

Vascular Leakage in Severe Dengue Virus Infections: A Potential Role for the Nonstructural Viral Protein NS1 and Complement

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

Methods. The major nonstructural dengue virus (DV) protein NS1 was tested for its capacity to activate human complement in its membrane-associated and soluble forms. Plasma samples from 163 patients with DV infection and from 19 patients with other febrile illnesses were prospectively analyzed for viral load and for levels of NS1 and complement-activation products. Blood and pleural fluids from 9 patients with DSS were also analyzed.

Results. 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 from patients with DSS.

Conclusions. Complement activation mediated by NS1 leads to local and systemic generation of anaphylatoxins and SC5b-9, which may contribute to the pathogenesis of the vascular leakage that occurs in patients with DHF/DSS.

Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are severe forms of dengue virus (DV) infection and are still one of the leading causes of morbidity and mortality in children of school age in tropical and subtropical regions. Major pathophysiological processes that distinguish DHF/DSS from mild dengue fe-

ver are abrupt onset of vascular leakage, hypotension, and shock, which are accompanied by thrombocytopenia and hemorrhagic diathesis in the absence of any characteristic histopathological vascular lesions [1, 2].

Four DV serotypes exist, and DHF/DSS occurs almost exclusively in patients who are reinfecting with a different virus serotype [3, 4]. An enigmatic dysfunction of the immune system then leads to enhanced viral replication. This has been proposed to be due to an

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antibody-mediated increase in viral uptake in target cells [5, 6] or to cross-depletion of protective CD8 lymphocytes [7]. High viral loads and levels of circulating viral antigens are consequently found in these patients [8–10].

Thirty years ago, accelerated complement consumption and a marked reduction in plasma complement components were observed during shock in patients with DSS [11, 12], which led to the proposal that complement activation plays an important role in disease pathogenesis [13, 14]. In the following decades, the thrust of international research shifted toward the possible role of lymphocytes and cytokines [6, 15, 16], and the cause of complement activation has remained unknown. Previously, we observed that surfaces of DV-infected cells bind DV antibodies, which leads to complement activation and cytokine secretion [17]. The search for the responsible viral antigen led to NS1, a 45-kDa nonstructural protein that resides in the plasma membrane of infected cells [18] and is also released in oligomeric form to the extracellular milieu [19]. NS1 is strongly immunogenic, and type-specific anti-NS1 antibodies play a role in protection against disease [20–23]. High levels of NS1 are found in the circulation of DV-infected patients during the acute phase of the disease [10, 24, 25].

We report that soluble NS1 (NS1s) and membrane-associated NS1 (NS1m) activate human complement, that plasma levels of NS1s and the terminal SC5b-9 complement complex correlate with disease severity, and that massive complement activation probably occurs at the sites of vascular leakage. Complement anaphylatoxins and the terminal SC5b-9 complement complex increase vascular permeability [26, 27], and SC5b-9 increases lung hydraulic conductivity [28]. A link thus emerges between NS1 level, complement activation, and the clinical manifestation of DHF/DSS.

PATIENTS AND METHODS

Patient enrollment and study design. A total of 182 patients admitted to the ward of Khon Khan Provincial Hospital, Thailand, between November 2001 and December 2003 who met the following 2 sets of criteria were enrolled in this prospective study. The first set of criteria included (1) age 1–15 years; (2) pyrexia for not more than 4 days, with no obvious source of infection; and (3) tourniquet test positivity or history of signs/symptoms of bleeding/hemorrhagic diathesis. These patients subsequently all tested positive for DV by reverse-transcription polymerase chain reaction (RT-PCR), virus isolation, and DV antibody ELISA profiling. The second set of criteria included (1) age 1–15 years and (2) <3 days of fever with no obvious source of infection. Patients who subsequently tested positive for DV were included in the dengue fever (DF) and DHF groups, whereas those with negative RT-PCR and DV antibody results were assigned to the control “other febrile illness” (OFI)

group. Blood specimens were collected daily in 5 mmol/L EDTA until 1 day after defervescence, and plasma samples were stored at -70°C . DV infection was confirmed by measuring anti-DV IgM/IgG and by RT-PCR [32]. Pleural fluid was aspirated only in patients experiencing severe respiratory difficulty, as a part of therapeutic measures to alleviate the pulmonary insufficiency caused by rapidly accumulated fluid.

Grading of DHF followed World Health Organization criteria [33]: DHF3 is assigned to patients with signs of circulatory failure, DHF2 is assigned to patients with spontaneous bleeding, and DHF1 is assigned to patients with fever who are tourniquet-test positive. Study day 0 was defined as the calendar day during which the patient’s temperature fell and stayed below 37.8°C . The study protocol was approved by the Ministry of Public Health (approval date, 7 May 2003), the Faculty of Medicine Siriraj Hospital (certificates of approval 156/2002 and 115/2004), and the Khon Khan Hospital (approval date, 31 October 2002). Informed consent was individually obtained from all subjects.

Reagents. IgG from pooled convalescent-phase serum (PCS) samples (hemagglutination titer, $\geq 1/25600$) and control serum samples (DV antibody-negative serum [DNS]) were purified by protein G column chromatography (Pharmacia). NS1-specific monoclonal antibody (MAb) clones 2G6, 1A4, 1B2, 1F11, 2E11, and 2E3 have been described elsewhere [29].

Cells and viruses. The swine fibroblast cell line (PsCloneD) and the insect cell line C6/36 were cultured at 37°C and 28°C , respectively, in L-15 medium (Life Technologies) containing 10% tryptose phosphate broth (Sigma) and 10% fetal bovine serum (FBS) (Hyclone). The human kidney epithelial cell line HEK-293T was grown in RPMI 1640 (GIBCO) containing 10% FBS, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin. DV serotype 1 (DEN-1), DEN-2, DEN-3, and DEN-4 (strain Hawaii, 16681, H-87, and H-241, respectively) were propagated in C6/36 cells [17].

