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Enzyme-Linked Immunosorbent Assay Specific to Dengue Virus Type 1 Nonstructural Protein NS1 Reveals Circulation of the Antigen in the Blood during the Acute Phase of Disease in Patients Experiencing Primary or Secondary Infections

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During flavivirus infection in vitro, nonstructural protein NS1 is released in a host-restricted fashion from infected mammalian cells but not vector-derived insect cells. In order to analyze the biological relevance of NS1 secretion in vivo, we developed a sensitive enzyme-linked immunosorbent assay (ELISA) to detect the protein in the sera of dengue virus-infected patients. The assay was based on serotype 1 NS1-specific mouse and rabbit polyclonal antibody preparations for antigen immunocapture and detection, respectively. With purified dengue virus type 1 NS1 as a protein standard, the sensitivity of our capture ELISA was less than 1 ng/ml. When a panel of patient sera was analyzed, the NS1 antigen was found circulating from the first day after the onset of fever up to day 9, once the clinical phase of the disease is over. The NS1 protein could be detected even when viral RNA was negative in reverse transcriptase-PCR or in the presence of immunoglobulin M antibodies. NS1 circulation levels varied among individuals during the course of the disease, ranging from several nanograms per milliliter to several micrograms per milliliter, and peaked in one case at 50 μg/ml of serum. Interestingly, NS1 concentrations did not differ significantly in serum specimens obtained from patients experiencing primary or secondary dengue virus infections. These findings indicate that NS1 protein detection may allow early diagnosis of infection. Furthermore, NS1 circulation in the bloodstream of patients during the clinical phase of the disease suggests a contribution of the nonstructural protein to dengue virus pathogenesis.

Dengue is one of the most serious mosquito-borne viral diseases in humans. It occurs in tropical areas and affects up to 100 million people each year, with a high mortality rate in children (9, 20). Infection with one of the four serotypes (1, 2, 3, and 4) of dengue virus may result in its classical form, a febrile illness (dengue fever [DF]). In some cases, the disease may be associated with more severe manifestations, such as hemorrhagic syndrome (dengue hemorrhagic fever) and hypovolemic shock, which often proves fatal (dengue shock syndrome) (1, 21). In order to provide timely information for the care of the patient, it is important to establish a diagnosis of dengue virus infection during the first few days of clinical symptoms. Furthermore, determination of the serotype of dengue virus is also important for the surveillance of DF. The introduction of a serotype never isolated in a region will result more or less rapidly in an epidemic of DF, in which case surveillance measures should be increased.

The major diagnostic methods currently available are viral RNA detection by reverse transcriptase PCR (RT-PCR) (15) or serological tests, such as an immunoglobulin M (IgM) capture enzyme-linked immunosorbent assay (ELISA) (MAC-ELISA) (12, 13). Early dengue diagnosis still remains a problem, as RT-PCR is an expensive method and is difficult to use on a large scale and MAC-ELISA does not provide early diagnosis, as the first detectable IgM appears only on days 4 to 5 of illness (31, 32). Serotype diagnosis may also be difficult, as it generally requires RT-PCR or virus isolation from viremic sera.

Dengue virus belongs to the genus *Flavivirus* of the family *Flaviviridae*. It is an enveloped, single-stranded, positive-sense RNA virus. The genome is approximately 11 kb long and encodes a polyprotein precursor of about 3,400 amino acid residues. Co- and posttranslational processing by cellular and viral proteases generates three structural proteins, C, prM, and E, and seven nonstructural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 (3, 24). NS1 is a highly conserved glycoprotein which appears essential for virus viability, although no precise function has yet been assigned to it. During cell infection, NS1 is found associated with intracellular organelles (16, 19, 33) or is alternatively transported through the secretory pathway to the cell surface (27). A soluble hexameric form may be released in a glycosylation-dependent fashion from infected mammalian cells but not from vector-derived mosquito cells (4, 5, 8, 23). Recently, NS1 was found circulating during the acute phase of the disease in sera from patients experiencing secondary but not primary infections (35).

