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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.

ภาคผนวก

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 ≥ 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% CO_2. 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 CM^2 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 H_2O_2 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. $1x10^6$ 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 containing10 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

$1x10^6$ 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 1h. 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/mm^2. 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 µg/ 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 µl normal human serum and buffer was added to give a total of 200 µl per sample. CH50 was determined after incubation at 37°C for 1 h. Data are displayed as the mean ± 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 µl of culture supernatants from DV-infected cells were mixed with purified antibodies from PCS and DNS at the given final concentrations and 25 µl normal human serum. CH50 was determined after 60 min, 37°C. Data are displayed as the mean ± 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 µg/ml) or PCS (20 µg/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 ± 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 ± SD from three independent experiments.

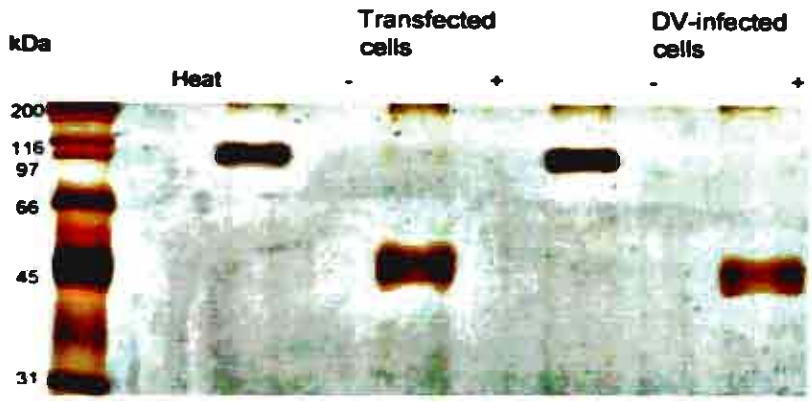
FIGURE 5. Viral load, levels of NS1, and terminal SC5b-9 complexes in the circulation of patients with DF and DHF/DSS. Plasma samples were assayed for dengue viral RNA levels using quantitative real time RT-PCR, and soluble NS1 and SC5b-9 complexes were quantified by ELISA. Disease day 0 was defined 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


kDa
Heat
Transfected cells
DV-infected cells
-
+
-
+
200
116
97
66
45
31

B

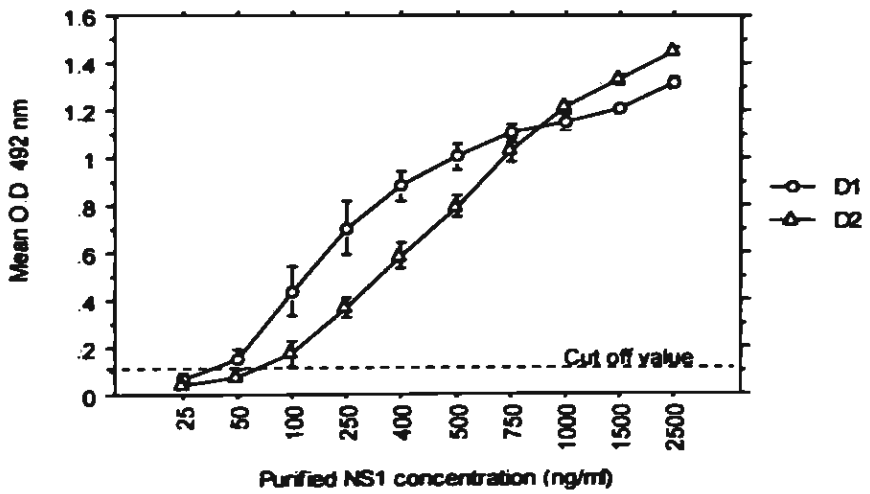
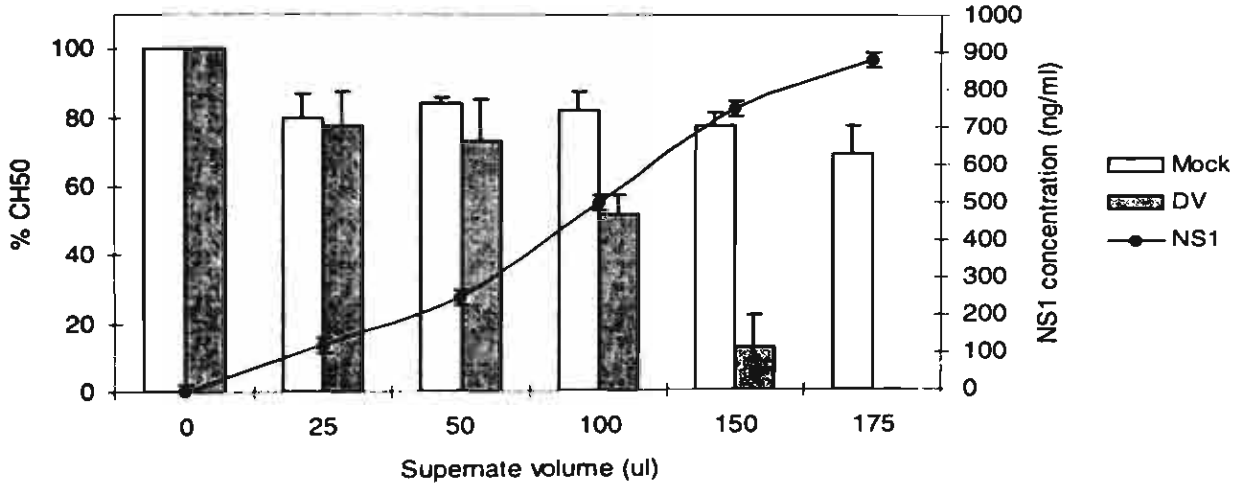

