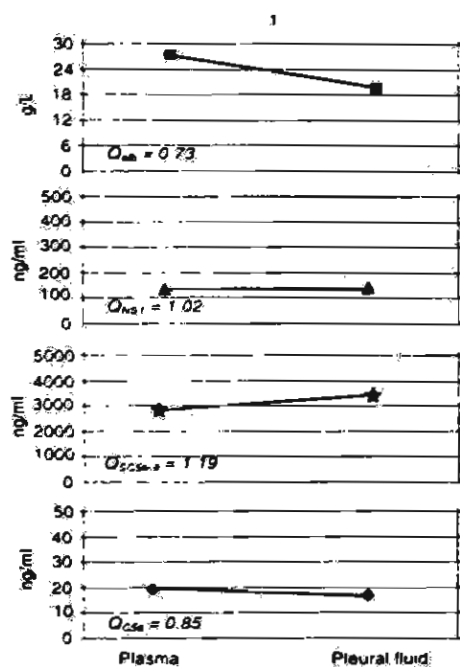


FIGURE 17 Measurements of albumin (■), NS1 (▲), SC5b-9 (★) and C5a (●) in EDTA-plasma and pleural fluids (PF) of nine children with DSS. The quotients between pleural fluid and plasma concentrations are shown for each case.

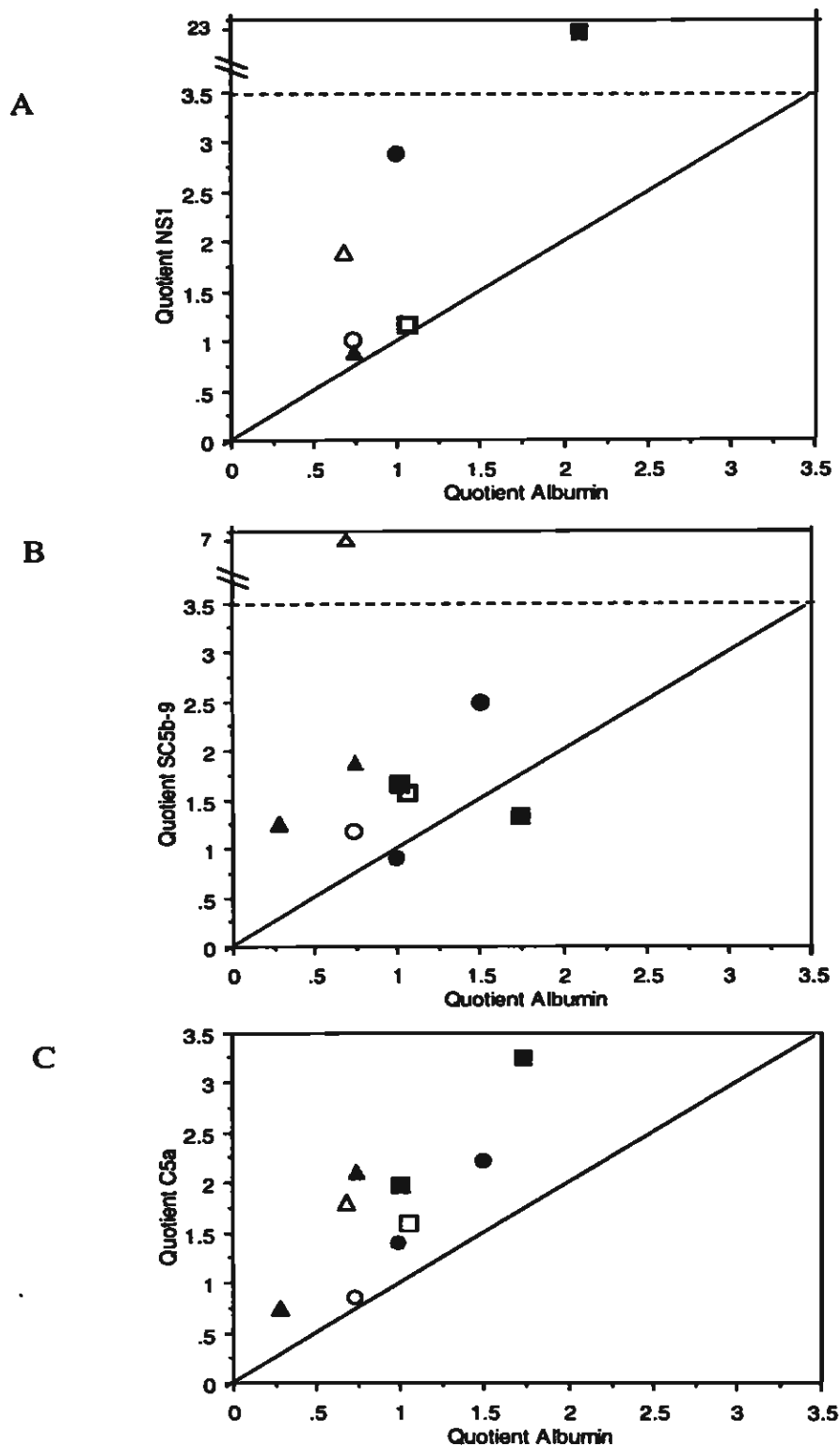


FIGURE 18 Relative accumulation of NS1, SC5b-9 and C5a in pleural fluids. The quotients between pleural fluid and plasma concentrations of these analytes are plotted against the respective albumin quotients. Each symbol represents one patient.

Discussion

The major finding of the present study is to show the capability of DV-NS1 protein in activating complement system via antibody dependent mechanism for membrane associated-NS1 and antibody independent mechanism for soluble NS1. NS1 specific antibodies, however, could enhance complement activation efficiency by soluble DV-NS1. Complement activation occurred to completion as demonstrated by the generation of C5b-9 complexes. It is the first study that demonstrated the capability to activate human complement system of dengue NS1 protein.

Massive complement activation is a hallmark of DHF/DSS (13, 42). Increasing of anaphylatoxins C3a levels was demonstrated in plasma of DSS patients during shock (14). The mechanism underlying complement activation has remained unknown. The fact that very small amounts of sIC could be detected in the circulation of patients during acute phase of the disease (14, 15) and that sIC is known to be a poor complement activator (16), it is unlikely that immune complexes are the major cause for complement activation in DHF/DSS. In 1990 Bhakdi et al proposed an alternative hypothesis for complement activation in DHF/DSS (18). High amounts of specific antibodies are generated in response to DV-infection due to an anamnestic response during secondary infection and at the same time most of DV-infected cells express relevant amounts of DV antigens on their surfaces so that the antibodies can bind to dengue viral antigens on the surface of infected cells forming large immune complexes which are capable of activating complement system. Consequently, an *in vitro* study demonstrated that surface of DV infected cells in the presence of anti dengue antibodies was able to activate the complement system and resulted in the deposition of C5b-9 complexes (19). In the present study, antibody-dependent complement activation of DV-infected cells was confirmed by immunofluorescence for the deposition of C3dg fragments on the surface of complement attacked cells (Fig. 4). Dose-dependent effect of dengue specific antibodies-induced complement activation on cell surfaces was demonstrated in Fig. 5.

However, what antigen(s) on the surface of DV-infected cell activate(s) complement is still unknown. Many studies in the past had been carried out to analyze type of dengue viral antigens expressed on the surface and in the cytoplasm of

various types of infected cells. During early period of the studies, DV antigens had been demonstrated to locate in the cytoplasm and plasma membrane of infected cells by using pulse chase radio labeling, membrane isolation, and staining by dengue specific polyclonal antibodies (23, 43-45). Later, when the technique for mAbs production was available, envelope (E), and NS1 protein of DV were shown to be major dengue antigens resided in the plasma membrane of infected cells (26). The results of kinetics study of NS1 expression in DV-infected HEK-293T cells using various clones of NS1-specific mAbs in the present study revealed that NS1 was expressed in considerable amounts on the surfaces and intracellular of DV-infected cells at 24 h after infection and a slight increase of NS1 expression at both sites at 48 h after infection was observed (Fig. 3A, B). Notably, the failure of 2C5 and 2H4 in reacting with intracellular but not surface-associated NS1 could reflect the difference in the conformation of NS1 at both sites. The difference in the staining of surface and cytoplasmic NS1 of the same mAb clones observed here was not due to that the specific epitopes seen by the particular mAbs were sensitive to the fixative used in the procedures of cytoplasmic staining prior to the permeabilization step because when infected cells were treated with the fixative but not with permeabilizing agent, the results were similar to that of conventional surface staining (data not shown). From the knowledge concerning NS1 biosynthesis, it has been demonstrated that after translation of dengue polyprotein, the cleavage of NS1 from C-terminus of E protein by cellular signalase in lumen of endoplasmic reticulum and from N-terminal of NS2A by host membrane-bound endoplasmic reticulum-resident protease immediately occurs. The newly synthesized monomeric NS1 could undergo membrane associated dimerization within 20-40 min (46) and is transported through the cellular secretory pathway to plasma membrane and released into extracellular compartment as a soluble hexameric form (27). Interestingly, NS1 was demonstrated to be present on the plasma membrane of cells stably expressed NS1 using glycosidylphosphatidylinositol (GPI) anchorage (47). Altogether, these findings could support the explanation that cell surface and cytoplasmic NS1 may have distinct structures and conformations.

The result of Fig. 6 demonstrated that binding of a mixture of NS1-specific mAbs to DV-infected cells could also induce complement activation to the same extent as dengue specific polyclonal antibodies purified from pooled convalescent

sera (PCS) (Fig. 5). These data indicated that surface-associated NS1 contributed to complement activation by DV-infected cells. However, the intensity of complement activation induced by individual clone of NS1 specific mAbs (1A4, 1B2, 1F11, and 2G6) was unequal (Fig. 6) indicating the variability in the efficiency to fix human complement of mouse mAbs. It has been shown that mouse mAbs of IgG2a isotype was able to fix human complement better than IgG2b and IgG1 (48) which could be account for the superior in the induction of complement activation of 2G6 and 1F11 isotype to that of 1B2 and 1A4 and both of which are IgG2a and IgG1 isotype respectively. In addition, the binding affinity between antigen and antibody was shown to affect the efficiency of complement activation (49). It is likely that each mAb binds to distinct epitopes present on a NS1 molecule. Complement activation of membrane associated NS1 was confirmed by using cells stably expressing NS1 (Fig. 7). The result showed that cells stably expressing NS1 were capable activating complement system in the presence of anti-DV antibodies. However, more than 10 times higher concentration of purified PCS was needed to elicit complement fixation on the surfaces of cells stably expressing NS1 than that used for the induction of complement activation by DV-infected cells (Fig.8A). There are two possible explanations for this observation. Firstly, dengue-specific antibodies in PCS reflects polyclonal response against DV-infection therefore it contains antibodies specific to dengue antigens other than NS1 which can bind to several types of dengue antigens expressed on the surface of infected cells. While NS1 is the only type of dengue antigens expressed on the surfaces of cells stably expressing NS1 therefore dengue specific antibodies in PCS can bind to a greater extent on DV-infected cellular surfaces than to the plasma membrane of cells stably expressing NS1. Secondly, anti-NS1 antibodies generated in patient's serum may react to natural NS1 expressed on the surfaces of DV-infected cells better than recombinant NS1 expressed on the surfaces of cells stably expressing NS1. Affinity of the binding between antigen and antibody in ICs has been shown to influence the efficiency of complement activation (49). Notably, complement activation on the surfaces of both cell types in the presence of equivalent concentrations of NS1-specific mAbs was in a similar extent (Fig. 7B). Capability of membrane-associated NS1 in activating the complement system was confirmed in a set of experiments showing the co-localization of NS1 and complement C3 fragments on the surfaces of complement attacked cells (Fig. 9).