In this study, we were interested in characterizing the period of time during which NS1 circulates in the sera of dengue

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virus-infected patients in comparison with viremia and appearance of IgM, in order to assess the potential of using NS1 detection as an early diagnostic tool. We developed an optimized capture ELISA for NS1 detection and used it to test an extensive number of well-characterized patient blood samples. We showed that NS1 circulates at high levels in the sera of patient experiencing primary as well as secondary infections during the entire clinical phase of illness and in the first few days of convalescence.

MATERIALS AND METHODS

NS1-specific polyclonal antibodies. Dengue virus type 1 NS1 protein, purified as previously described (8), was fixed on an amphoteric nylon membrane (Nytran; Schleicher & Schuell) according to the manufacturer's instructions. Ascitic fluids from mice immunized against dengue virus type 1, 2, 3, and 4 strains were incubated on this membrane for 1 h at room temperature. After three washes in phosphate-buffered saline (PBS), bound antibodies were eluted at a basic pH of 11.6 for 20 min at 4°C. The eluted antibodies were concentrated by ultrafiltration in PBS.

Monospecific anti-NS1 polyclonal antibody was raised in a male rabbit immunized with four subcutaneous injections of 40 μg of purified dengue virus type 1 NS1 at 1-week intervals. For the first injection, the protein was mixed with complete Freund's adjuvant; for the others, it was mixed with incomplete Freund's adjuvant.

Capture ELISA for detection of the NS1 protein. Microtitration plates (Nunc Maxisorp, Nunclon Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 μl (per well) of purified mouse anti-NS1 polyclonal antibodies diluted (1/500) in PBS. Wells were washed three times with PBS-Tween buffer (PBS, 0.05% Tween 20) prior to 30 min of saturation at room temperature with 100 μl (per well) of PBS-Tween-milk buffer (PBS, 0.05% Tween 20, 3% milk). After wells were washed as described above, human sera or purified dengue virus type 1 NS1 diluted in PBS-Tween buffer was added to wells (40 μl/well) and incubated for 2 h at room temperature. Sera were initially tested at 1/10 and then serially diluted threefold during the assay. The wells were washed again and incubated for 1 h at 37°C with 50 μl (per well) of anti-NS1 rabbit antiserum (diluted 1/500 in PBS-Tween-milk buffer). The wells were washed again and incubated for 1 h at 37°C with 50 μl (per well) of peroxidase-conjugated goat anti-rabbit antiserum (Jackson Immunoresearch) (diluted 1/5,000 in PBS-Tween-milk buffer). After three further washes, each well received 50 μl of a freshly prepared 3,3′, 5,5′-tetramethylbenzidine solution (TMB microwell peroxidase substrate system; Kirkegaard & Perry Laboratories). The color reaction was stopped after 5 min with 50 μl (per well) of 2.5 N sulfuric acid, and the plates were examined at 450 nm. Negative controls were measured when the reaction was carried out in the absence of antigen. Absorbance values were corrected by using the mean value of the negative controls.

Serum specimens. Different sera were tested for the presence of NS1 by using our capture ELISA. A total of 127 sera were collected from 61 dengue virus type 1-infected patients during an epidemic in French Guiana in 1996 to 1997 (30), and another 20 were obtained from 10 patients recruited by the Centre de Biologie Médicale Spécialisé (CBMS) of Pasteur Institute after travel in tropical countries.

For the French Guiana samples, diagnosis of dengue virus infection was based on MAC-ELISA, RT-PCR, or virus isolation in cell cultures. These sera were collected between days 0 and 66, day 0 corresponding to the onset of fevers. Daily blood specimens were recovered from four patients during the first 5 days of illness. All of these patients developed classical DF.

For the patients recruited by the CBMS, dengue virus diagnosis was confirmed with paired sera (acute and convalescent phases) by MAC-ELISA and hemagglutination inhibition (HI) tests. No clinical information was available for these patients.