Mean O.D 492 nm
1.6
1.4
1.2
1
.8
.6
.4
.2
0
Cut off value
25
50
100
250
400
500
750
1000
1500
2500
Purified NS1 concentration (ng/ml)
D1
D2

FIGURE 2.

A


% CH50
NS1 concentration (ng/ml)
Supernate volume (ul)
0
25
50
100
150
175
Mock
DV
NS1

B

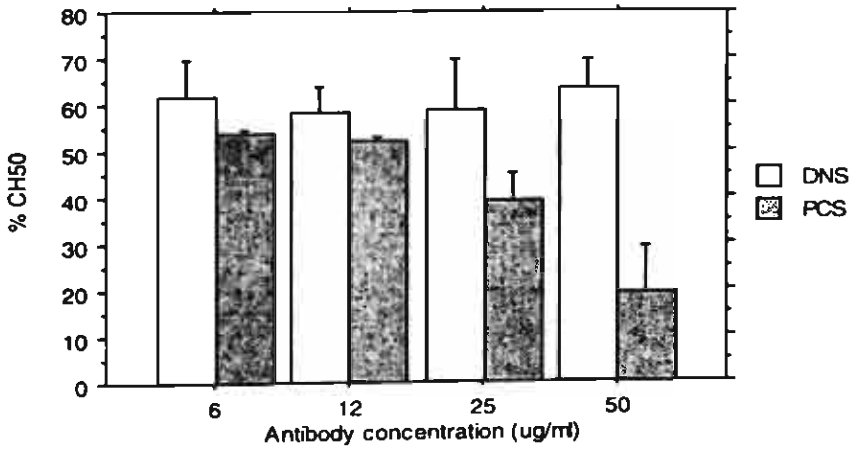

% CH50
Antibody concentration (ug/ml)
6
12
25
50
DNS
PCS

FIGURE 3.