Two HEK-293T cell lines expressing NS1 were conventionally generated [30]. Cells were transfected with pcDNA3.1/Hygro (Invitrogen) containing the coding sequence of NS1s (the complete sequence of NS1) or NS1m (the complete NS1 sequence connected to the 26-aa region at the N-terminus of NS2A).

NS1 purification. Fibroblast monolayers were infected with DEN-1, -2, -3, and -4 at an MOI of 1 and were cultured in protein-free medium (Ultradoma). Culture supernatants were harvested 3 days later, centrifuged at 200,000 g, and subjected to immunoaffinity chromatography with a column prepared with anti-NS1 MAb 2G6.

For isolation of NS1s from transfected cells, culture supernatants were harvested every 3 days and replaced with fresh medium. Supernatants were passed through a $0.2\text{-}\mu\text{m}$ filter before immunoaffinity chromatography. Purified NS1 was passed over a protein G column twice to remove any traces of contaminating antibodies.

NS1 ELISA. Microtiter plates (Nunc) were coated with MAb 2E11 (5 $\mu\text{g}/\text{mL}$) overnight at 4°C. After blocking with PBS containing 15% FBS, wells were washed 5 times with PBS/0.05% Tween-20. Samples (100 μL) were added to each well and incubated for 1 h at room temperature. After 5 washes, 100 μL of MAb 2E3 (50 $\mu\text{g}/\text{mL}$) was added to each well and incubated for 1 h. The ELISA was developed conventionally, using horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma).

Assay for fluid-phase complement activation. Cell supernatants or purified NS1s from DV-2 were incubated with 12.5% normal human serum (NHS) (final concentration) in a 0.2-mL total assay volume in the presence or absence of anti-DV antibodies for 60 min at 37°C. Heat-inactivated serum or serum containing 10 mmol/L EDTA served as a negative control. Hemolytic complement titers (CH_{50}) were determined in the conventional manner. 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 an MOI of 10 and were harvested 24 h after infection. DV-infected cells or cells expressing NS1m (1×10^6) were incubated with purified PCS, DNS, a mix of anti-NS1 MAbs, or isotype controls, in the presence of 12.5% NHS. Washed cells were incubated with a MAb against C3dg, provided by P. J. Lachmann, or against SC5b-9 complexes (Quidel); this was followed by staining with fluorescein isothiocyanate (FITC)-labeled rabbit $\text{F}(\text{ab}')_2$ anti-mouse immunoglobulin. Double staining for C3 and NS1 was performed by first reacting cells with a mix of anti-NS1 MAbs at 37°C for 1 h. After 1 wash, cells were fixed with 2% paraformaldehyde for 10 min and incubated with rabbit anti-human C3c and C3d (Dakopatts). Stainings were developed with FITC-conjugated swine anti-rabbit immunoglobulins (Dako) and Cy3-conjugated goat anti-mouse immunoglobulin (Jackson Immuno Research Laboratories). Washed cells were resuspended in 50% fluorescent mounting medium (Dako) and observed under a Zeiss LSM 510 META confocal microscope (Carl Zeiss).

Quantitative RT-PCR. RNA was extracted from DV-infected cell supernatants or patients' plasma by use of QIAamp Viral RNA Mini Kit (Qiagen), aliquoted, and stored at -70°C . Viral RNA was quantified by a single-tube 1-step real-time RT-PCR using a LightCycler instrument and software version 3.5 (Roche Molecular Biochemicals), as described by Shu et al. [31].

Measurement of anaphylatoxins. C3a and C5a were quantified using a commercial cytometric bead array kit (Becton Dickinson).

Statistical analysis. Data analysis was performed using SAS (version 8.1; SAS Institute). Viral load, NS1 level, and SC5b-9 level are presented as mean and SD and, in cases of skewness, as median and range. The χ^2 test and 1-way analysis of variance

were used to compare differences in sex and age, respectively, between groups of patients.

Since viral load, NS1 level, and SC5b-9 level were repeatedly observed over time and there were some missing data, a mixed model (MixMod) was employed as a multivariate statistical method. The purpose was to test the differences in patterns of viral load, NS1 level, and SC5b-9 level over time between different groups of patients. Under a random intercept model, mixed models with day ($-3, -2, -1, 0, 1, \text{ and } 2$) as a quantitative variable were fitted. Only patients with data on at least 3 consecutive days were included in the analysis. Since viral load, NS1, and SC5b-9 were positively skewed, a common log transformation was applied. Residual analysis was performed to assure that model assumptions were satisfied. All analyzed P values were 2-sided.