Antibody dependent complement activation of membrane associated NS1 was proven to be efficient leading to the formation of membrane attack C5b-9 complexes (Fig. 10). Plasma membrane deposition of C5b-9 complexes was unable to cause cell lysis since cells harboring membrane C5b-9 were negatively stained with propidium iodide, an indicator for cell viability. Similar observation was previously described in the complement activation by DV-infected endothelial cells (19). This is not surprising since nucleated cells are relatively resistant to complement-mediated lysis due to homologous restriction and repair mechanism (50). Resistant to complement-mediated lysis was also reported in HIV-infected cells (51-53).

Antibody dependent complement activation of membrane-associated NS1 occurred through both classical and alternative pathway since the addition of 10mM EGTA/MgCl₂ could partially inhibit C3dg deposition on the surfaces of complement attacked cells (Fig. 11A-B). Antibody dependent alternative pathway activation of the complement system has similarly been observed in cells infected with measles and bovine respiratory syncytial viruses (54, 55). Antibody has been proposed to facilitate the deposition of C3b on the surfaces of complement attacked cells either by directly binding to C3b or by masking complement control proteins (56, 57).

As shown in Fig. 12, culture supernatants from DV-infected cells were capable of activating complement system in a dose dependent manner independent of dengue-specific antibodies. The activation may be induced by dengue virions or any secreted soluble viral proteins since supernatants from mock-infected cells did not activate complement (Fig. 12A). As discussed earlier, it is tempting to speculate that NS1 is the key antigen responsible for complement activation in DV-infected culture supernatants since the protein is being extracellularly released in considerable amounts in an oligomeric form. Soluble NS1 purified from culture supernatants of DV-infected cells or cells stably expressing soluble NS1 (rNS1s) using immuno-affinity chromatography was able to fix complement in the absence of specific antibodies (Fig. 15A, B). Direct activation of soluble but not surface-associated NS1 may be related to the oligomeric stage of the molecules when being secreted into extracellular milieu (27). The spontaneous complement activation of soluble NS1 may occur by the direct binding of NS1 to complement C1q and mannan-binding lectin (MBL), the initiator of classical and lectin pathway respectively. In fact,

soluble gp 41 or synthetic peptides of gp 41 of HIV were able to directly bind the C1q subunit in the absence of specific antibodies (58). Additionally, the rgp120 subcomponent of HIV-1 glycoprotein could also activate complement system in an antibody-independent manner (59). The direct binding of human complement components to viral proteins expressed on cellular surfaces was also observed in various types of infection. HIV-1 gp41 and HTLV-1 gp21 envelope proteins could bind C1q and trigger antibody-independent activation of the classical pathway on the surface of infected cells (60-62). A purified preparation of Epstein-Barr virus (63) and Cytomegalovirus infected fibroblast (64) was shown to activate the classical pathway of human complement in the absence of antibodies, which resulted from the direct interaction of C1 with unidentified viral protein. Moreover, direct binding of MBL to various viral proteins has also been reported. Binding of MBL to HIV-1 gp 120 or the corresponding envelope protein gp 110 of HIV-2 was shown to activate the MBL pathway of complement activation, independently of C1q and antibodies (65). The role of MBL in complement activation was also proposed in Herpes simplex viruses and influenza viruses (66). A recent study demonstrated direct activation of the cell surface expression of measles virus (MV) fusion (F) protein in activating the alternative complement pathway (67). The mechanism of spontaneous complement activation of soluble dengue NS1 awaits for further studies.

Not unexpectedly, complement activation by DV-infected cultures was remarkably enhanced by anti-dengue antibodies (Fig. 12B) or by NS1 specific antibodies (Fig. 13A, B). A mixture of several clones of NS1 specific mAbs elicited the highest complement activation as demonstrated in Fig 13A. The results were similar to that of Fig. 6 in which a mixture of NS1 specific mAbs induced complement activation on the surfaces of DV-infected cells with the greatest intensity compared with that of individual clone of NS1 mAbs at equal concentrations. Dose dependent effect of NS1 specific mAbs in enhancing complement activation of DV-infected cultures was shown in Fig. 11B and 12B respectively. It is generally known that DHF, the severe form of dengue disease, occurs almost exclusively in secondary immune response and that vascular leakage is the key event that differentiates DHF from DF, therefore, the enhancement of NS1 specific antibodies in complement activation by both membrane-associated and soluble NS1 may partly contribute to the development of DHF during secondary dengue infection.

NS1-mediated complement activation occurred to completion both on cells and in fluid phase, so that membrane bound C5b-9 and soluble C5b-9 complexes were generated. Membrane bound C5b-9 can trigger cellular reactions and production of inflammatory cytokines (68, 69) while SC5b-9 can independently provoke other local and systemic effect (32, 70, 71).

A novel finding here was that plasma SC5b-9 levels followed a similar course and also appeared to correlate with the severity of the disease. Levels of NS1 and especially SC5b-9 were significantly higher in shock than in non-shock cases. A major challenge for the future will be to identify the major sites of DV-infection and to examine for the local presence of complement activation products at these sites. According to one report, DV antigen is present in alveolar macrophages and endothelial cells of the lung (72). This would fit nicely with our finding that pleural fluids from patients with DSS contain high levels of NS1 and SC5b-9, and that quotients formed between SC5b-9 in pleural fluids versus plasma are higher than the corresponding albumin ratios. It would thus follow that complement activation occurs locally at these sites. In line with this contention, the anaphylatoxins C3a and C5a were also detected at high levels in pleural fluids. While anaphylatoxins bind to cells and are also rapidly inactivated *in vivo*, the terminal SC5b-9 complex is stable. The half-life in plasma is approximately 1 h (73, 74), but it is probably considerably longer in closed compartments. SC5b-9 has been shown to enhance endothelial permeability *in vitro* and *in vivo* at a concentration of just a few micrograms per milliliter (32). These concentrations were reached in the pleural fluids of 8 of the 9 patients in this study.

A unifying concept can thus now be formulated to explain the pathogenesis of vascular leakage in DHF/DSS. An antibody response to a primary infection generates non-neutralizing antibodies against heterotypic dengue viruses. Viral replication is augmented due to immunological enhancement during secondary infections, and NS1 then becomes a key element that determines the course of the disease. The protein is released in copious amounts from infected cells. It is probably identical to the soluble viral antigen that was reported in 1970 to bind anti-DV antibodies and activate guinea pig complement (20, 22, 75). At the same time, antibodies against NS1 direct complement attack to the infected cells, causing generation of membrane-damaging C5b-9 and by-stander SC5b-9 complexes. DV infection could also induce the

production of inflammatory cytokines, and IL-8 and RANTES have been found in high concentrations in pleural fluids of DSS-patients (19). Complement activation products and cytokines may synergize locally to incur massive vascular leakage that is the hallmark of DSS.

The present findings fulfill a number of early predictions that were made on the pathogenesis of DHF/DSS (18). Pending availability of bedside assays, it should become possible to establish whether plasma levels of NS1 and/or SC5b-9 can serve as predictive markers, allowing patients at high risk for developing vascular leakage to be identified prior to manifestation of the catastrophic events that claim the lives of so many children around the globe.

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OUTPUTS

1. A manuscript entitled “Vascular Leakage in Severe Dengue Infections: a Role for the Nonstructural Viral Protein NS1 and Complement” submitted to the Journal of Immunology
2. Development of NS1 capture ELISA which is capable of detecting NS1 protein in clinical specimens from all 4 DV-serotypes.
3. Patent of the idea that combination of NS1 and SC5b-9 may not only identify DV-infected patients during early febrile phase but also predict the development of severe DV-infection, DHF/DSS.
4. Graduate training for one M.Sc student of the Department of Immunology Faculty of Medicine Siriraj Hospital, Mahidol University.

บทความ

SUBMITTED MANUSCRIPT

**Vascular leakage in severe Dengue virus infections: a role for the
non-structural viral protein NS1 and complement**

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Summary

Background: Severe vascular leakage and shock are the major causes of death in patients with dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS). Complement activation was proposed to be a key underlying event 30 years ago, but the cause of complement activation has remained unknown to the present day.

Methods: The major nonstructural DV-protein NS1 was tested for its capacity to activate human complement in its cell-bound and soluble form. Plasma samples from 163 patients with DV-infection and from 19 patients with other febrile illnesses (OFI) were prospectively analyzed for levels of viremia, NS1, and complement activation products. Blood and pleural fluids from 9 patients with DSS were also analyzed.

Findings: Soluble NS1 activated complement to completion, and activation was enhanced by polyclonal and monoclonal antibodies against NS1. Complement was also activated by cell-associated NS1 in the presence of specific antibodies. Plasma levels of NS1 and terminal SC5b-9 complexes correlated with disease severity. Large amounts of NS1, complement anaphylatoxin C5a and the terminal complement complex SC5b-9 were present in pleural fluids of DSS patients.

Interpretation: Complement activation mediated by NS1 leads to local and systemic generation of anaphylatoxins and SC5b-9. High concentrations of these activation products may be directly responsible for vascular leakage occurring in DHF/DSS patients.

Relevance to practice: Massive complement activation in DV infections is triggered by NS1 both on cell surfaces and in the circulation. Measurements of NS-1 and SC5b-

9 in plasma may render it possible to identify patients at risk of developing vascular leakage and shock.

Key words: Dengue hemorrhagic fever, shock, vascular leakage, nonstructural protein-1, complement anaphylatoxin, SC5b-9 complement complex.

Introduction

Dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) are severe forms of dengue virus (DV) infection and still one of the leading causes of morbidity and mortality in children of school age in tropical and subtropical regions. Major pathophysiological processes which distinguish DHF/DSS from mild dengue fever are abrupt onset of vascular leakage, hypotension and shock, which are accompanied by thrombocytopenia and hemorrhagic diathesis (76). If the crisis is overcome, recovery is rapid and complete. The pathogenetic mechanisms underlying DHF/DSS are incompletely understood. The rapid onset of plasma leakage and brief manifestation of disease, the remarkably rapid recovery with no clinical sequelae (76), and the fact that no characteristic histopathological vascular lesions have been found (77), suggest that short-lived pharmacological mediators play major roles. Another unique feature in DHF/DSS is that ascites and pleural effusion are the only two sites that account for most of the plasma leakage (76).

Four DV-serotypes exist and DHF/DSS occur almost exclusively in patients suffering from a re-infection with a different virus serotype (4, 78). An enigmatic dysfunction of the immune system then leads to enhanced viral replication. This has been proposed to be due to antibody-mediated increase of viral uptake in target cells (5, 79), or to cross-depletion of protective CD8 lymphocytes (7). High levels of viremia and of circulating viral antigens are consequently found in these patients (39, 41, 80).

Thirty years ago, accelerated complement consumption and marked reduction of plasma complement components were observed in DSS patients during shock (13, 81), which led to the assumption that complement activation plays an important role in disease pathogenesis (14, 18). In the following decades the thrust of international research shifted towards the possible role of lymphocytes and cytokines (79, 82, 83), and the significance of complement receded to the background. Thereby, the important issue regarding the cause of complement activation has remained untouched. In a previous investigation, we observed that surfaces of DV-infected cells bind DV-antibodies, which leads to complement activation and cytokine secretion (19). The search for the responsible viral antigen led to NS1, a 45 kD non-virion associated protein that is synthesized in the endoplasmic reticulum and exported along the cellular secretory pathway (26). NS1 resides in the plasma membrane of infected cells (26) and is also released in oligomeric form to the extracellular milieu (27). NS1 is strongly immunogenic and anti-NS-1 antibodies play a role in protection against disease (84-87). However, protection is afforded only by type-specific antibodies. High levels of anti-NS1 antibodies are found in the circulation of DV-infected patients during the late-acute and convalescent phase (88-91). Moreover, high levels of soluble NS1 have been detected in the blood of DHF/DSS patients during the acute phase of the disease (28, 29, 80).