The paired sera from the CBMS and French Guiana patients were classified in the group of primary dengue virus infection when the HI titer of the convalescent-phase serum (i.e., recovered at least 7 days after the first fevers) was ≤1:640 or in the group of secondary infection when the acute- or convalescent-phase serum HI titer was ≥1:1,280.

RESULTS

Characteristics of the NS1 capture ELISA. In order to establish a sensitive and polyvalent NS1 capture ELISA, we used immunopurified mouse anti-NS1 polyclonal antibodies for antigen NS1 capture. NS1-specific rabbit antiserum was applied as the secondary detection antibody. Once the antibody dilutions were maximized, the sensitivity of the NS1 capture ELISA was determined by probing replicates of serially purified dengue virus type 1 NS1 of known concentrations. A sample was considered positive if the optical density (OD) was greater than twice the mean value of the negative controls. Using these criteria, the limit of dengue virus type 1 NS1 detection with our capture ELISA reached approximately 1 ng/ml (Fig. 1). The linear portion of the standard curve ranged from 1 to 100 ng/ml and was used to estimate the dengue virus type 1 NS1 levels in patient sera (see below). This assay was at least 10 times less sensitive for the detection of NS1 proteins of other viral serotypes than for the detection of NS1 of dengue virus type 1 (data not shown).

Detection of the NS1 protein in sera from dengue virus type 1 infected patients. We examined a panel of sera from dengue virus type 1-infected patients with our NS1 capture ELISA. The sera were collected from patients experiencing DF during an epidemic in French Guiana in 1996 to 1997. The day on which the sample was recovered was designated day 0, corresponding to the onset of fevers, but the duration of illness for each patient was not available. Sera collected up to day 7 after the onset of symptoms were referred to as acute-phase sera. Convalescent-phase sera were sera collected beyond day 7.

The sera were tested at different dilutions in our capture ELISA. In Fig. 2, values are reported for a 1/10 dilution, except for ambiguous sera, which were tested at a 1/2 dilution. NS1 could be detected from day 0 until day 9 but not in any sera recovered after this date (Fig. 2). The percentage of NS1-positive sera was determined for each day: 80% at day 1, 60% at day 2, and 100% at days 4 and 5 (Table 1). At day 6, 80% of the sera were still found positive. NS1 was scarcely detected between days 7 and 9 and was never detected beyond day 10.

On the same day and for the same dilution of serum tested, the ODs were highly variable from one individual to another. For example, at day 4, the OD varied between 0.21 and 1.45, variability which remained important throughout the clinical phase.

Using the same panel of sera, we compared the detection of NS1 with the detection of dengue virus-specific IgM (Table 1). IgM was detected by the MAC-ELISA, which is currently used for dengue diagnosis. The NS1 antigen was detectable at earlier times than IgM, from day 0 onward for NS1 compared to day 3 or 4 for IgM. Between days 3 and 9, NS1 and IgM were detected concomitantly; from day 11, only IgM persisted in the various blood samples. The appearance of IgM did not seem to prevent NS1 detection, as observed at day 4 or 5, for example.

Daily study of individual patients infected with dengue virus type 1 during the acute phase of the disease. Blood samples were taken daily during the first 5 days of the disease for four patients. We tested the level of secretion of NS1 in each sample with our NS1 capture ELISA and found that all the samples were positive for NS1 (Fig. 3). For the same patient, the OD measured by the capture ELISA varied according to time. For patient 3, the OD was 0.78 at day 1, peaked to 1.56 at day 3, and decreased to 0.79 at day 5. The NS1 secretion profiles were similar for patients 1 and 4 but were somewhat different