RESULTS

Study participants. The overall male-to-female ratio among the 182 patients was 1:1, and the mean \pm SD age was $9.6 \pm$

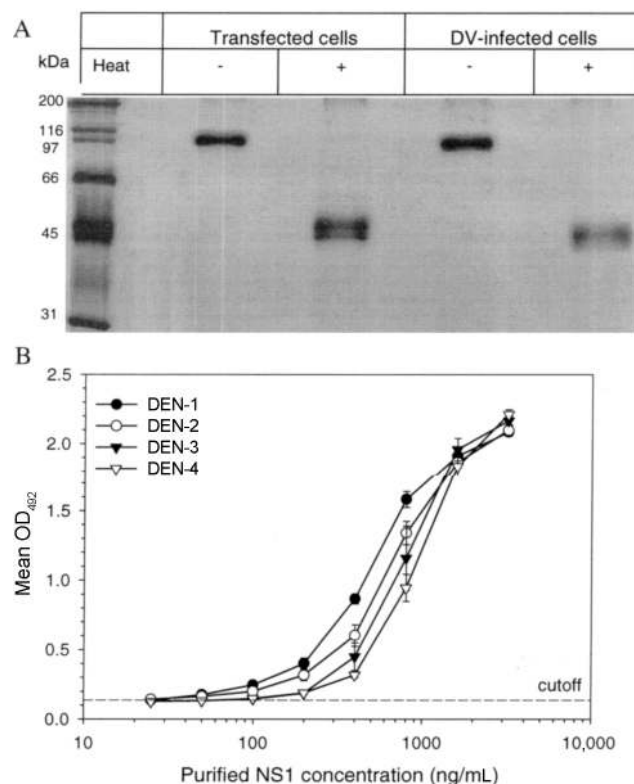


Figure 1. A, SDS-PAGE of purified soluble NS1 from dengue virus (DV)-infected cells and from cells stably expressing NS1. Purified NS1 was unheated or heated (at 95°C for 3 min) before SDS-PAGE. Markers are shown. B, Standard curves for NS1 capture ELISA with purified NS1 from DEN-1, -2, -3, and -4. Data points represent the mean and SD for 3 replicates. The cutoff value was set at twice the mean optical density value for negative control samples (mean \pm SD, 0.103 ± 0.025).

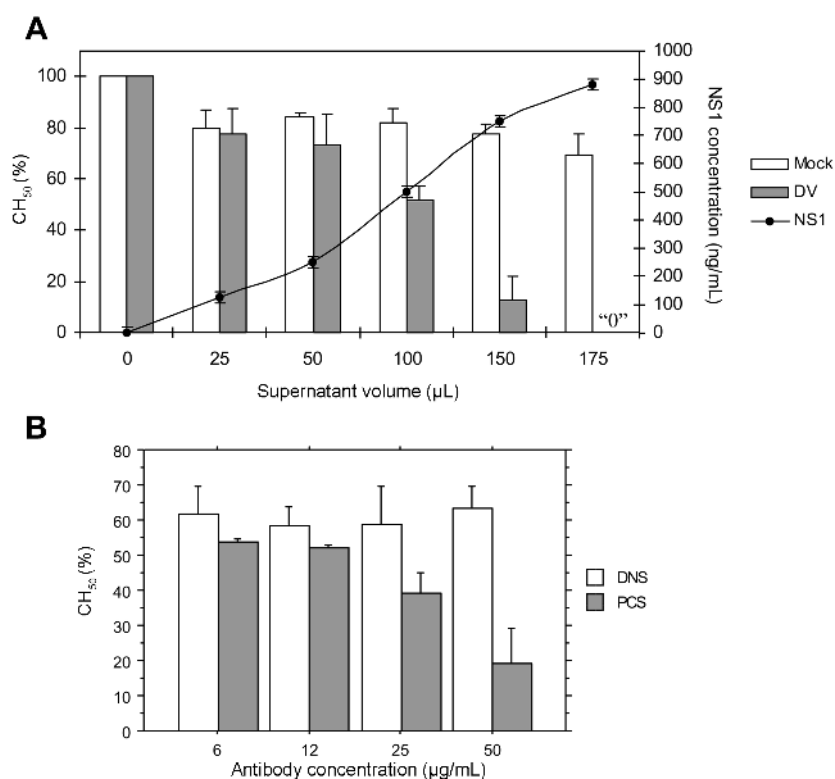


Figure 2. Complement activation by supernatants of dengue virus (DV)-infected cells. *A*, Dose dependency of spontaneous complement activation. The given amounts of culture supernatants were mixed with 25 μL of normal human serum (NHS), and buffer was added to give a total of 200 μL /sample. CH_{50} was determined after incubation at 37°C for 1 h. Data are displayed as the mean \pm SD of the percentage CH_{50} over serum controls from 3 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. Culture supernatants (100 μL) from DV-infected cells were mixed with purified antibodies from pooled convalescent-phase serum (PCS) and DV antibody-negative serum (DNS) at the given final concentrations and 25 μL of NHS. CH_{50} was determined after 60 min at 37°C. Data are displayed as the mean \pm SD of percentage CH_{50} over serum controls from 3 independent experiments. “0” indicates that the value of CH_{50} in the DV experiment was not detectable.

3 years (range, 2–15 years; median, 9 years). There were no major differences in sex ratios and mean ages of patients in each group. There were 49 patients with DF, 44 with DHF1, 44 with DHF2, 26 with DHF3/DSS, and 19 with OFIs. DV RT-PCR results were positive for 151 (92.6%) of 163 patients infected with DV. The virus types identified were DEN-1 ($n = 87$), DEN-2 ($n = 52$), DEN-3 ($n = 6$), and DEN-4 ($n = 6$). Secondary infection was diagnosed in 148 patients (90.8%), and primary infection was diagnosed in 15 patients (9.2%). Of the primary infection cases, 67% were classified as DF ($n = 10$), and the rest were classified as DHF1 ($n = 2$), DHF2 ($n = 2$), or DHF3 ($n = 1$).

Purification of NS1s. Figure 1A depicts SDS-PAGE of purified NS1. As described by Winkler et al. [18], the 80-kDa dimeric form was converted to the 40-kDa monomer by heating. The same bands appeared in Western blots with NS1-specific MAbs (data not shown). The mean \pm SD NS1 level 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 $\mu\text{g/L}$ of culture from DV-infected cells and from NS1-transfected cells, respectively.