We discovered that soluble and cell-bound NS1 activate human complement, and that plasma levels of soluble NS1 protein and the terminal SC5b-9 complement complex correlate with disease severity. Large amounts of complement activation products and soluble NS1 were found in the pleural fluids of DSS patients, indicative of massive complement activation occurring at the sites of vascular leakage. Complement anaphylatoxins as well as the terminal SC5b-9 complement complex

increase vascular permeability (31, 92) and SC5b-9 increases lung hydraulic conductivity (93). A link thus emerges between NS1 load, complement activation and the clinical manifestation of DHF/DSS.

Methods

Reagents

Purified Ig fractions from pooled convalescent sera (PCS) (hemagglutination titer $\geq 1/25600$) and control sera without DV antibodies (DV antibody-negative sera, DNS) were obtained using protein G column affinity chromatography (Pharmacia). NS1 specific monoclonal antibodies (mAb) clone 2G6, 1A4, 1B2, 1F11, 2E11, and 2E3 have been previously described (34).

Cells and viruses

The swine fibroblast cell line (PsCloneD), and C6/36, a cell line from *Aedes albopictus* were cultured at 37°C and 28°C, respectively, in L-15 medium (Life Technologies) containing 10% tryptose phosphate broth (Sigma, St. Louis, MO), 10% FCS (Hyclone). The human kidney epithelial cell line HEK-293T was grown in RPMI 1640 (GIBCO) containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in humidified air containing 5% CO₂. Dengue virus serotype 1, 2, 3, and 4 (strain Hawaii, 16681, H-87, and H-241) were propagated in C6/36 cells. Preparation of virus stocks and virus titrations were performed as previously described (19).

Two HEK-293T cell lines expressing NS1 used in this study have been generated according to the standard protocol with minor modifications (94). 5×10^5 cells were transfected with 5 µg of pcDNA3.1/Hygro (Invitrogen, Carlsbad, CA) encoding for soluble NS1 (NS1s) or membrane-associated NS1 (NS1m). The transfected cell cultures were assessed for NS1 expression on day 3 post-transfection by indirect immunofluorescence assay and by ELISA of supernatants. Stable clones were maintained in RPMI 1640-10% FBS medium containing 100 µg/ml hygromycin B. Cells transfected with the empty vector were used as negative controls.

Immunoaffinity purification of NS1

Monolayers of swine fibroblasts cultured in 162 CM² tissue culture flasks (COSTAR) were infected with dengue virus serotype 2 (strain 16681) at a multiplicity of infection (MOI) of 1. After infection, the cells were cultured in protein free medium (Ultradoma). Culture supernatants were harvested 3 days later, centrifuged at 200,000xg to remove virions and subjected to immunoaffinity chromatography with a column prepared with anti-NS1 mAb 2G6. Antibodies were purified from ascitic fluid using Protein G Sepharose columns and coupled to CNBr-activated Sepharose beads (Pharmacia). NS1 was detected by ELISA and purity was checked by SDS-PAGE and Western blot. For isolation of soluble NS1 from transfected cells (NS1s), culture supernatants were harvested every three days and replaced with fresh medium. Supernatants were passed through a 0.2 µm cut-off membrane prior to immunoaffinity chromatography. Purified NS1 was passed over a protein G column twice in order to remove any traces of contaminating antibodies, the absence of which was ascertained by ELISA measurements.

NS1 capture ELISA

Microtiter plates (Nunc) were coated with anti-NS1 mAb 2E11 (5µg/ml) overnight at 4°C. After blocking with PBS containing 15% FBS, wells were washed 5 times with PBS containing 0.05% Tween-20. 100µl of samples were added to each well and incubated for 1 h at room temperature (RT). After 5 washes, 100µl of anti-NS1 mAb 2E3 (50µg/ml) were added to each well and incubated for 1 h at RT. The ELISA was developed conventionally using horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG (SIGMA) and O-phenyldiamine H₂O₂ substrates.

Assay for fluid phase complement activation

Cell supernatants or purified NS1s were incubated with 12.5% normal human serum (final concentration) in the presence or absence of purified anti-DV antibodies. The total volume of the assay was 0.2 ml. Heat inactivated serum (HI) or serum containing 10 mM EDTA served as negative controls. After 60 min at 37°C, samples were serially diluted and hemolytic complement titers (CH50) were determined in the conventional manner (95). SC5b-9 measurements were performed using a commercial ELISA from Quidel.

Assay for complement activation on cells

HEK-293T cells were infected with DV-2 at a MOI of 10 and harvested at 24 h post infection. 1×10^6 DV-infected or cells expressing membrane-bound NS1 (NS1m) were incubated with purified PCS, DNS, a mix of anti-NS1 mAbs, or isotype controls in the presence of 12.5% normal human serum. Heat inactivated serum or serum containing 10 mM EDTA served as negative controls. Washed cells were incubated with a mAb against C3dg provided by Dr. P.J. Lachmann, or against SC5b-9 complexes (Quidel), followed by staining with FITC-labeled rabbit F(ab')₂ anti-mouse Ig.

Double immunofluorescent staining of C3 and NS1

1×10^6 DV-infected or cells expressing membrane-associated NS1 (NS1m) were incubated with 12.5% normal human serum in the presence of a mix of anti-NS1 mAbs at 37°C for 1 h. After one wash, cells were fixed with 2% paraformaldehyde at RT for 10 min. Fixed cells were incubated with rabbit anti-human C3c and C3d (DAKOPATTS a/s, Denmark) followed by staining with FITC-conjugated swine anti-rabbit immunoglobulins (DAKO, Denmark) and Cy3-conjugated goat anti-mouse Ig (Jackson Immuno Research Laboratories, Inc., West Grove, PA). Washed cells were resuspended in 50% fluorescent mounting medium (DAKO) and observed under a

Zeiss LSM 510 META confocal microscope (Carl Zeiss, Germany). Excitation and detected emission wavelengths were 488 nm and 505-530 nm for FITC, or 543 nm and 560-615 nm for Cy3. Photography was performed by using an image capture program (LSM 510 software version 3.2, Carl Zeiss).

Quantitative RT-PCR of dengue viral genome

RNA was extracted from DV-infected cell supernatants or patients' plasma using QIAamp Viral RNA Mini Kit (QIAGEN), aliquoted, and stored at -70° C. Levels of dengue viral RNA were subsequently quantified by a single tube one-step real-time RT-PCR using a LightCycler instrument and software version 3.5 (Roche Molecular Biochemicals, Germany) as described by Shu *et al* (36).

Measurement of complement fragments in clinical specimens

The anaphylatoxins C3a, and C5a were quantified by flow cytometry using a commercial cytometric bead array kit (Becton Dickinson). SC5b-9 was quantified with the ELISA from Quidel.

Patient enrolment and study design

Pediatric patients admitted to the ward of Khon Khan Provincial Hospital, Thailand between November 2001 and December 2003 with the clinical diagnosis of dengue infection (DF or DHF) and the following criteria were included in the study: age 1 to 15 years, pyrexia not more than 4 days with no obvious source of infection, Tourniquet test positive, history of signs/symptoms of bleeding/hemorrhagic diathesis). At the time of enrolment, subjects and their parents were interviewed by a study nurse to collect demographic data and medical history. Blood specimens were taken daily until one day after defervescence. Plasma aliquots were collected in 5 mM EDTA containing vacuum tubes (Becton Dickinson, Cat.No.367661) and stored at -70°C. Diagnosis of dengue infection was confirmed by measuring anti-DV

IgM/IgG and by virus identification by RT-PCR (37). Aspiration of pleural fluid has been conducted as part of treatment to relieve excess fluid collected within the pleural cavity, only in patients experiencing respiratory difficulty.

Clinical diagnosis and grading of DHF followed the WHO criteria (38). Study day 0 was defined as the calendar day during which the temperature fell and stayed below 37.8 °C. Evidence of plasma leakage included a peak of hematocrit value more than 20% above the value at the convalescent visit, a pleural effusion demonstrated on the chest radiograph, or detection of ascites on physical examination.

Thrombocytopenia was defined as a count of $\leq 100,000/\text{mm}^3$. Any subject with serological or virological evidence of acute dengue infection who did not meet the criteria for DHF was assigned to the DF group. Subjects were diagnosed as having OFI (other febrile illnesses) when there was no clinical evidence for a bacterial infection and no serological or virological evidence for DV-infection. The study protocol has been approved by the Ministry of Public Health (approval date, 7th May 2003), the Faculty of Medicine Siriraj Hospital (certificate of approval, 156/2002 and 115/2004), and the Khon Khan hospital (approval date, 31st October 2002). Informed consent was individually obtained from all subjects.

Statistical analysis

Data analysis was performed using software package StatView for Windows version 5.0 (SAS Institute Inc., NC). First, the mean and standard deviation (SD) of NS1, viral load, and SC5b-9 were presented for selected subgroups. Median and range were also displayed when the data were highly skewed. The aim of the statistical analysis was to investigate whether patients with DF, DHF grade 1, 2, and 3 differed regarding the NS1, viral load and SC5b-9. Kolmogorov Smirnov test was used to test for normality. Comparisons between DF and DHF (any group) were done

by t-test if the distribution of the variables was comparable to a normal distribution; otherwise the Mann Whitney test was used. Multiple comparisons were performed using ANOVA. $P \leq 0.05$ was considered to be statistical significant. All analyzed P values were 2-sided.

Results

Purification of soluble NS1 from DV infected cells and from cells expressing NS1

An SDS-PAGE of the NS1 preparations is shown in Fig. 1A. The electrophoretic behavior of the protein was as previously described by Winkler et al (26). The 80 kD dimeric form of NS1 was converted to the monomer (40 kD) by heating. Slight heterogeneity of the bands could be due to small variations in glycosylation. Immunoreactivity with NS-specific mAbs yielded the same bands in Western blots (data not shown). The concentration of NS1 in 3-day supernatants of infected cells ranged from 900.3 ± 46.7 to 1029.4 ± 62.4 ng/ml, and the yield of NS1 was 334 ± 87.5 and 237.4 ± 38.5 μ g/ 1 liter culture from DV-infected cells and from NS1 transfected cells, respectively.

NS1 capture ELISA

Establishment of the NS1 ELISA was achieved using IgM mAb 2E11 and IgG mAb 2E3 for antigen capture and detection, respectively. Both antibodies cross-react with NS1 from all four DV serotypes. The ELISA could detect NS1 in DV-1, 2, 3, and 4 infected culture supernatants. Using purified NS1 derived DV-1 and DV-2 infected cells as standards, the detection limit was found to be approximately 50 ng/ml (Fig. 1B). The assay was considered positive if the optical density (OD) was greater than twice the average value of the negative controls, i.e. 0.103 ± 0.025 . To determine the effect of plasma components on the sensitivity of NS1 detection, purified DV-2-NS1 was serially diluted in healthy dengue non-immune human plasma or in buffer. The effect of human plasma on NS1 detection was found to be negligible.