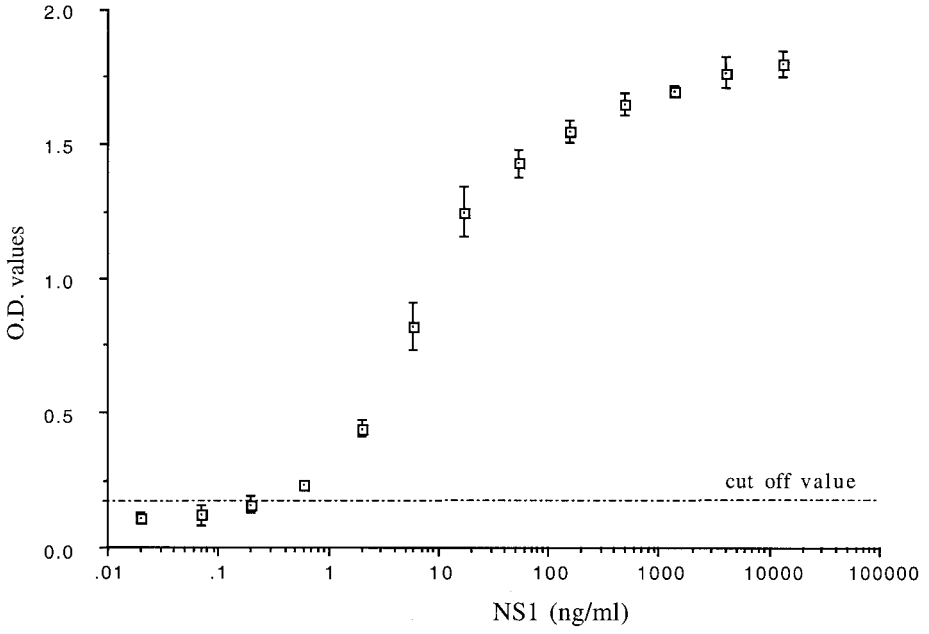

FIG. 1. Standard capture ELISA with purified dengue virus type 1 NS1. NS1 was serially diluted in PBS-Tween buffer. Data points represent the mean and standard deviation for three replicates corrected by the mean value of the negative controls. The cutoff value was set at twice the average value of the negative controls.

for patient 2. For this patient, the highest OD (0.92) was measured at day 4 and remained at that level at day 5.

We compared the circulation of NS1 in serum with the detection of IgM and the period of viremia demonstrated by positive RT-PCR. NS1 was detected concomitant with viremia but was still detectable even when RT-PCR was negative, as was the case for patients 3 and 4 at day 5 (Fig. 3). As shown in Table 1, NS1 was present in sera before IgM but also was present during the first days of the appearance of these antibodies.

Comparison of NS1 secretion levels between patients experiencing primary or secondary infections. We determined for 29 patients from French Guiana or individuals returning from travel in areas in which dengue is epidemic whether they had