NS1 capture ELISA. Antibodies employed in the capture ELISA cross-reacted with NS1 from all 4 DV serotypes. The detection limit for NS1 was found to be ~ 50 ng/mL from DV-1 and DV-2, 120 ng/mL for DV-3, and 160 ng/mL for DV-4 (figure 1B).

Activation of complement by NS1s. Supernatants from DV-infected cells, but not from mock-infected cells, dose dependently consumed complement in pooled human serum (figure 2A). Addition of purified immunoglobulin fractions from PCS but not control DNS enhanced complement consumption (figure 2B). An increase in complement activation was also observed when a mix of MAbs against NS1 was employed (data not shown).

NS1s purified from supernatants of infected cells also activated complement and caused a decrease in CH_{50} similar to that caused by the unfractionated culture supernatants. Complement activation occurred to completion with formation of

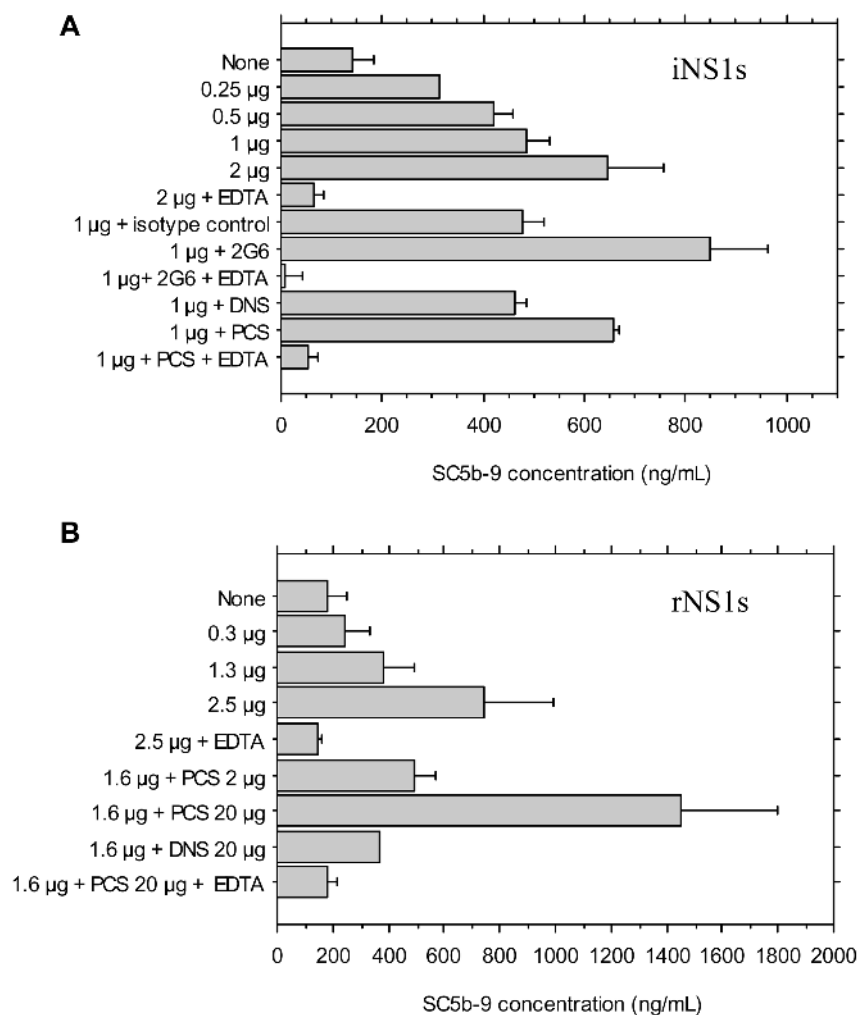


Figure 3. Activation of complement to completion by purified dengue virus (DV) NS1 protein. *A*, Purified soluble NS1 from DV-infected cells (iNS1s) at the given final concentrations was incubated in 12.5% normal human serum in the presence or absence of NS1-specific monoclonal antibody 2G6 (10 μ g/mL) or pooled convalescent-phase serum (PCS) (20 μ g/mL) at 37°C for 1h, and the SC5b-9 level was measured. *B*, Purified soluble NS1 from NS1 stably expressing cells (rNS1s) at the given final concentrations, tested for complement activation, as described in panel *A*. Equivalent concentrations of isotype control antibody and DV antibody–negative serum (DNS) were used as controls. EDTA (10 mmol/L) was added to inhibit complement activation for negative controls. Data are displayed as the mean \pm SD from 3 independent experiments.

SC5b-9 (figure 3A) and was enhanced by NS1-specific MAbs and by purified immunoglobulin fractions from PCS but not by isotype-control antibodies or by purified immunoglobulin from DNS (figure 3A). Similar results were obtained with purified recombinant NS1s from transfected cells (figure 3B). Fractions from the protein G columns containing little or no NS1 had no complement-consuming activity.

Antibody dependency of complement activation by cell-associated NS1. NS1 was expressed on the surface of DV-infected cells and on NS1m-transfected cells (figure 4A). When DV-infected cells were incubated with 12.5% NHS, no complement activation was observed, as was evident from negative staining for C3dg (data not shown) and C5b-9 (figure 4D) on cell surfaces. However, the presence of purified antibodies

against NS1 triggered complement activation, as evidenced by colocalization of complement C3 and NS1 on the cells (figure 4B). Similar results were obtained with purified immunoglobulin from PCS (data not shown). Antibody-dependent complement activation was induced by all 4 clones of NS1-specific MAbs tested but not by isotype control antibodies. Colocalization of NS1 and C3dg was also observed after antibody-dependent complement activation on NS1m-transfected cells (figure 4C). Parallel immunofluorescent staining for C5b-9 revealed its deposition on the plasma membrane of DV-infected (figure 4D) and transfected (data not shown) cells.