Supernatants of DV-infected cells activate complement

Supernatants from DV-infected cells containing approximately 900 ng/ml NS1, but not from mock-infected cells dose-dependently consumed complement independent of specific antibodies (Fig. 2A). Addition of purified Ig fractions from pooled convalescent sera of DV-infected patients (PCS) but not control DV-antibody-negative sera (DNS) enhanced complement consumption (Fig. 2B). Similar enhancement was also observed when a mix of mAbs against NS1 was employed (data not shown).

Purified NS1 activates complement to completion

Purified NS1 also activated complement and caused a fall in CH_{50} similar to unfractionated culture supernatants from DV-infected cells. Complement activation occurred to completion with the formation of SC5b-9 complexes (Fig. 3). Activation was enhanced by either NS1 specific mAbs or by purified Ig fractions obtained from pooled convalescent sera of DV-infected patients (PCS) but not by isotype-control antibodies or by purified Ig from control DV-antibody-negative sera (DNS) (Fig. 3). Similar results were obtained with purified NS1 from transfected cells (data not shown).

Complement activation by cell-associated NS1 is antibody-dependent

Expression of NS1 antigens on the surfaces of DV-infected cells and on cells stably expressing membrane associated-NS1 (NS1m) was demonstrated by immunofluorescent staining and by flow cytometry (Fig. 4A). When DV-infected cells were incubated with 12.5% NHS, no complement activation was observed as evident from negative staining for C3dg (data not shown) and C5b-9 on cell surfaces (Fig. 4D). However, the presence of purified antibodies against NS1 triggered complement activation on the cells as evidenced by the co-localization of complement C3 and NS1 (Fig. 4B). Similar results were obtained with purified Ig from pool

convalescent sera (data not shown). Antibody dependent complement activation was induced by all 4 clones of NS1 specific mAbs tested but not with isotype control antibodies. Complement consumption by membrane associated NS1 was confirmed using cells stably expressing the membrane bound form of NS1 and co-localization of NS1 and C3dg was again observed following complement activation in the presence of NS1-specific antibodies (Fig. 4C). Parallel immunofluorescent staining for C5b-9 revealed its deposition on the plasma membrane of both DV-infected (Fig. 4D) and transfected cells (data not shown).

DV-RNA, soluble NS1, and complement activation products in clinical specimens

A total of 182 patients admitted to the pediatric ward of Khon Khan Provincial Hospital between November 2001 and December 2003 were enrolled into this prospective study. The overall male-to-female ratio was 1:1, and the overall mean age was 9.6 ± 3 (range 2-15, median 9) years. There were no major differences in male-to-female ratios and mean ages of patients in each group. According to the WHO criteria, the final diagnosis was DF for 49 patients and DHF for 114, including 44 of grade 1, 44 of grade 2 and 26 cases of grade 3 or dengue shock syndrome (Table 1). The residual 19 cases were diagnosed as OFI.

Dengue RT-PCR was positive for 151 out of 163 patients (92.6%). Virus types identified were DEN-1 ($n = 87$), DEN-2 ($n = 52$), DEN-3 ($n = 6$), and DEN-4 ($n = 6$). A total of 148 patients (90.8%) were diagnosed as secondary infection, while 15 patients (9.2%) had a primary dengue infection.

Viremia levels during acute phase of illness were compared over time. Highest viremia levels were detected early in clinical illness for all groups of dengue-patients and gradually declined to undetectable levels on day + 1 in DF or on day + 2 in DHF (Fig. 5A). Similar delayed virus removal from the circulation of DHF

compared with DF patients has been observed earlier (39, 40). Viral clearance in patients with shock was significantly slower than in non-shock patients; mean viremia levels at day +1 of shock and non-shock cases were 62.9 ± 206 (range, 0-545 PFU/ml) and 8.9 ± 61 PFU/ml (range, 0-869 PFU/ml) respectively ($p < .05$). In confirmation of a previous report (41), mean levels of dengue viral RNA were higher in DHF grade 3 patients than in patients with DF or DHF grade 1 and 2 and reached statistical significance at day -1 ($p < .001$).

Unlike these kinetics of DV RNA levels, plasma NS1 levels were relatively lower in the early febrile days and peaked at day -2 (Fig. 5B). In three patients, plasma NS1 levels were extremely high at day -2 (3911, 3974, and 4474 ng/ml) and these values were not included in the statistical analysis. Mean levels of soluble NS1 in DHF (383.9 ± 620 ng/ml; range, 60-4151 ng/ml; median, 166.3 ng/ml) were higher than those of DF patients (181.6 ± 120 ng/ml; range, 78-895 ng/ml; median, 137.7 ng/ml) during acute illness (disease day ≤ 0 , $p = .003$). At day -3, -2, and -1, mean NS1 levels were 162.7 ± 61 (range, 95-267 ng/ml; median, 148.7 ng/ml), 211.4 ± 96 (range, 91-384 ng/ml; median, 218.3 ng/ml), 203.1 ± 164 ng/ml (range, 80-896 ng/ml; median, 140 ng/ml) for DF and 433.4 ± 306 (range, 141-1028 ng/ml; median, 357.4 ng/ml), 500 ± 400 (range, 99-1616 ng/ml; median, 361 ng/ml), and 438.1 ± 640 ng/ml (range, 88-3181 ng/ml; median, 183.9 ng/ml) for DHF respectively ($p = .01$, $.01$, and $.05$). At the time of maximum leakage or shock (day 0), mean levels of NS1 in DHF3 (666.2 ± 1274 ng/ml; range, 77-4152 ng/ml; median, 134.5 ng/ml) were significantly higher than in the non-shock cases (DHF2 [mean, 305.9 ± 593 ng/ml; range, 60-3785 ng/ml; median, 157.9 ng/ml; $p = .01$], DHF1 [mean, 172.2 ± 160 ng/ml; range, 83-1004 ng/ml; median, 124.4 ng/ml; $p = .001$], and DF [mean, 163.6 ± 98 ng/ml; range, 78-538 ng/ml; median, 131.9 ng/ml; $p < .001$]). Unlike viral load, enhanced levels of NS1 in

DHF3 patients at day -1 were at the borderline of significance compared with patients of other groups. At day +1, soluble NS1 was cleared from the circulation in almost all cases (Fig. 5B).

Levels of the terminal complement complex SC5b-9 were measured in the same blood samples (Fig. 5C). SC5b-9 plasma concentrations were significantly higher in DHF patients (mean, 306.9 ± 174 ng/ml) as compared to DF (mean, 225.3 ± 97 ng/ml; $p < .001$) and OFI (mean, 170.3 ± 57 ng/ml; $p < .001$) patients during acute illnesses (disease day < 2). There was also a significant difference between SC5b-9 levels in DF compared to patients with OFI ($p = .02$). At day -1, there was a correlation trend between SC5b-9 levels and disease severity: mean values were highest for DHF3 and lowest for DF (Fig. 5C). The difference in mean levels of SC5b-9 in shock and non-shock cases was statistically significance ($p < .05$).

Soluble NS1 and complement activation products in pleural fluids of DSS patients

NS1, complement anaphylatoxins C3a and C5a, and SC5b-9 were measured in pleural fluids and in plasma of 9 patients with DSS. Samples were collected at the day of shock or 1-2 days later. Identification of viral RNA was also performed using nested RT-PCR. The results are depicted in Fig. 6. Soluble NS1 was detected in 6 pleural specimens, while only 4 of these were positive for DV. In 3 cases with undetectable NS1 in both plasma and pleural fluids, the specimens were collected after the day of shock (7-9, Fig. 6). In eight cases (1-8, Fig. 6), the quotients between albumin concentrations in pleural fluids versus plasma were 0.7- 1.5, typical of exudates. In one case, the quotient was approximately 0.28, indicative of considerable transudation (9, Fig. 6). The specimens in this case were obtained several days after shock.

NS1 concentrations displayed large variations. In four cases, (1-4, Fig. 6), concentrations ranging from 116-120 ng/ml and 122-337 ng/ml were found in plasma and pleural fluids respectively. In all cases, levels in pleural fluids were equivalent (1, Fig. 6) or higher than in plasma (2-4, Fig. 6). In one case (5, Fig. 6), the NS1 level was relatively low in plasma and 20-fold higher in the pleural fluid. In case 6 (Fig. 6), concentrations were very high (about 2000 ng/ml) in both plasma and pleural fluid. NS1 was not detectable in case 7-9 (Fig. 6).

Pleural fluid concentrations of SC5b-9 were markedly higher than the plasma concentrations in all but one case where the levels were equivalent (4, Fig. 6). Mean SC5b-9 levels in pleural fluids were 2575.9 ± 1121 ng/ml; range, 627-4865 ng/ml; median, 2312.5 ng/ml, and were significantly higher than plasma concentrations (1546.3 ± 943 ng/ml, range, 394-2935 ng/ml; median, 1722 ng/ml; $p=.04$). A similar trend was found for C5a: levels of this anaphylatoxin in pleural fluids were 47.4 ± 61.1 ng/ml; range, 7-227 ng/ml; median, 23 ng/ml, and were also greater than in plasma (25.6 ± 33.9 ng/ml; range, 5-114 ng/ml; median, 15 ng/ml; $p=.34$).

When quotients obtained for NS1, SC5b-9 and C5a shown above were plotted against the respective quotients for albumin in the individual patients, almost all plotted values came to lie above the diagonal, which indicated relative accumulation of the analytes, probably due to their local generation at the site of leakage (Fig. 7).

Discussion

The concept that a detrimental immune response to a heterotypic infecting dengue virus is key to the development of DHF/DSS, first advanced 40 years ago, has been confirmed by epidemiological studies (4, 78). Two major, mutually non-exclusive mechanisms for immunological enhancement of infection have been proposed. The first envisages non-neutralizing, cross-reactive antibodies against DV to enhance uptake of the virus into susceptible cells (5, 79). The second states that DV-specific CD8 lymphocytes undergo apoptotic depletion upon confrontation with cells infected with the heterotypic virus (7). In both cases, loss of immunological control over viral replication ensues; indeed, it has been shown that the severity of disease correlates with levels of viremia (39, 41, 80).

However, viral load alone does not explain how and why vascular leakage should occur. Since the advent of the cytokine era, overproduction of these mediators by DV-infected cells or by activated lymphocytes has been widely thought to assume central importance (82, 83). Yet, measurements of proinflammatory cytokines in dengue infection have not uncovered any characteristic pattern (9, 82, 96-102). Moreover, endotoxin- and superantigen-mediated shock, diseases known to be caused by over-production of inflammatory cytokines, follow very different clinical courses. A major issue relates to the abruptness of leakage onset and disease-termination in DHF/DSS, which suggests the involvement of rapidly generated mediators with short biological half-lives whose production is triggered directly by the virus or by a viral protein. Complement naturally emerges as a prime candidate. Further to anaphylatoxins C3a and C5a, which are classical inducers of vascular leakage (31), the fluid phase SC5b-9 terminal complex has also been found to directly enhance

endothelial permeability via the induction of bradykinin and platelet activating factor (PAF) (92).

The discovery that massive complement activation occurs in DSS patients was made over 30 years ago, and it may appear surprising that the cause of this potentially catastrophic event has never been assiduously sought. The present study aspires to provide a solution to the puzzle. It is proposed that NS1, the major non-structural dengue virus protein, is the important trigger for complement activation. Expression of NS1 on the surface of infected cells results in antibody-binding and complement attack as shown in experiments with DV-infected cells and with cells expressing NS1. Activated C3 co-localized with NS1, and C5b-9 complexes were generated. Furthermore, NS1 released from infected cells can directly activate complement in the fluid phase. This spontaneous activation may be related to the oligomeric state of the molecule and is enhanced in the presence of NS1 antibodies as shown in experiments utilizing unfractionated supernatants of DV-infected cells, or of cells expressing NS1, as well as in experiments employing purified NS1. NS1-mediated complement activation occurs to completion both on cells and in the fluid phase, so that membrane-bound C5b-9 complexes and soluble SC5b-9 complexes are generated. Membrane-bound C5b-9 possibly triggers cellular reactions and the production of inflammatory cytokines (68, 103), while SC5b-9 can independently provoke other local and systemic effects (70, 71, 92, 93, 104).