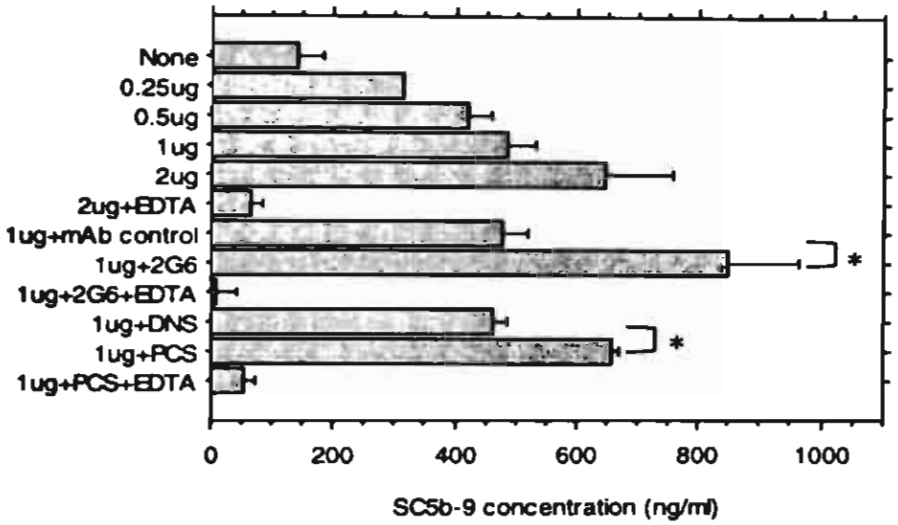
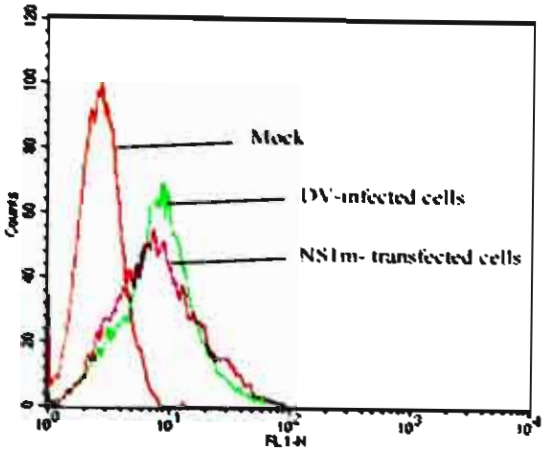
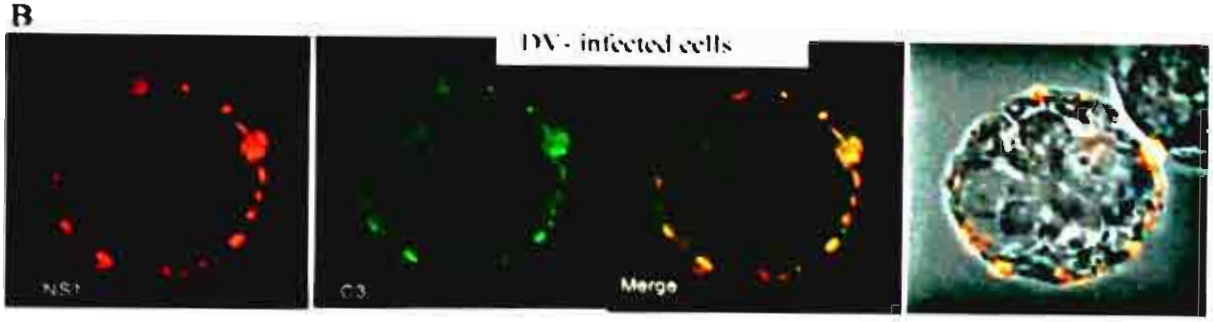

None
0.25ug
0.5ug
1ug
2ug
2ug+EDTA
1ug+mAb control
1ug+2G6
1ug+2G6+EDTA
1ug+DNS
1ug+PCS
1ug+PCS+EDTA
*
*
0
200
400
600
800
1000
SC5b-9 concentration (ng/ml)

FIGURE 4.