DV RNA, NS1s, and complement activation products in clinical specimens. Measurements of DV RNA, NS1s, and SC5b-9 were performed in a blinded manner. Figure 5 displays

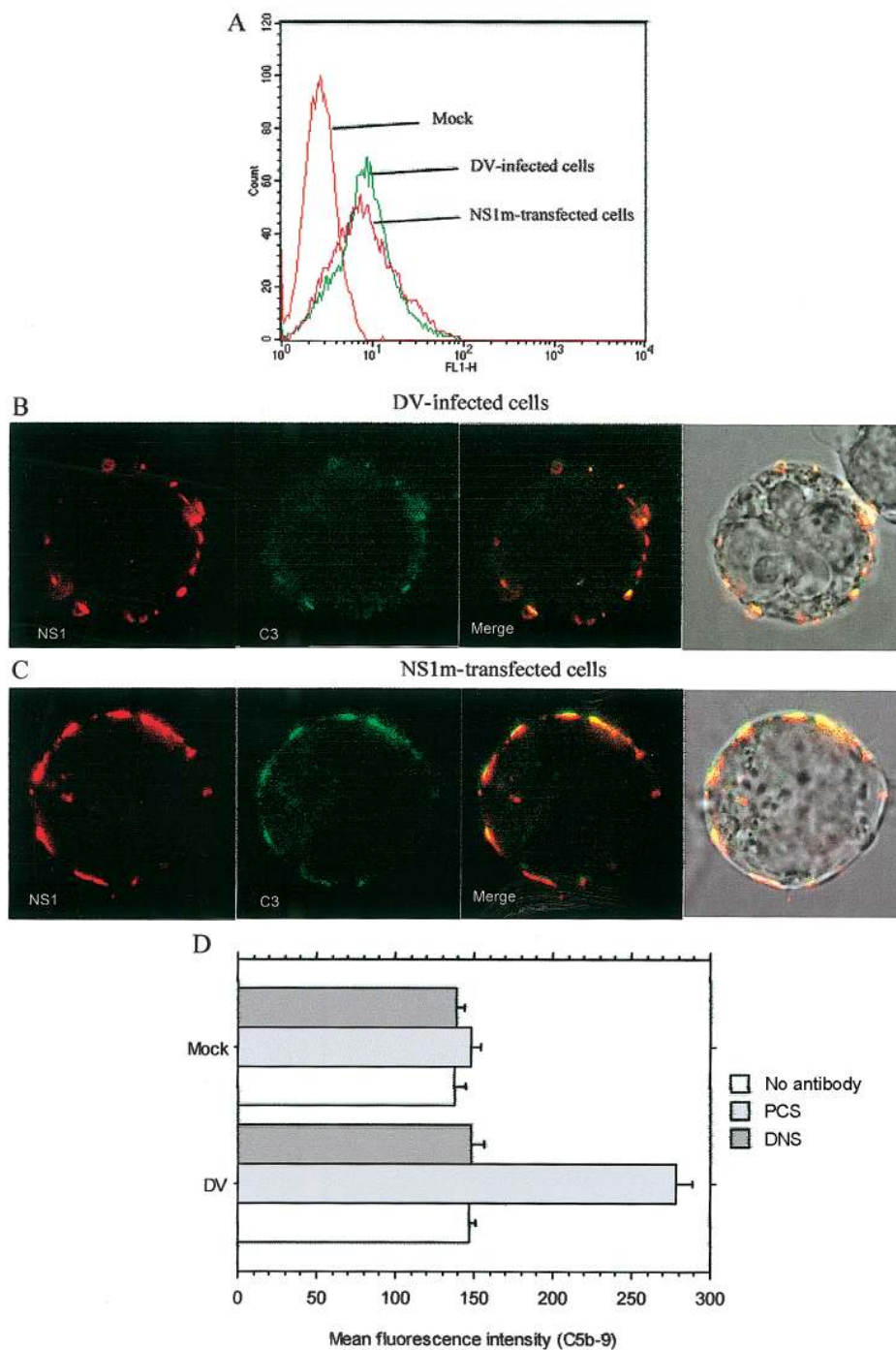


Figure 4. Activation of complement to completion by membrane-associated NS1 (NS1m) in the presence of NS1-specific antibodies. *A*, Surface expression of dengue virus (DV) NS1 on DV-infected cells and on cells stably expressing NS1. Cells were stained with NS1-specific monoclonal antibody (MAb) 1A4, followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin. Histogram plots were determined from data acquired from 5000 events in viable cells. The representative set of histograms is derived from 1 of 3 independent experiments. *B* and *C*, Colocalization of NS1 and complement C3 fragments on the surfaces of complement-attacked cells. DV-infected cells or cells expressing NS1m were incubated with 12.5% normal human serum (NHS) in the presence of a mix of purified NS1-specific MAbs. After 1 h at 37°C, cells were stained with fluorescent-conjugated secondary antibodies and observed by confocal microscopy. NS1 (Cy3; red) and complement (FITC; green) colocalized on the membranes. *D*, Formation of C5b-9 on cells. Mock- or DV-infected cells were incubated with purified antibodies from pooled convalescent-phase serum (PCS) and DV antibody-negative serum (DNS) in the presence of 12.5% NHS. The deposition of membrane attack complexes was detected by flow cytometry after 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 3 independent experiments.