Thus, a single virus protein, by virtue of its high expression on the surface of infected cells and its release to the fluid phase, may play a major role in the pathogenesis of vascular leakage due to its complement activating capacity. That the described processes indeed occur in patients is indicated by the results of NS1 and SC5b-9 determinations. High levels of NS1 were detected in plasma of patients with

DHF/DSS with an apparent peak at day -2. These findings stood in accord with a recent report, in which similar concentrations of NS1 were measured on early illness days (80). A novel finding here was that plasma SC5b-9 levels followed a similar course and also appeared to correlate with the severity of the disease. Levels of NS1 and especially SC5b-9 were significantly higher in shock than in non-shock cases. A major challenge for the future will be to identify the major sites of DV-infection and to examine for the local presence of complement activation products at these sites. According to one report, DV antigen is present in alveolar macrophages and endothelial cells of the lung (72). This would fit nicely with our finding that pleural fluids from patients with DSS contain high levels of NS1 and SC5b-9, and that quotients formed between SC5b-9 in pleural fluids versus plasma are higher than the corresponding albumin ratios. It would thus follow that complement activation occurs locally at these sites. In line with this contention, the anaphylatoxins C3a and C5a were also detected at high levels in pleural fluids. While anaphylatoxins bind to cells and are also rapidly inactivated *in vivo*, the terminal SC5b-9 complex is stable. The half-life in plasma is approximately 1 h (73, 74), but it is probably considerably longer in closed compartments. SC5b-9 has been shown to enhance endothelial permeability *in vitro* and *in vivo* at a concentration of just a few micrograms per milliliter (92). These concentrations were reached in the pleural fluids of 8 of the 9 patients in this study.

A unifying concept can thus now be formulated to explain the pathogenesis of vascular leakage in DHF/DSS. An antibody response to a primary infection generates non-neutralizing antibodies against heterotypic dengue viruses. Viral replication is augmented due to immunological enhancement during secondary infections, and NS1 then becomes a key element that determines the course of the disease. The protein is

released in copious amounts from infected cells. It is probably identical to the soluble viral antigen that was reported in 1970 to bind anti-DV antibodies and activate guinea pig complement (22, 75, 105). At the same time, antibodies against NS1 direct complement attack to the infected cells, causing generation of membrane-damaging C5b-9 and by-stander SC5b-9 complexes. DV infection could also induce the production of inflammatory cytokines, and IL-8 and RANTES have been found in high concentrations in pleural fluids of DSS-patients (19). Complement activation products and cytokines may synergize locally to incur massive vascular leakage that is the hallmark of DSS.

The present findings fulfill a number of early predictions that were made on the pathogenesis of DHF/DSS (18). Pending availability of bedside assays, it should become possible to establish whether plasma levels of NS1 and/or SC5b-9 can serve as predictive markers, allowing patients at high risk for developing vascular leakage to be identified prior to manifestation of the catastrophic events that claim the lives of so many children around the globe.

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Table 1. Age and sex of children enrolled from November 2001 to December 2003

Diagnosis	Number	Mean age (years)	Male to female ratio
DF	49	9.12	1.04:1
DHF I	44	10.07	1.44:1
DHF II	44	10.11	1.2:1
DHF III	26	9.31	0.63:1
OFI	19	9	0.46:1
Total	182		

OFI, other febrile illness; DF, dengue fever; DHF I, II, and III, dengue hemorrhagic fever grade 1, 2, and 3.

Figure legends

FIGURE 1. (A) SDS-PAGE of purified soluble NS1 from DV-infected cells and from cells stably expressing NS1. Purified NS1 was unheated or heated (95°C, 3 min) prior to SDS-PAGE. Markers are shown. (B) Standard curves for NS1 capture ELISA with purified NS1 from DV-1 and 2. Data points represent the mean and standard deviation for three replicates. Cut off value was set at twice the mean OD value for negative controls samples (0.103 ± 0.025).

FIGURE 2. Complement activation by supernatants of DV-infected cells. (A) Dose-dependency of spontaneous complement activation. The given amounts of culture supernatants were mixed with 25 μ l normal human serum and buffer was added to give a total of 200 μ l per sample. CH50 was determined after incubation at 37°C for 1 h. Data are displayed as the mean $\pm$ SD of percent CH50 over serum controls from three independent experiments. Final concentrations of NS1 in the samples are given on the second y axis. (B) Enhancement of complement activation by DV-specific antibodies. 100 μ l of culture supernatants from DV-infected cells were mixed with purified antibodies from PCS and DNS at the given final concentrations and 25 μ l normal human serum. CH50 was determined after 60 min, 37°C. Data are displayed as the mean $\pm$ SD of percent CH50 over serum controls from three independent experiments.

FIGURE 3. Purified dengue NS1 protein activates complement to completion. Purified soluble NS1 from DV-infected cells at the given final concentrations was incubated in 12.5% normal human serum in the presence or absence of NS1-specific mAb 2G6 (10 μ g/ml) or PCS (20 μ g/ml) at 37°C for 1h, and SC5b-9 was measured. Equivalent concentrations of isotype control Ab and DNS were used as controls. 10 mM EDTA was added to inhibit complement activation for negative controls. Data are displayed as the mean $\pm$ SD from three independent experiments. * $p < .05$.

FIGURE 4. Membrane-associated NS1 activates complement to completion in the presence of NS1 specific antibodies. (A) Surface expression of dengue NS1 on DV-infected cells and on cells stably expressing NS1. Cells were stained with NS1 specific mAb 1A4 followed by FITC-conjugated anti-mouse Ig. Histogram plots were determined from data acquiring from 5000 events of viable cells. The representative set of histograms is derived from one of three independent experiments. (B and C) Co-localization of NS1 and complement C3 fragments on the surfaces of complement attacked cells. DV-infected or cells expressing membrane-associated NS1 (NS1m) were incubated with 12.5% normal human serum in the presence of a mix of purified NS1 specific mAbs. After 1h at 37°C, cells were stained with fluorescent conjugated secondary antibodies and observed under confocal microscopy. NS1 (red, Cy3) and complement (green, FITC) co-localized on the membranes. (D) Formation of C5b-9 on cells. Mock or DV-infected cells were incubated with purified antibodies from PCS or DNS in the presence of 12.5% normal human serum. Deposition of membrane attack complexes was detected by flow cytometry following staining with a mAb against C5b-9 and FITC-conjugated secondary antibodies. Analysis was performed on 5000 viable cells. Data are displayed as the mean $\pm$ SD from three independent experiments.

FIGURE 5. Viral load, levels of NS1, and terminal SC5b-9 complexes in the circulation of patients with DF and DHF/DSS. Plasma samples were assayed for dengue viral RNA levels using quantitative real time RT-PCR, and soluble NS1 and SC5b-9 complexes were quantified by ELISA. Disease day 0 was defined as the calendar day during which the temperature fell and stayed below 37.8 °C. Plasma samples from patients with acute febrile diseases other than dengue (OFI-other febrile illness) were also used as controls.

FIGURE 6. Measurements of albumin (■), NS1 (▲), SC5b-9 (★) and C5a (◆) in EDTA-plasma and pleural fluids (PF) of nine children with DSS. The quotients between pleural fluid and plasma concentrations are shown for each case.

FIGURE 7. Relative accumulation of NS1, SC5b-9 and C5a in pleural fluids. The quotients between pleural fluid and plasma concentrations of these analytes are plotted against the respective albumin quotients. Each symbol represents one patient.

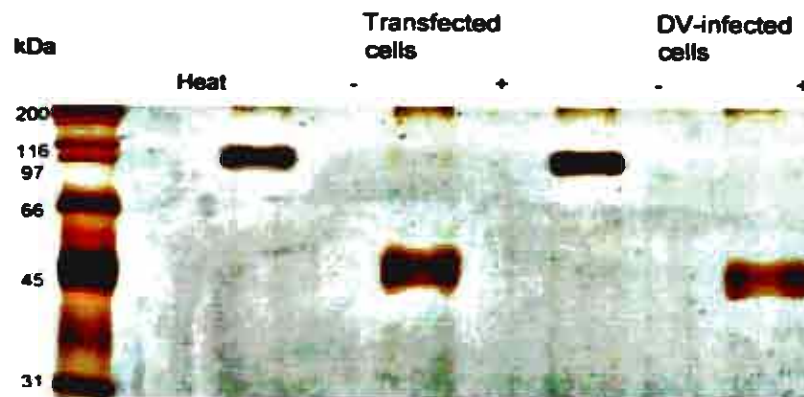
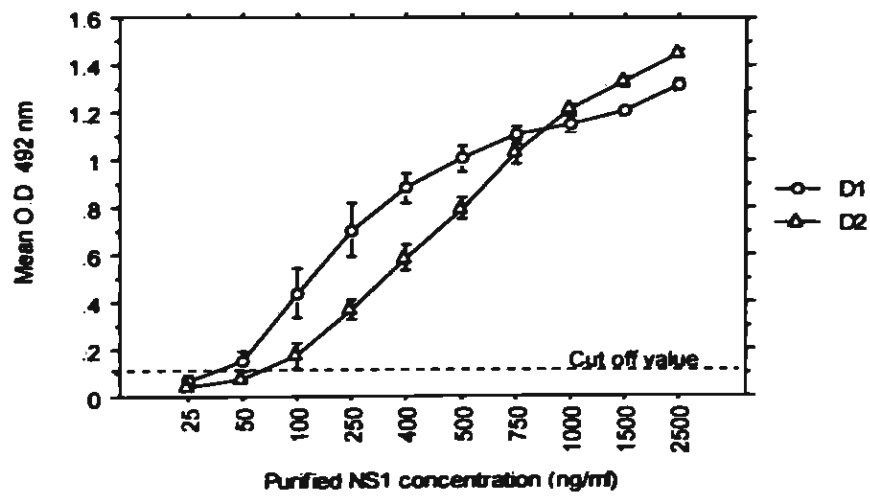
FIGURE 1.**A****B**