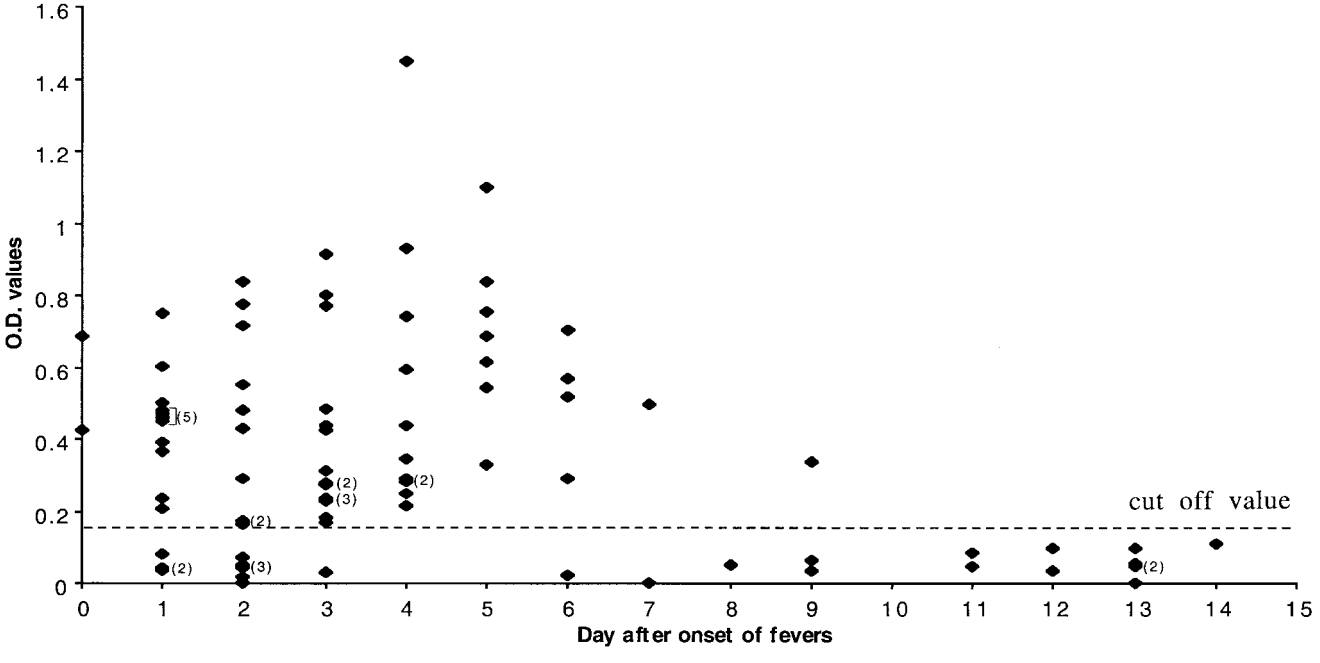

FIG. 2. NS1 antigen capture in the sera of dengue virus type 1-infected patients. Data points represent the OD of sera tested at a dilution of 1/10 and corrected by using the mean value of the corresponding negative controls. Ambiguous sera were tested at 1/2. The numbers of sera with similar ODs are indicated with a number in parentheses near the representatives data point.

TABLE 1. Comparison between IgM (MAC-ELISA) and NS1 (capture ELISA) detection in the sera of dengue virus type 1-infected patients

No. of days after onset of fever	No. of sera that were[a]:				% of NS1+ sera[b]
	NS1+		NS1−		
	IgM−	IgM+	IgM−	IgM+	
0	2				—
1	12		3		80
2	8	1	6		60
3	10	4	1		93
4	4	6			100
5	1	6			100
6		4		1	80
7		1		1	—
8				1	—
9		1		2	—
11–66				33	0

[a] The number of sera positive (+) or negative (−) in each of the two assays is reported day by day, from the onset of fevers at day 0 onward.
[b] The percentage of NS1-positive sera was calculated for each day, except when the number of tested sera was not significant (—).

TABLE 2. Similarity of NS1 concentrations in sera of patients experiencing primary or secondary dengue virus infections

Type of infection[a]	Phase[b]	No. of sera positive/ no. tested	NS1 concn (μg/ml)[c]
Primary	Acute	10/13	0.04–2
	Convalescent	1/13	0.04
Secondary	Acute	14/16	0.01–2
	Convalescent	0/15	

[a] Determined as primary when the HI titer was ≤ 1:640 in convalescent-phase serum or as secondary when the HI titer was ≥1:1,280 in acute- or convalescent-phase serum.
[b] Two blood samples were taken for each patient, one during the acute phase of the disease and the second beyond day 7, except for one patient whose serum HI titer was ≥1:1,280 at day 5.
[c] NS1 concentrations were determined by comparison of the ODs of diluted serum samples with the ODs in a standard curve determined in parallel.

experienced a primary or a secondary infection. Two samples were recovered for each patient, one during the febrile phase of the disease and the second during the convalescent phase, except for one patient who could be classified as having a secondary infection from day 5. Thirteen had developed a primary infection, among which 10 had NS1-positive sera. Among 16 secondary infections in dengue virus type 1-infected patients, we found 14 NS1-positive sera (Table 2). We calculated in each case the serum NS1 concentration by comparing the ODs of appropriately diluted fractions with the ODs falling in the linear portion of the standard curve. NS1 levels ranged from 0.04 to 2 μg/ml in primary sera and from 0.01 to 2 μg/ml in secondary sera.