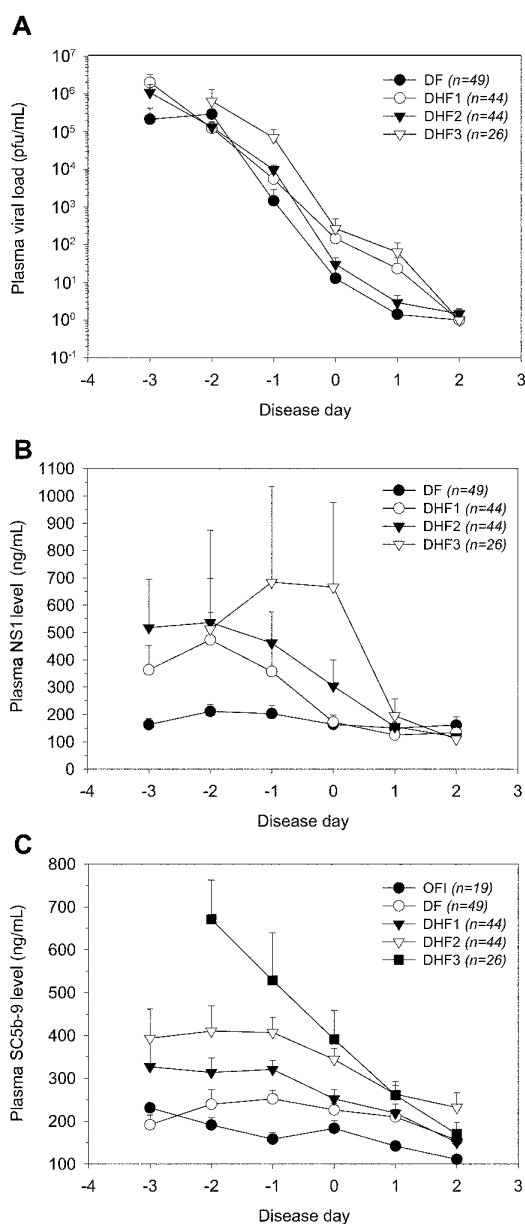


Figure 5. Viral loads, NS1 levels, and terminal SC5b-9 complexes in the circulation of patients with dengue fever (DF) and dengue hemorrhagic fever (DHF) and dengue shock syndrome. Plasma samples were assayed for dengue virus (DV) RNA levels by use of quantitative real-time reverse-transcription polymerase chain reaction, 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 (other febrile illnesses [OFIs]) were also used as controls. Plots show the mean and SE. NS1 levels were not detectable in patients with OFIs.

cross-sectional analysis of the mean and SD of each variable (viral load, NS1 level, and SC5b-9 level) over time, based on all available data. The highest viral loads were detected early during clinical illness in all patients and gradually declined to undetectable levels on day +1 in patients with DF or on day

+2 in patients with DHF (figure 5A), in accordance with previous reports [9, 34]. The mean \pm SD level of NS1s in patients with DHF (383.9 \pm 620 ng/mL) was higher than that in patients with DF (181.6 \pm 120 ng/mL) during acute illness (figure 5B). NS1 was undetectable in patients with OFIs. Similarly, mean \pm SD plasma SC5b-9 levels were higher in patients with DHF (306.9 \pm 174 ng/mL) than in patients with DF (225.3 \pm 97 ng/mL) or OFIs (170.3 \pm 57 ng/mL) during acute illness (figure 5C).

Because of missing viral load, NS1 level, and SC5b-9 level data at some time points, a mixed model of multivariate statistical analysis was applied. Only patients with at least 3 consecutive measurements were included in the analysis. This resulted in 83 patients and 301 observations for viral load, 89 patients and 319 observations for NS1 level, and 85 patients and 312 observations for SC5b-9 level. Figure 6 displays plots of predicted viral load, NS1 level, and SC5b-9 level obtained from the quadratic mixed model against time.

Viral load for patients with DF, DHF1, DHF2, or DHF3 decreased over time (figure 6A). There was a statistically significant difference in plasma viral load between patients with DF and some groups of patients with DHF (DF vs. DHF1, $P = .0856$; DF vs. DHF2, $P = .0087$; DF vs. DHF3, $P = .0599$) and between patients with DF and all patients with DHF ($P = .0035$). However, no difference in viral load was found among the different groups of patients with DHF (DHF1 vs. DHF2, $P = .1790$; DHF1 vs. DHF3, $P = .3577$; DHF2 vs. DHF3, $P = .4999$).

NS1 levels significantly decreased over time in all groups of patients with DHF, whereas, in patients with DF, NS1 levels increased slightly during early acute illness, with a peak at day -1 or 0, and then gradually decreased (figure 6B). However, no statistically significant difference was observed among patients with the 3 types of DHF (DHF1 vs. DHF2, $P = .2811$; DHF1 vs. DHF3, $P = .3158$; DHF2 vs. DHF3, $P = .9123$). The pattern of change in NS1 levels over time in patients with DF was significantly different from that in patients with DHF1 or DHF2 (DF vs. DHF1, $P = .0002$; DF vs. DHF2, $P = .0042$) but not patients with DHF3 ($P = .0761$), which might have been due to the small sample size of this group. When all types of DHF were combined, a highly significant difference was found between patients with DF and patients with DHF ($P < .0001$). Importantly, levels of NS1 during the febrile phase (days -3 to -1) could be used to differentiate between patients with DF and all groups of patients with DHF (day -3 and -2, $P < .0001$; day -1, $P = .021$).

SC5b-9 levels exhibited a similar pattern in patients with DF and patients with DHF, reaching a peak at day -1 and day -2 for those with DF and those with DHF, respectively (figure 5C). No complement activation was found in patients with OFIs. Comparisons of SC5b-9 levels between patients with DF and all patients with DHF and between patients with DF and patients with OFIs revealed a statistical difference ($P < .0001$).