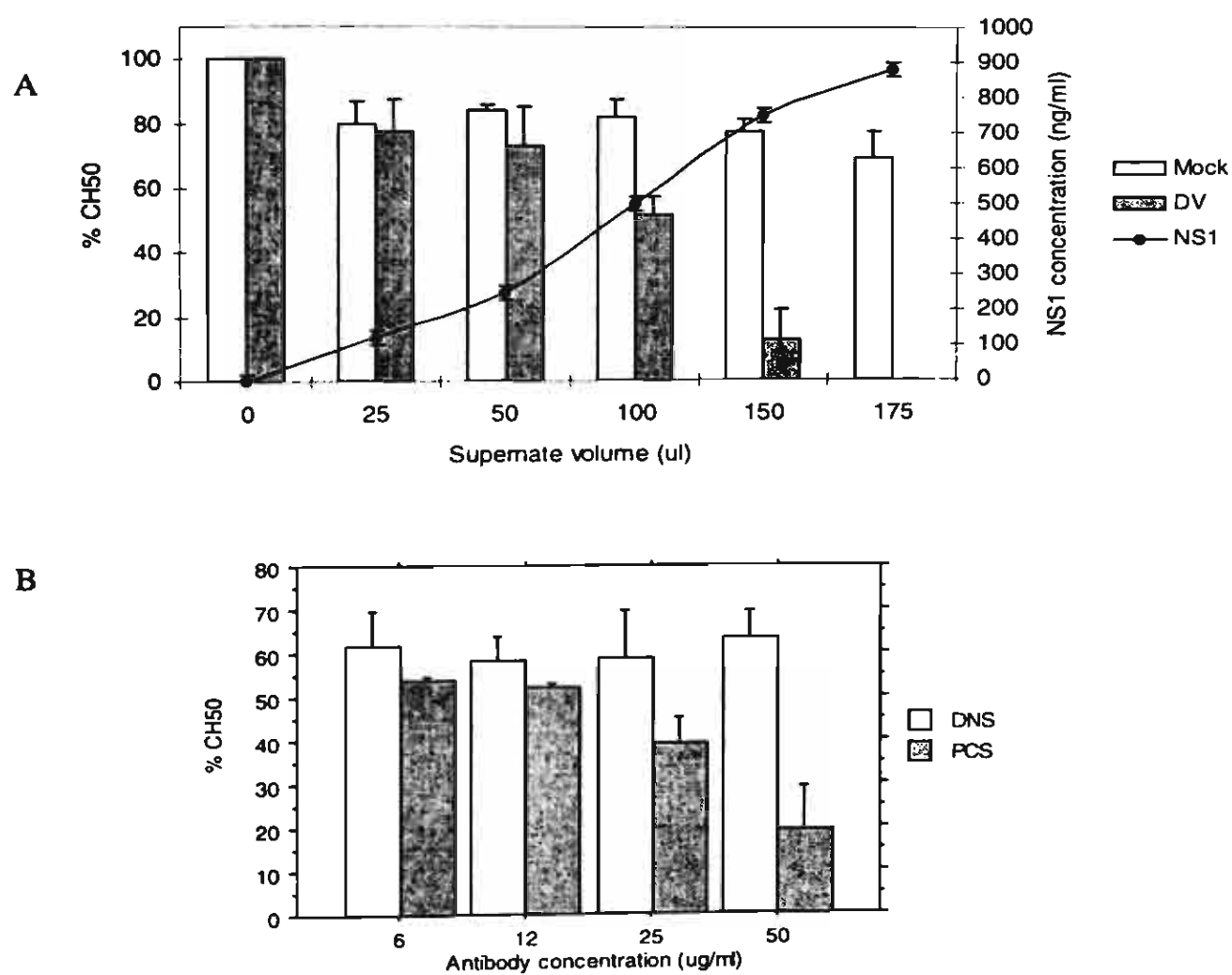
FIGURE 2.

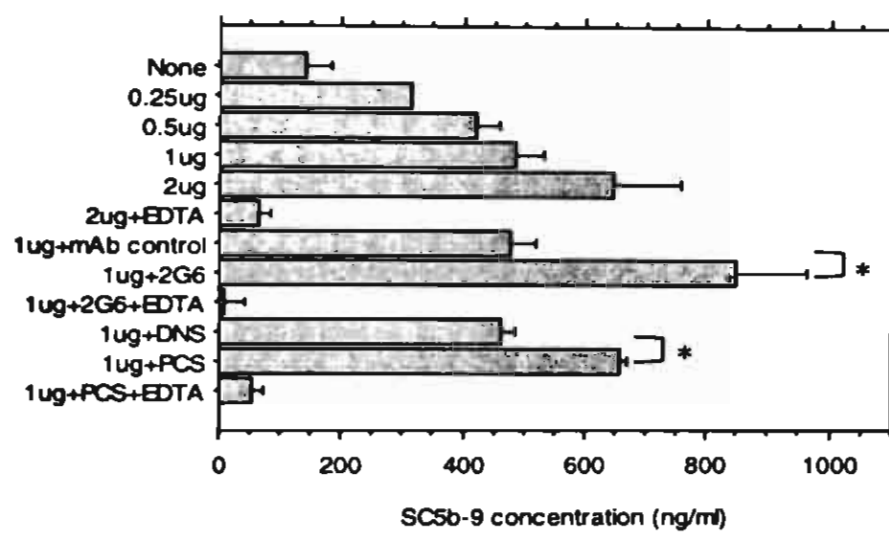
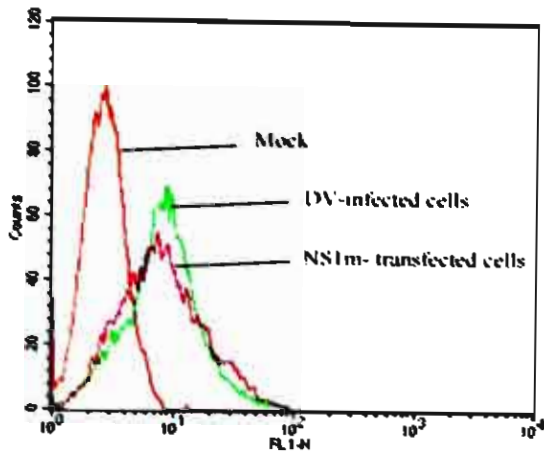
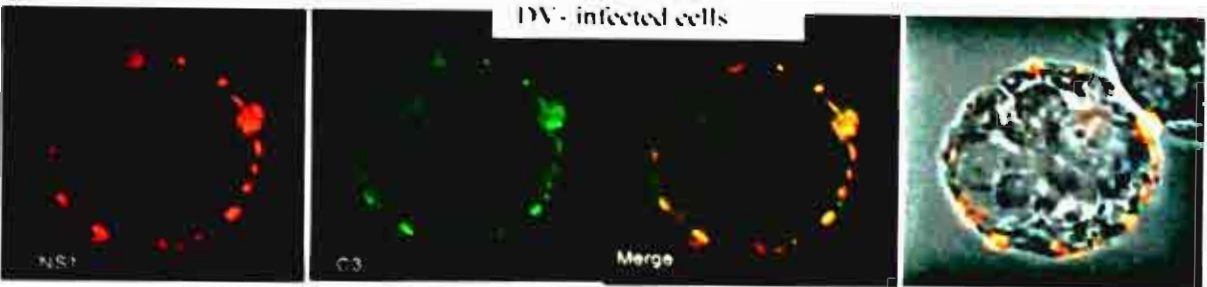
FIGURE 3.

FIGURE 4.

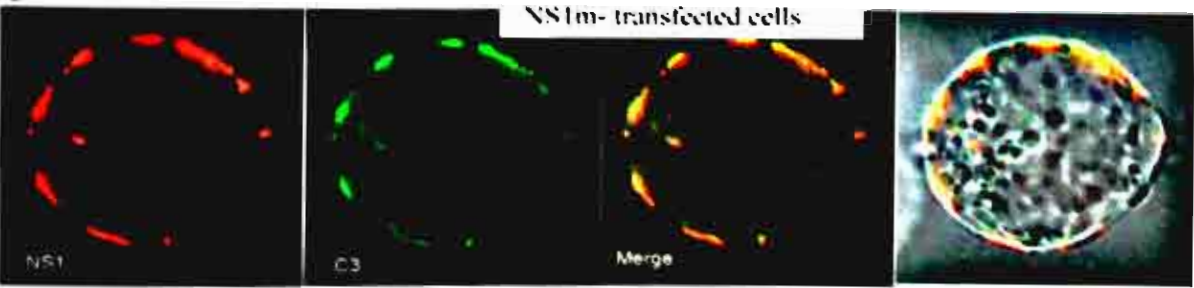
A



B



C



D

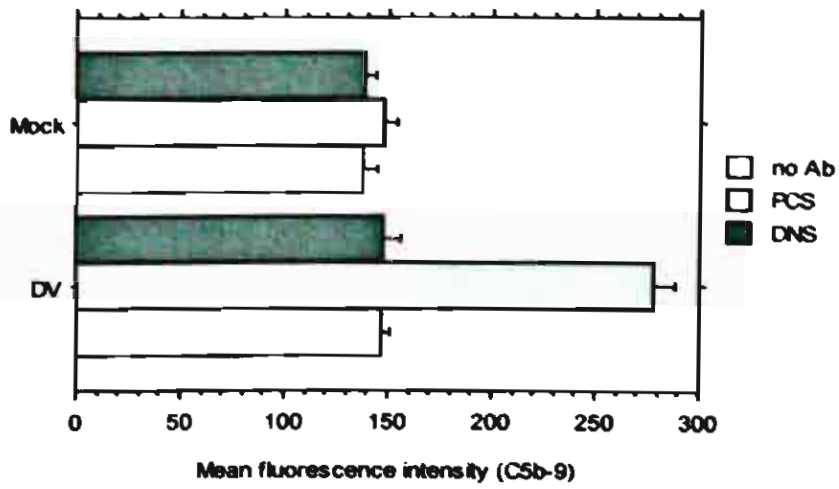


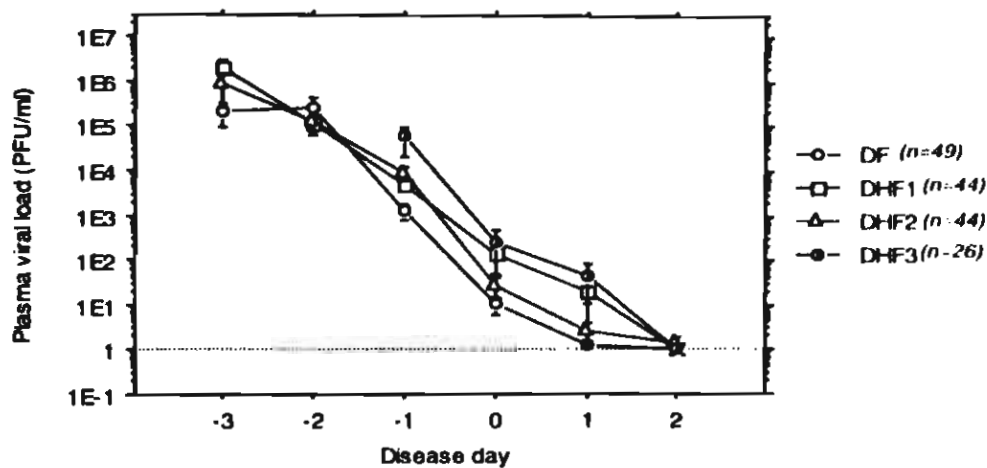
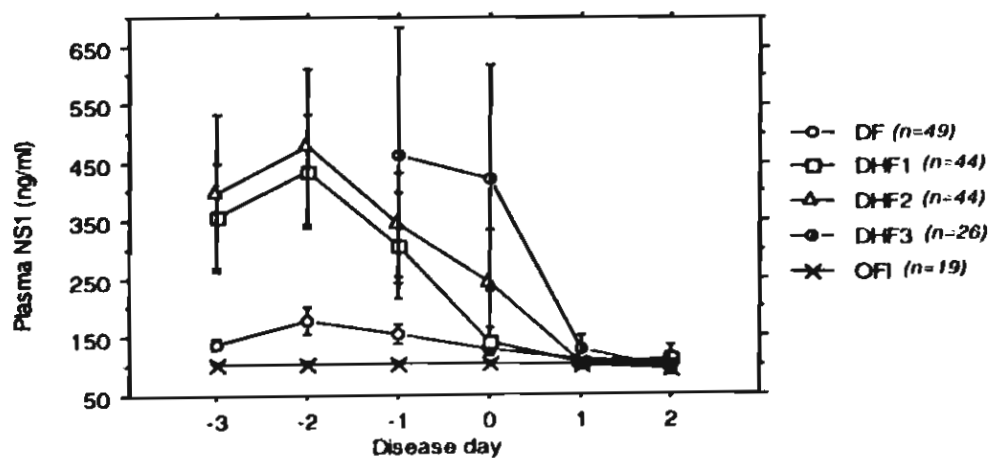
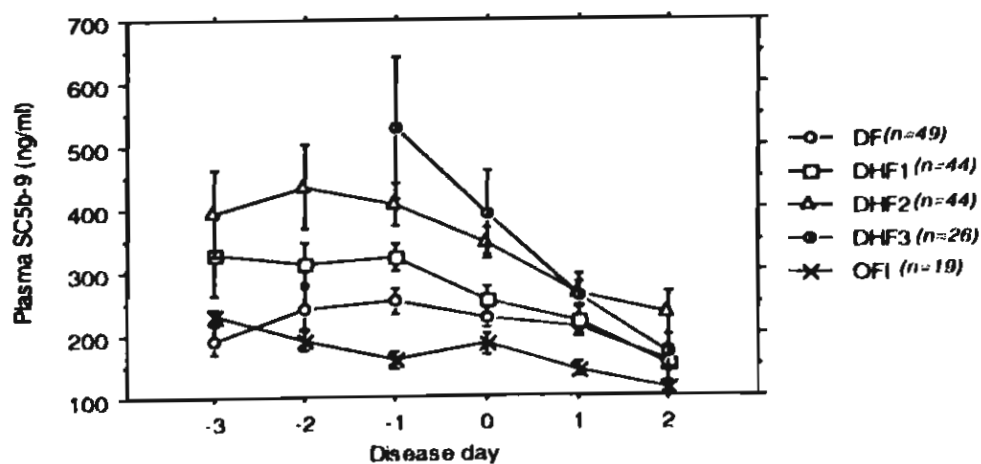
FIGURE 5.**A****B****C**