DISCUSSION

In order to analyze the circulation of nonstructural protein NS1 in the serum of dengue virus type 1-infected patients, we developed a capture ELISA using a polyclonal antibody approach. Mouse and rabbit polyclonal antibodies were used, respectively, for the capture and detection of NS1 to widen the spectrum of strain recognition. The tool proved to be well

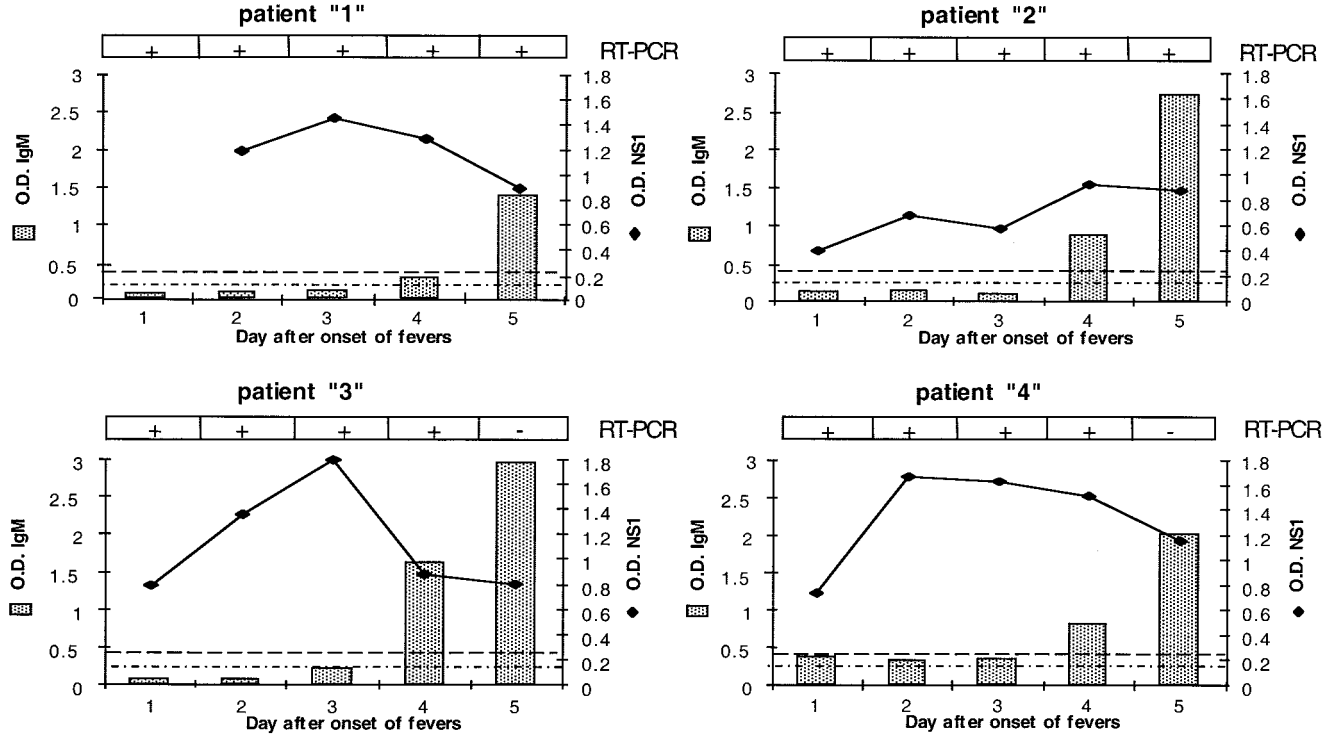

FIG. 3. Daily follow-up of serum-NS1 in dengue virus type 1-infected patients. For each serum, we report the RT-PCR results; the IgM and NS1 ODs, corrected by the mean value of the corresponding negative controls; and the IgM (– – – –) and NS1 (-·-·-·) cutoff values.