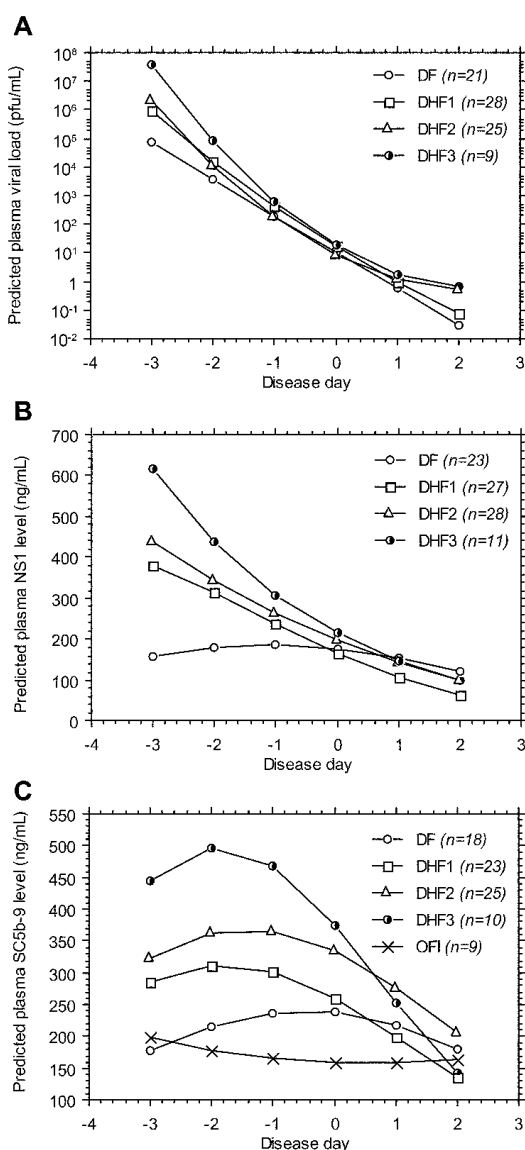


Figure 6. Predicted viral loads (A), NS1 levels (B), and SC5b-9 levels (C) from a mixed-model analysis. DF, dengue fever; DHF, dengue hemorrhagic fever; OFI, other febrile illness.

Levels of SC5b-9 observed during acute illness were highly correlated with disease severity—that is, the levels in patients with DHF3 were greater than those in patients with DHF2, and the levels in patients with DHF2 were greater than those in patients with DHF1 (DHF3 vs. DHF2, $P = .0052$; DHF2 vs. DHF1, $P = .0507$). Similar to NS1 levels, SC5b-9 levels in the febrile phase of DHF were significantly higher than those in patients with DF (day -3, $P = .0001$; day -2, $P < .0001$; day -1, $P = .0004$). Unlike NS1 levels, SC5b-9 levels could still be used to differentiate between DF and DHF at the day of defervescence (day 0, $P = .0183$).

NS1s and complement activation products in pleural fluids from patients with DSS. NS1, C3a and C5a, and SC5b-9 were

measured in pleural fluids and in plasma from 9 patients with DSS. Samples were collected at the day of shock or 1–2 days later. The results are shown in table 1. Pleural fluid levels of NS1 were similar to (patients 1–3) or higher than (patients 4–6) those in plasma. Pleural fluid levels of SC5b-9 were markedly higher than the plasma levels in all but 1 case. The mean \pm SD SC5b-9 level in pleural fluids was 2575.9 ± 1121 ng/mL (range, 627–4865 ng/mL; median, 2312.5 ng/mL) and was significantly higher than the mean \pm SD level in plasma (1546.3 ± 943 ng/mL; range, 394–2935 ng/mL; median, 1722 ng/mL) ($P = .04$). A similar trend was found for C5a: the mean \pm SD level of this anaphylatoxin in pleural fluids was 474 ± 61.1 ng/mL (range, 7–227 ng/mL; median, 23 ng/mL) and was also greater than that in plasma (25.6 ± 33.9 ng/mL; range, 5–114 ng/mL; median, 15 ng/mL) ($P = .34$). Results obtained for C3a were erratic, with no recognizable pattern (data not shown).

When the pleural fluid-to-plasma ratios of NS1, SC5b-9, and C5a levels were plotted against the respective quotients for albumin, almost all plotted values came to lie above the diagonal. This indicated relative accumulation of the analytes, probably as a result of their local generation at the leakage site (figure 7).

DISCUSSION

Two mutually nonexclusive mechanisms for immunological enhancement of infection have been proposed. The first involves nonneutralizing, cross-reactive antibodies against DV enhancing the uptake of the virus into susceptible cells [5, 6]. The second involves DV-specific CD8 lymphocytes undergoing apoptotic depletion when confronted with cells infected with the heterotypic virus [7]. In both cases, a loss of immunological

Table 1. Measurements of NS1, C5a, SC5b-9, and albumin in EDTA-treated plasma (P) and pleural fluid (PF) from 9 children with dengue shock syndrome.

Patient	NS1 level, ng/mL		C5a level, ng/mL		SC5b-9 level, ng/mL		Albumin level, g/L	
	P	PF	P	PF	P	PF	P	PF
1	117	139	10	16	876	1369	13.6	12.9
2	120	122	20	17	2935	3516	26.7	19.5
3	2333	2058	10	21	1801	3353	3.3	2.5
4	116	337	5	7	691	627	9.8	9.6
5	141	3234	15	49	1722	2307	10.3	17.3
6	117	220	28	50	394	2764	29.5	20.6
7	ND	ND	19	14	1838	2270	13.4	3.7
8	ND	ND	9	20	750	1869	1	1.5
9	ND	ND	114	227	2910	4865	1.2	1.2

NOTE. ND, not detectable.