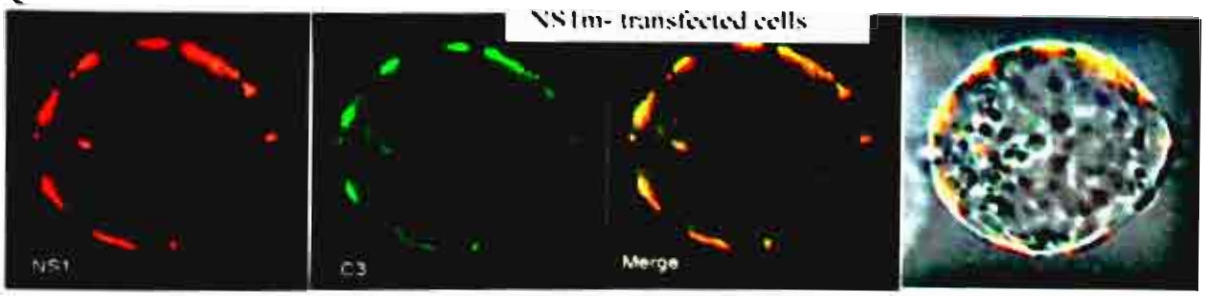
A



B



C



D

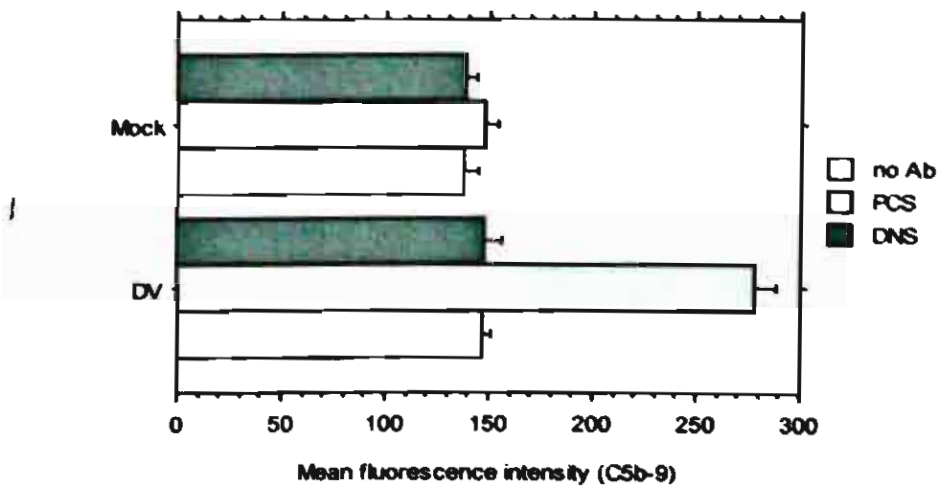
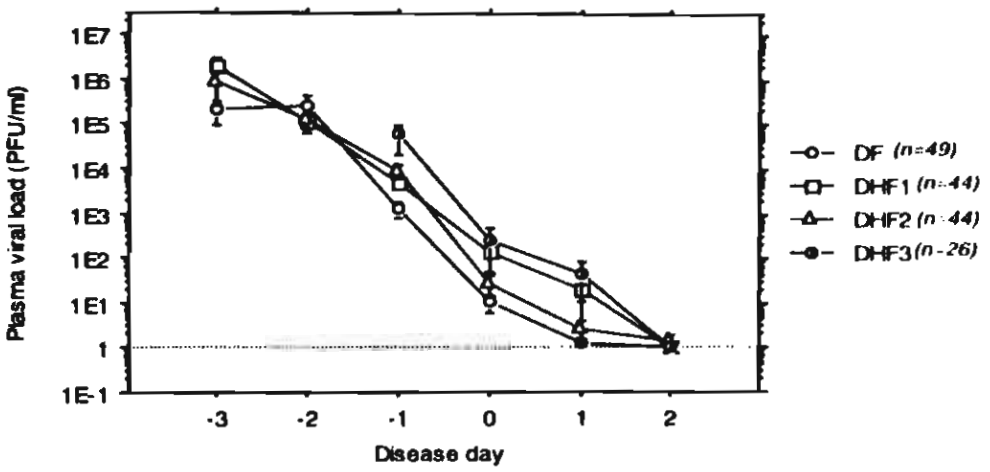
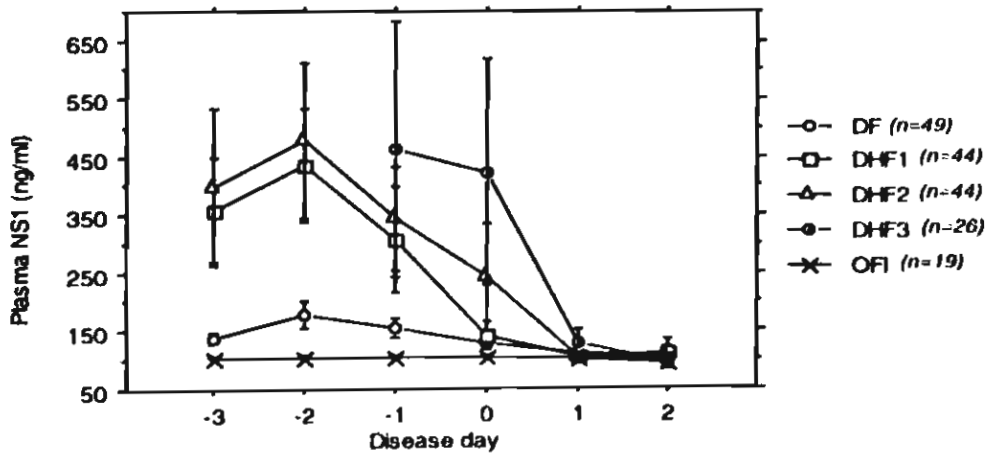


FIGURE 5.