FIGURE 6

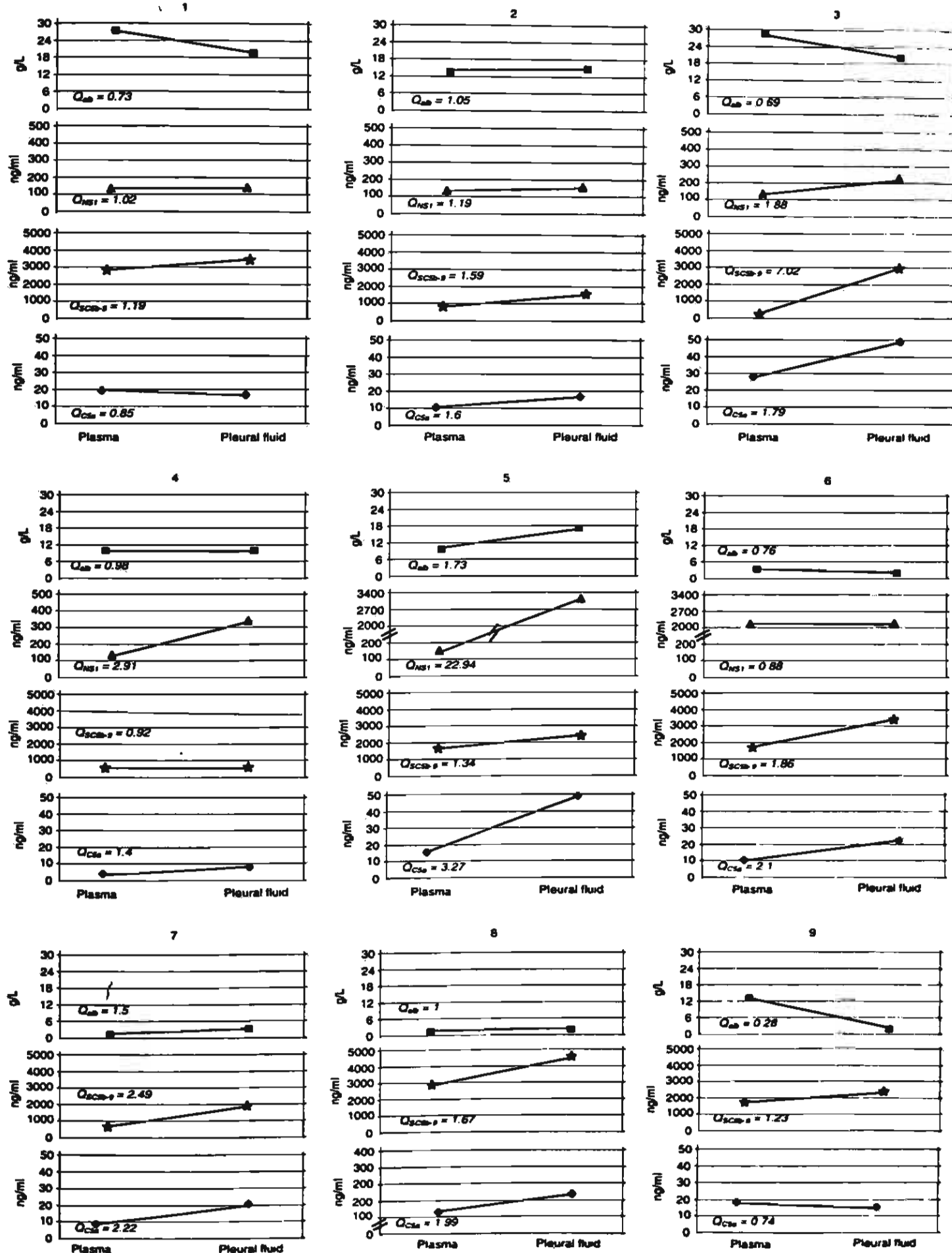
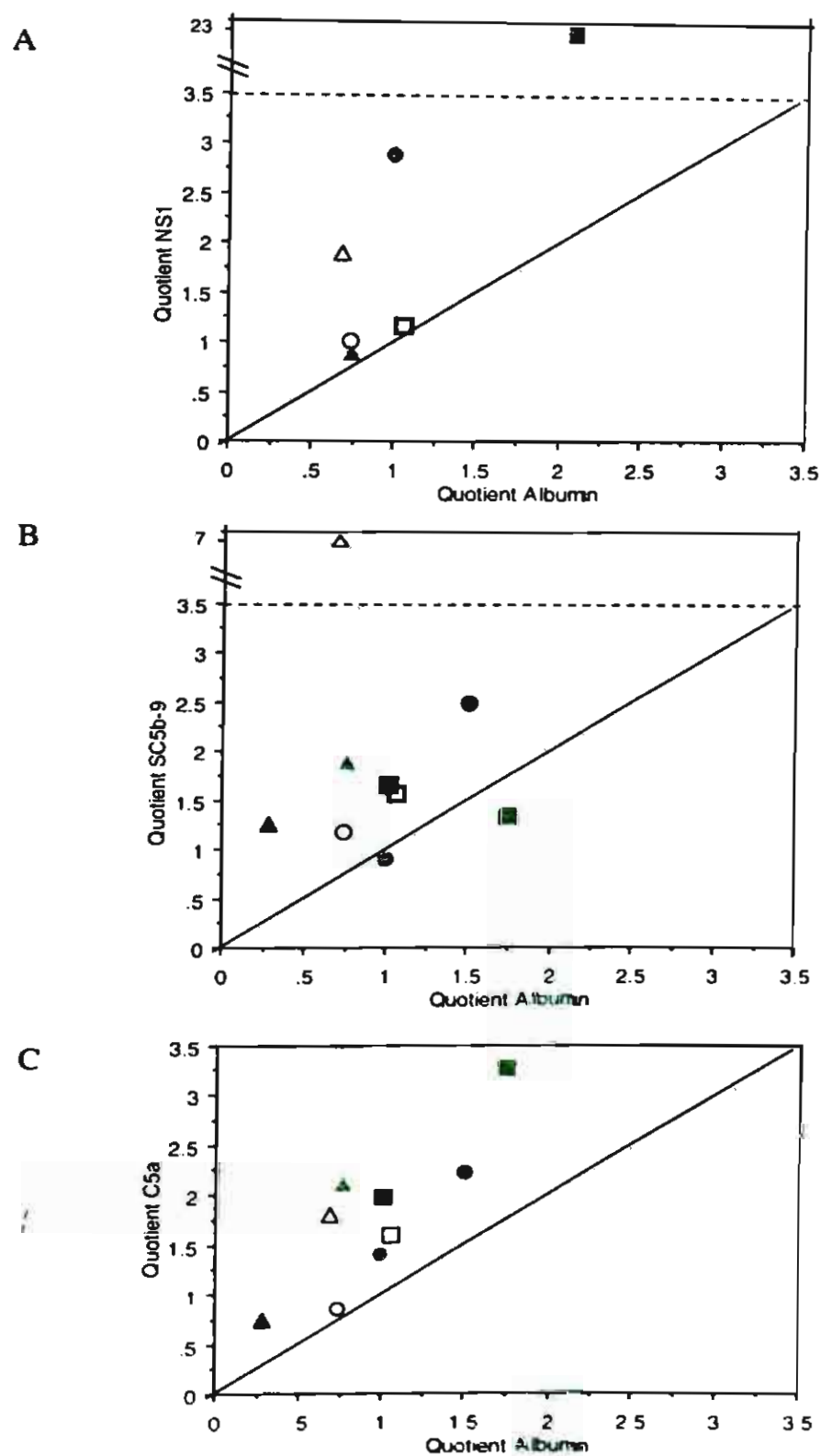


FIGURE 7



POSTER PRESENTATION

ABSTRACT

ESTABLISHMENT OF ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA) FOR THE DETECTION OF DENGUE VIRAL NONSTRUCTURAL PROTEIN-1 IN PLASMA/SERA OF DENGUE INFECTED PATIENTS

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Identification of viable viruses or viral genome in clinical specimens is a gold standard method for dengue serotype determination and diagnosis confirmation. Requirement of technical expertise, well-equipped laboratory, and high cost limit its field and bedside utilization. Nonstructural protein-1 (NS1), a non-virion associated viral product expressed in the form of glycosidylphosphatidyl inositol membrane linkage on the surfaces of infected cells and extracellularly secreted hexameric complexes, has apparently become a potential diagnostic marker due to its significantly detectable level in blood circulation of dengue infected patients. Therefore, ELISA for the detection of NS1 antigen has been developed based on a pair of dengue serotype cross reactive anti-NS1 monoclonal antibodies of IgM and IgG isotype for antigen capture and detection respectively. By employing various combinations among three clones of dengue serotype cross-reactive anti NS1 monoclonal antibodies, 2E11 (IgM), 2E3 (IgG), and 1F11 (IgG), the pair of 2E11 and 2E3 was proven to have the best detection capability for NS1 proteins from all dengue serotypes while absolutely gives very low background O.D when testing with mock-infected supernatant. Immunoaffinity-purified NS1 derived from dengue 2 virus infected cells is used as protein standard to establish the sensitivity for NS1 detection of 5 ng/ml. The linear portion of the standard curve ranges from 5 to 250 ng/ml. Application of human plasma or serum to the purified NS1 reveals minimal interference with NS1 detection capability from serum components at the test dilutions routinely used. The developed ELISA for NS1 detection could become a useful additional diagnostic test for dengue virus infection.