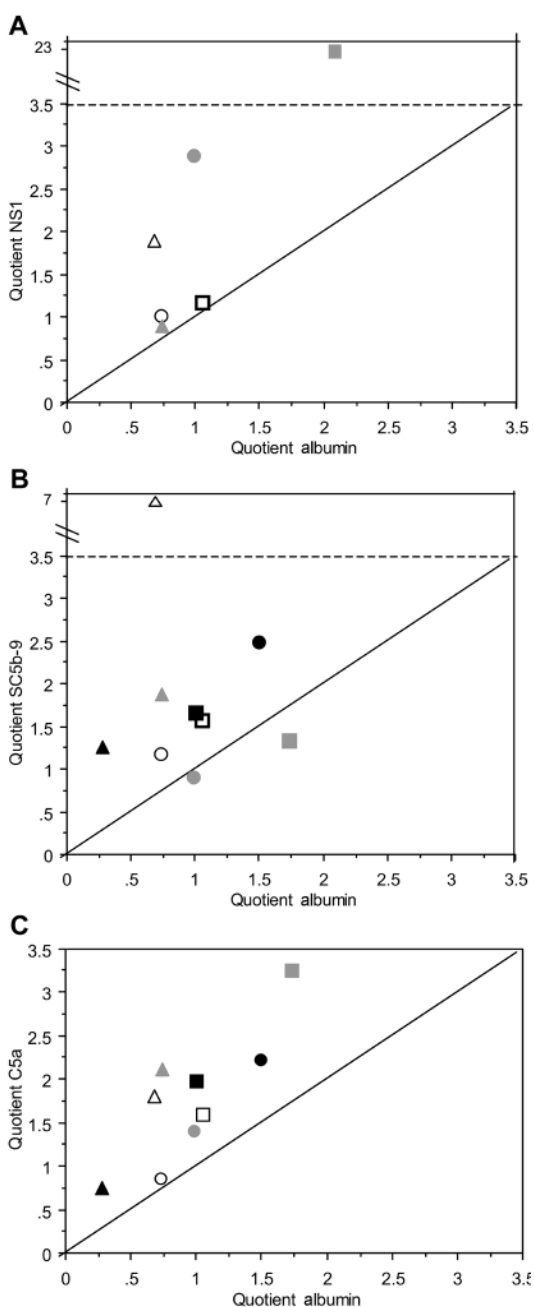


Figure 7. Relative accumulation of NS1, SC5b-9, and C5a in pleural fluids. The quotients between pleural fluid and plasma levels of these analytes are plotted against the respective albumin quotients. Each symbol represents 1 patient.

control over viral replication ensues. Indeed, the severity of disease does correlate with viral load [8–10].

However, viral load alone does not explain why vascular leakage should occur. In general, overproduction of cytokines by DV-infected cells or by activated lymphocytes is widely believed to be critical [15, 16], and the possible relevance of complement has received virtually no attention since 1973 [11]. We now propose that NS1, the major nonstructural DV protein,

is an important trigger for complement activation. Expression of NS1 on infected cells may result in binding of heterotypic, nonneutralizing antibodies and complement attack. Furthermore, NS1 released from infected cells can directly activate complement in the fluid phase. NS1-mediated complement activation occurs to completion. Membrane-associated C5b-9 might trigger cellular reactions and the production of inflammatory cytokines [35], and SC5b-9 can independently provoke other local and systemic effects [27, 28, 36–38].

In accordance with results of a recent study in which similar concentrations of NS1 were measured during early illness [10], high levels of NS1 were detected in plasma from patients with DHF/DSS during the febrile phase. A novel finding here was that plasma SC5b-9 levels followed a similar course and appeared to correlate with disease severity. A major challenge for the future will be to identify the major sites of DV infection and to assess the local presence of complement activation products. According to one report, DV antigen is present in alveolar macrophages and endothelial cells of the lung [39], which 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 follows that local complement activation likely occurs at these sites, and C5a was indeed detected at high levels in pleural fluids. Although anaphylatoxins bind to cells and are also rapidly inactivated *in vivo*, the terminal SC5b-9 complex is stable. The half-life in plasma is ~1 h [40, 41], but it is probably considerably longer in closed compartments. SC5b-9 enhances endothelial permeability *in vitro* and *in vivo* at a concentration of just a few micrograms per milliliter [27]. These concentrations were reached in the pleural fluids from 8 of the 9 patients in this study.

A unifying concept can thus be formulated to explain the pathogenesis of vascular leakage in DHF/DSS. The antibody response to a primary infection generates nonneutralizing antibodies against heterotypic DVs. Viral replication is augmented because of immunological enhancement during secondary infections. Although it is not excluded that other viral proteins may contribute to complement activation, all of the results of the present study implicate a major role for NS1. The protein is released in copious amounts from infected cells and is probably identical to the soluble viral antigen that was reported in 1970 to bind anti-DV antibodies and activate guinea pig complement [42, 43]. At the same time, antibodies against NS1 direct complement attack to the infected cells, causing generation of membrane-damaging C5b-9 and bystander SC5b-9 complexes. DV infection may also induce the production of inflammatory cytokines, and interleukin-8 and RANTES have been found in high concentrations in pleural fluids from patients with DSS [17]. Complement-activation products and cy-

tokines may synergize locally to incur vascular leakage. 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 before the manifestation of the catastrophic events that claim the lives of so many children around the globe.

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