A


Plasma viral load (PFU/ml)
1E7
1E6
1E5
1E4
1E3
1E2
1E1
1
1E-1
-3
-2
-1
0
1
2
Disease day
DF (n=49)
DHF1 (n=44)
DHF2 (n=44)
DHF3 (n=26)

B


Plasma NS1 (ng/ml)
650
550
450
350
250
150
50
-3
-2
-1
0
1
2
Disease day
DF (n=49)
DHF1 (n=44)
DHF2 (n=44)
DHF3 (n=26)
OFI (n=19)

C

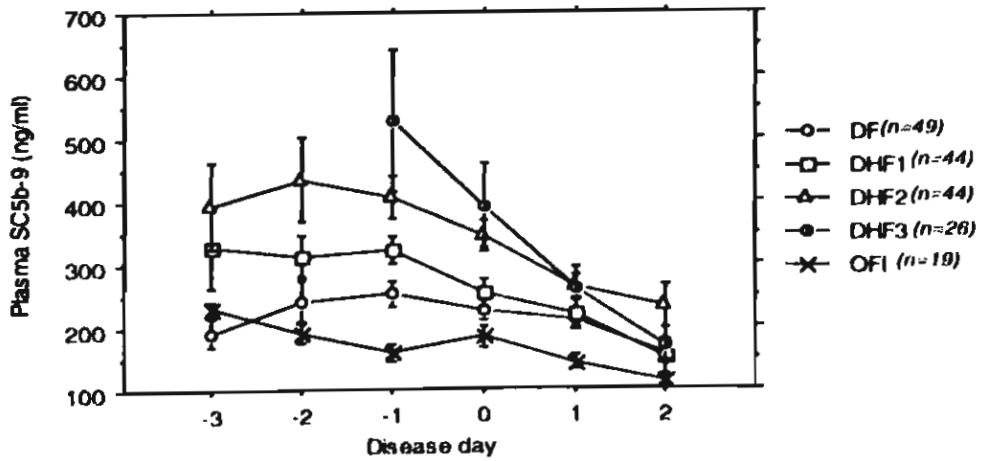

Plasma SC5b-9 (ng/ml)
700
600
500
400
300
200
100
-3
-2
-1
0
1
2
Disease day
DF(n=49)
DHF1 (n=44)
DHF2 (n=44)
DHF3 (n=26)
OFI (n=19)