INTRODUCTION

Identification of viable viruses or viral genome in clinical specimens is a gold standard method for dengue serotype determination and diagnosis confirmation. Requirement of technical expertise, well-equipped laboratory, and high cost limits its field and bedside utilization. Nonstructural protein-1 (NS1), a non-virion associated viral product expressed in the form of glycosidylphosphatidyl inositol membrane linkage on the surface of infected cells and extracellularly secreted hexameric complexes, has apparently become a potential diagnostic marker due to its significantly detectable level in blood circulation of dengue infected patients.

OBJECTIVE

To establish of Enzyme Linked Immunosorbent Assay (ELISA) for the detection of dengue viral nonstructural protein-1 in plasma/sera of dengue infected patients

MATERIALS AND METHODS

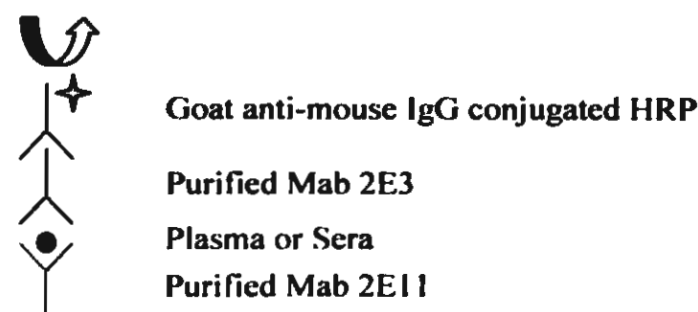


FIGURE 1. The diagram of pattern and reagents used in NS1 capture ELISA

NS1 capture ELISA has been developed based on a pair of dengue serotype cross reactive anti-NS1 monoclonal antibodies of IgM and IgG isotype for antigen capture and detection respectively. By employing various combinations among three clones of dengue serotype cross-reactive anti NS1 monoclonal antibodies, 2E11 (IgM), 2E3 (IgG), and 1F11 (IgG), the pair of 2E11 and 2E3 was proven to have the best detection capability for NS1 protein from all dengue serotypes while absolutely gives very low background O.D. when testing with mock-infected supernatant. Therefore, the pair of 2E11 and 2E3 was selected for further development of capture ELISA. Format of NS1 capture ELISA was illustrated in Fig. 1.

Optimization of purified NS1 specific monoclonal antibodies used for ELISA

To establish an ELISA for detection of NS1 antigen, the optimal concentration of purified monoclonal antibody used for NS1 capture and detection was explored. NS1 specific monoclonal antibody clone 2E11 was coated on 96-well microtiter plate at concentration 3, 5, 7 and 9 $\mu\text{g/ml}$ while various concentration of anti-NS1 monoclonal antibody clone 2E3 ranging from 5-200 $\mu\text{g/ml}$ were tested for NS1

detection capability. The optimal concentration of 2E11 and 2E3 selected for further experiments was 7 and 150 μ g/ml respectively as shown in Fig. 2.

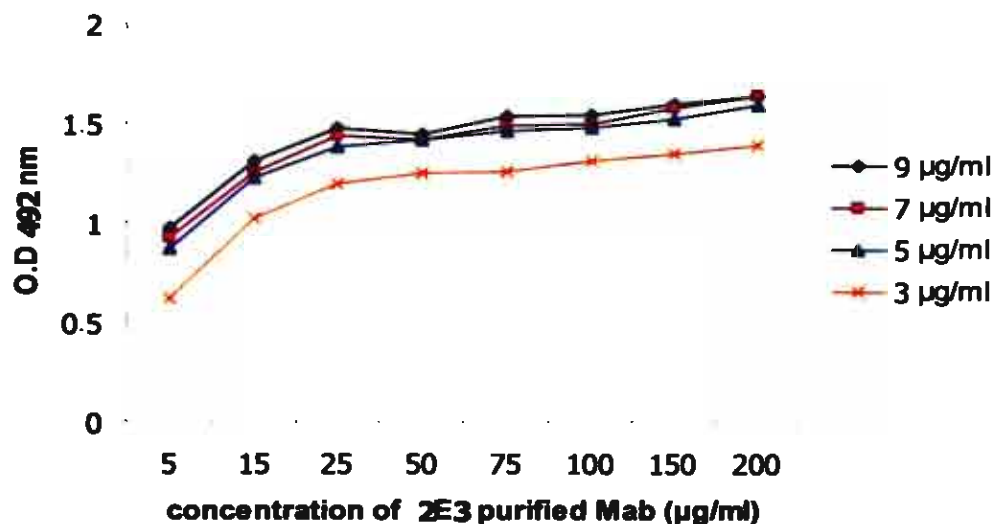


FIGURE 2. Checker board analysis to acquire the optimized concentrations of purified NS1 specific monoclonal antibody clone 2E11 and 2E3 used in NS1 capture ELISA. Purified monoclonal clone 2E11 was coated at the dilution of 3, 5, 7 and 9 μ g/ml as indicated in the legend. Various concentrations of purified monoclonal clone 2E3 ranging from 5 to 200 μ g/ml as indicated in the X axis were tested at each concentration of 2E11 purified monoclonal antibody using dengue infected culture supernatant.

Characteristics of the NS1 capture ELISA

In order to establish a sensitivity of NS1 capture ELISA, immunoaffinity-purified NS1 derived from dengue virus serotype 2 was used as standard antigen. The linear portion of the standard curve ranged from 50 to 1000ng/ml (Fig. 3). The sensitivity of the assay was approximately 50ng/ml.

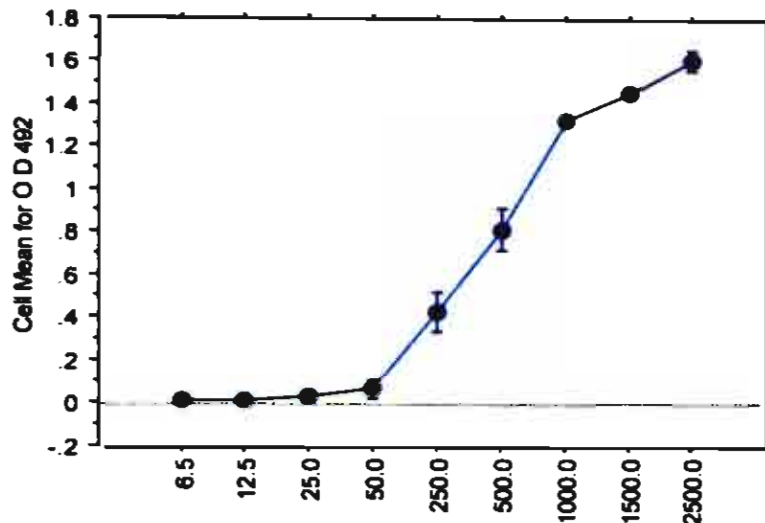


FIGURE 3. Standard curve for NS1 capture ELISA with purified dengue virus type 2 NS1. NS1 was serially diluted in PBS containing 15% fetal bovine serum. Data points represent the mean and standard deviation for three replicates.

The developed NS1 capture ELISA based on serotype cross reactive monoclonal antibodies is capable of detecting NS1 secreted from all 4 dengue serotypes

In order to test whether our developed ELISA is capable of detecting NS1 protein derived from all four dengue virus serotypes, culture supernatants of each dengue serotypes were two fold serially diluted and subjected to NS1 capture ELISA as described above. As shown in Fig. 5, NS1 from all dengue serotypes gave considerable high absorbance (> 1.5) at undiluted specimens and produced sigmoid curve in a similar manner of standard curve using immunoaffinity purified NS1 derived from dengue serotype 2 (Fig. 4). In order to provide quantitative estimates of NS1 derived from all four dengue serotypes, purified NS1 from each serotypes are being prepared to establish appropriate standard curves.

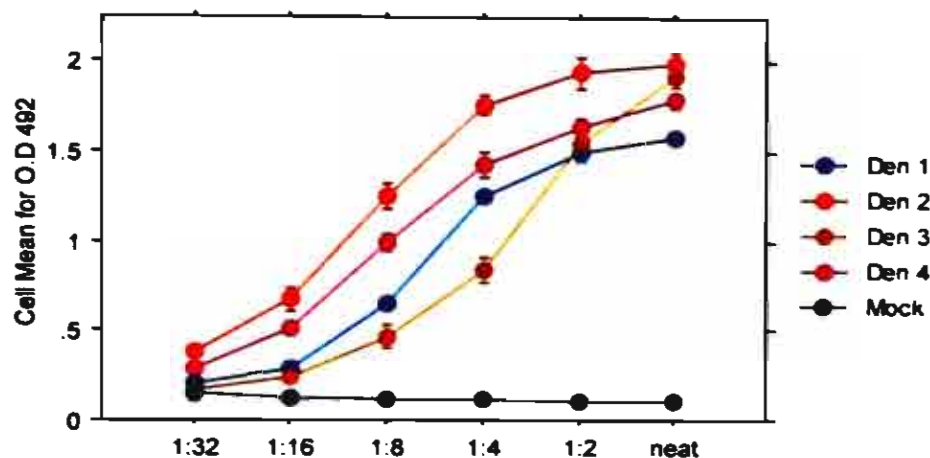


FIGURE 4. NS1 capture ELISA is capable of detecting secreted NS1 of all 4 dengue serotypes. Mock or dengue infected culture supernatant derived from all 4 serotypes was two fold serially diluted and subjected to NS1 capture ELISA. Data points represent the mean and standard deviation for three replicates.

Effect of human serum components on the detection of NS1 by the developed NS1 capture ELISA

To determine the effect of plasma components on the sensitivity of NS1 detection, dengue virus serotype 2 supernatant was serially diluted in PBS containing 0.1% bovine serum albumin or in normal human plasma. The effect of human plasma components on the NS1 detection capability was minimal at a dilution of 1:5 (data not shown) and negligible at a 1:10 dilution which is routinely used as demonstrated in Fig. 5.

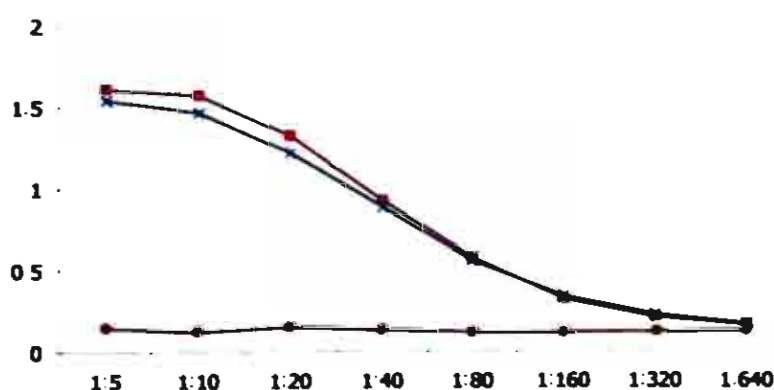


FIGURE 5. Effect of human serum or plasma components on the detection of NS1 in the capture ELISA. Dengue serotype 2 infected supernatant was serially diluted in the absence (■) or presence (×) of normal human plasma at dilution 1:10. Normal human plasma at the dilution of 1:10 in buffer alone served as negative control for the test (●).

CONCLUSION

The detection of viral nonstructural protein-1 by ELISA was developed. The assay was based on a pair of dengue serotype cross reactive anti-NS1 antibodies for antigen capture and detection. With purified dengue virus type 2 NS1 as a protein standard, the sensitivity of an ELISA was approximately 50ng/ml. The linear portion of the standard curve ranged from 50 to 1000ng/ml. Normal human plasma at the dilution of routinely used had no effect on NS1 detection capability by capture ELISA. The developed ELISA for NS1 detection could become a useful additional diagnostic test for dengue virus infection.