FIGURE 6

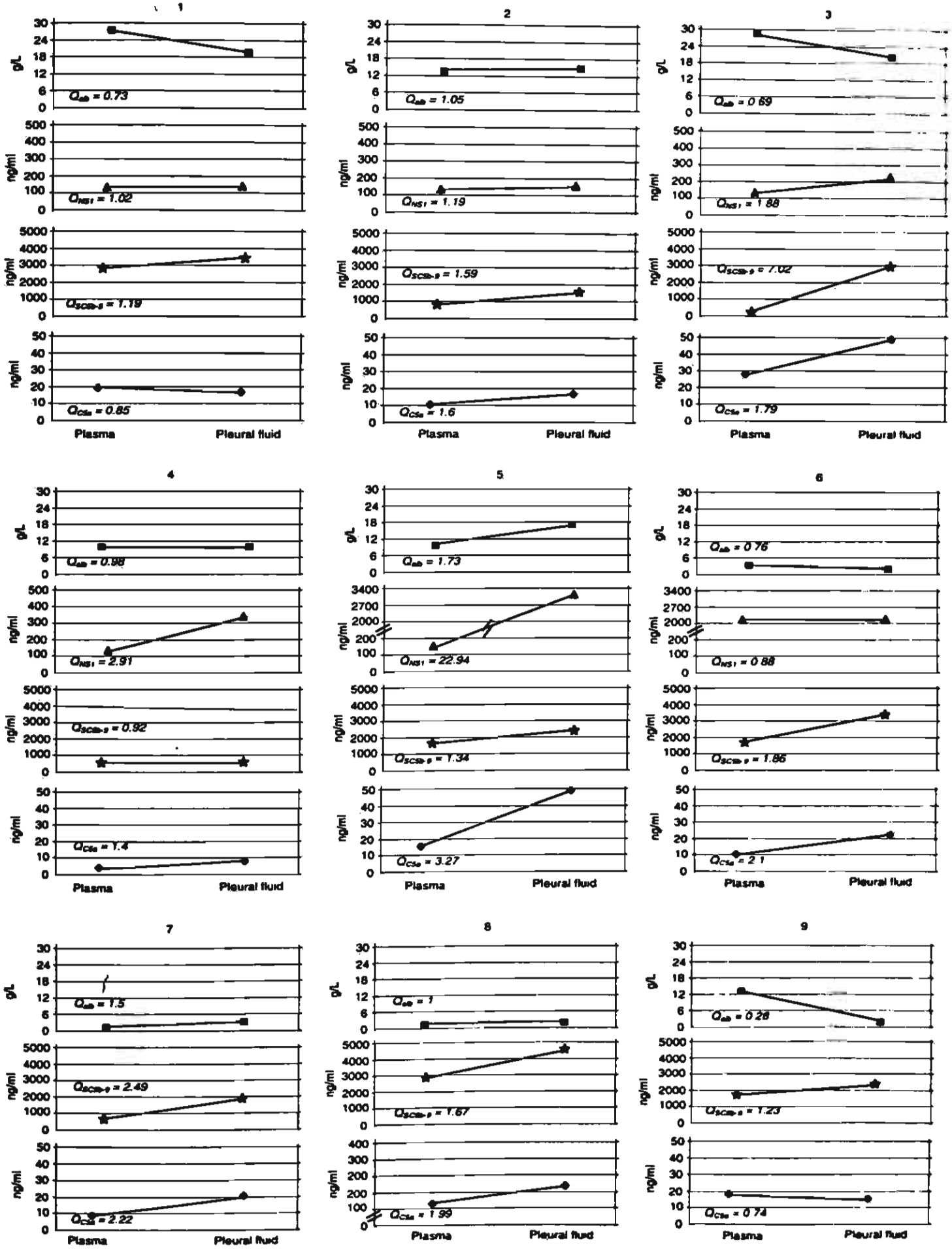
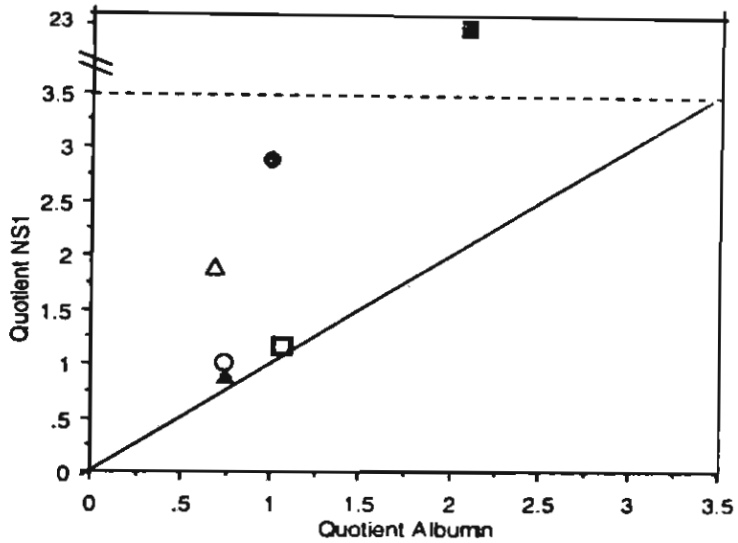
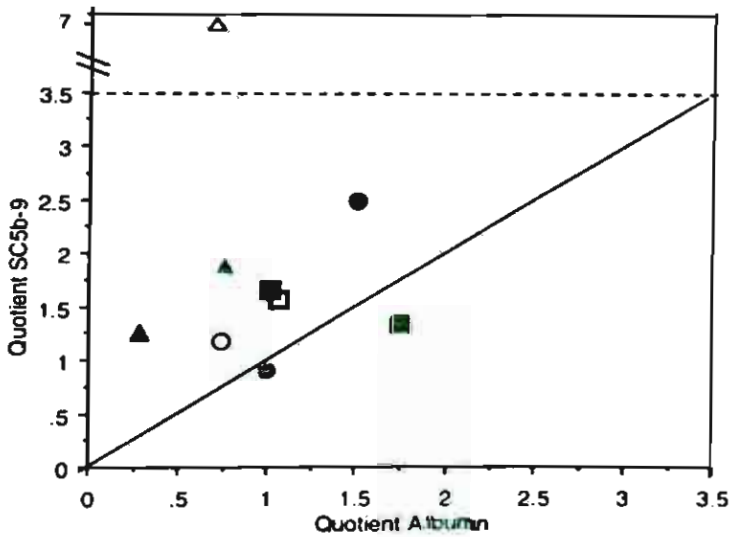


FIGURE 7

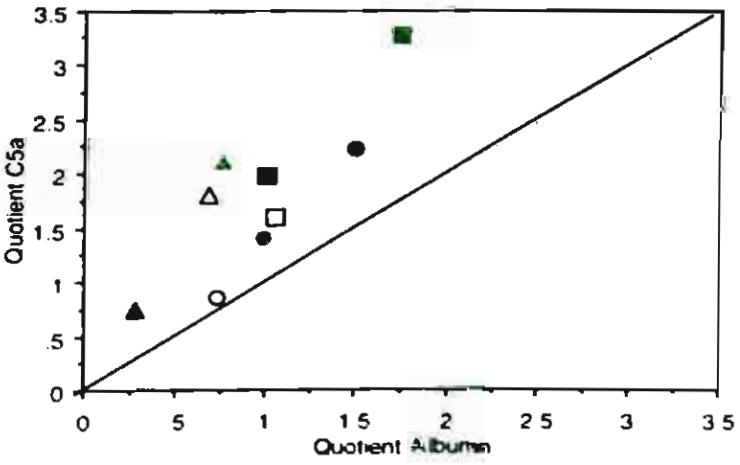
A


Quotient NS1
Quotient Albumin
0
.5
1
1.5
2
2.5
3
3.5
23

B


Quotient SC5b-9
Quotient Albumin
0
.5
1
1.5
2
2.5
3
3.5
7

C


Quotient C5a
Quotient Albumin
0
.5
1
1.5
2
2.5
3
3.5

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

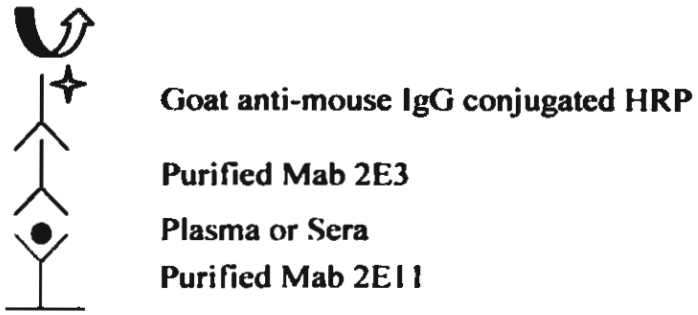

FIGURE 1. The diagram of pattern and reagents used in NS1 capture ELISA

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 9μg/ml while various concentration of anti-NS1 monoclonal antibody clone 2E3 ranging from 5-200μg/ml were tested for NS1

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

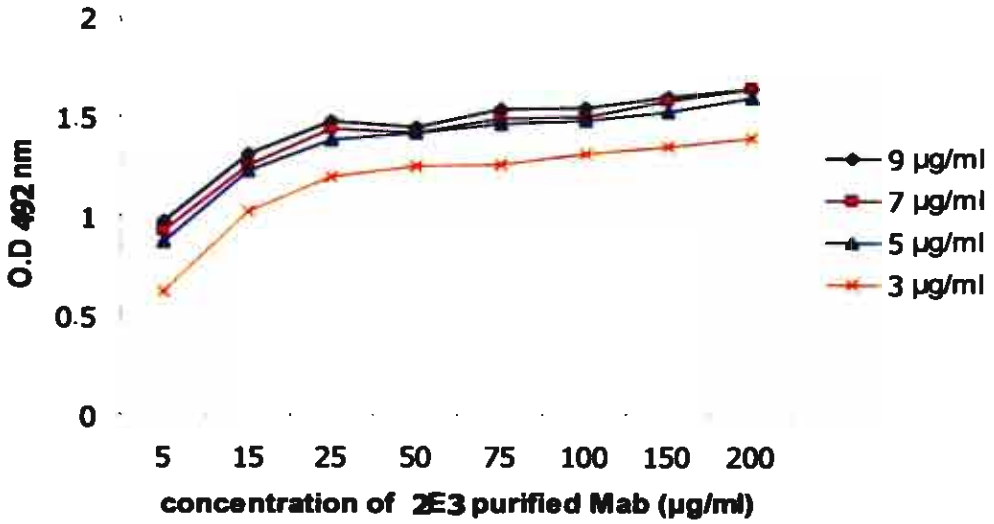

FIGURE 2. Checker board analysis to acquire the optimized concentrations of purified NS1 specific monoclonal antibody clone 2E11 and 2E3 used in NS1 capture ELISA. Purified monoclonal clone 2E11 was coated at the dilution of 3, 5, 7 and 9µg/ml as indicated in the legend. Various concentrations of purified monoclonal clone 2E3 ranging from 5 to 200µg/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.

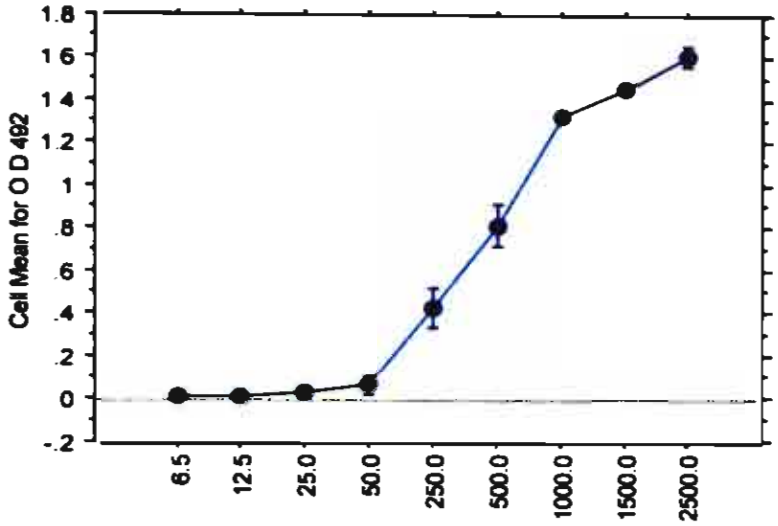

FIGURE 3. Standard curve for NS1 capture ELISA with purified dengue virus type 2 NS1. NS1 was serially diluted in PBS containing 15% fetal bovine serum. Data points represent the mean and standard deviation for three replicates.

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.

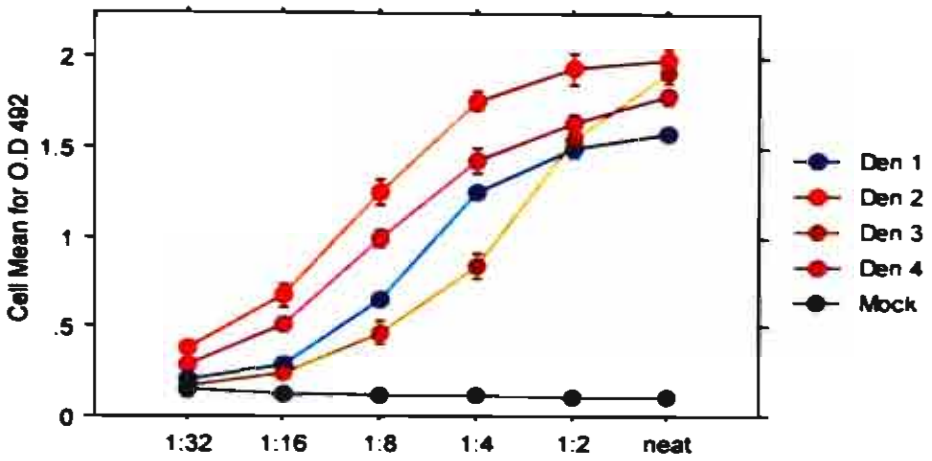

FIGURE 4. NS1 capture ELISA is capable of detecting secreted NS1 of all 4 dengue serotypes. Mock or dengue infected culture supernatant derived from all 4 serotypes was two fold serially diluted and subjected to NS1 capture ELISA. Data points represent the mean and standard deviation for three replicates.

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.

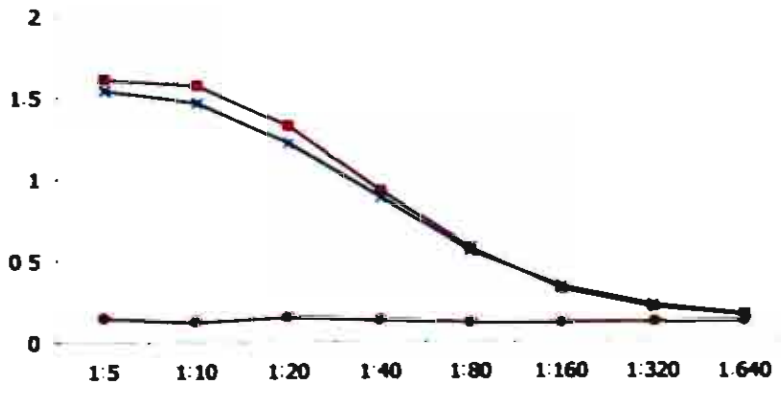

FIGURE 5. Effect of human serum or plasma components on the detection of NS1 in the capture ELISA. Dengue serotype 2 infected supernatant was serially diluted in the absence (■) or presence (▲) of normal human plasma at dilution 1:10. Normal human plasma at the dilution of 1:10 in buffer alone served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