adapted to serotype 1 NS1. Using a positive threshold of twice the average signal of negative controls, we were able to detect 1 ng of purified dengue virus type 1 NS1 protein standard per ml. This detection level was not obtained for NS1 of other viral serotypes, for which the sensitivity of the assay was at least 10 times lower. The discrepancy may be due to the fact that we raised and purified polyclonal antibodies against serotype 1 NS1. These may react less efficiently against other serotypes because the homology in the amino acid sequence of NS1 does not exceed 80% among dengue viruses. Such observations have been reported for monoclonal antibodies against dengue virus type 2 NS1 (6, 35).

A cohort of dengue virus-infected patients was recruited over a period of 18 months (1996 to 1997) in French Guiana, during which serotypes 1 and 2 cocirculated. All patients had DF, but none developed the severe forms of the disease, including hemorrhage and shock. Dengue virus type 1-infected patients were selected on the basis of virus isolation or RT-PCR (15). Sera were recovered between days 0 and 66 after the onset of fevers. Using our capture ELISA, we could demonstrate the presence of NS1 in some samples as early as day 0 and up to day 9 (Fig. 2), a period of time which covers the entire clinical phase of the disease, usually no longer than 1 week. Apart from the results on day 2, the percentage of positive sera exceeded 80% from days 1 to 6. Nearly all sera were found positive for NS1 antigen between days 3 and 5 (Table 1), the critical period of illness preceding defervescence, which generally occurs at about day 5 (31). Between days 10 and 66, none of the sera had detectable levels of NS1, presumably once the antibody response to complex circulating viral antigens was high enough. Accordingly, all the convalescent-phase sera tested by HI contained dengue virus-specific antibodies.

Dengue virus-specific IgM first appeared at about day 3 and was prominent from day 5 onward. However, IgM did not seem to interfere with NS1 detection during the clinical phase (Table 1). Although some authors have demonstrated NS1-specific IgM directed against an N-terminal peptide within days following the first symptoms (11), others have not reported any specific IgM directed against the native form of secreted NS1 (29), nor did we find any when analyzing acute-phase sera (data not shown). Interestingly, in some acute-phase sera from dengue virus-infected patients with secondary infections, NS1-specific IgG could be detected either by Western blotting (14) or by an ELISA (29). Several of our acute-phase sera did contain NS1-specific IgG and were also positive for NS1 antigen. However, a lack of NS1 detection within the clinical phase invariably correlated with detectable anti-NS1 IgG (data not shown).

Four dengue virus type 1-infected patients were monitored daily over a 5-day period by RT-PCR, MAC-ELISA, and our NS1 antigen capture ELISA. Like the results for the previous panel of sera, dengue virus-specific IgM was undetectable before day 4. NS1 circulated on a continuous basis throughout the study, with a peak between days 2 and 4 (Fig. 3). In some cases, protein detection was still possible once viremia (revealed by positive RT-PCR) turned out negative, suggesting that NS1 may circulate at higher levels than virus particles. This suggestion was also made in a previous study showing that dengue virus detection by an ELISA with an anti-envelope E protein monoclonal antibody was as sensitive as virus isolation in cell cultures (17). Thus, our NS1 capture ELISA may allow early serospecific diagnosis of dengue virus infection and may represent a useful alternative to currently available methods (34). This test may be extended to other flavivirus infections. For example, with appropriate antibodies raised specifically against NS1 from yellow fever virus (generous gift from J. J. Schlesinger) (26), we were able to detect NS1 in sera from patients infected with this virus (kindly provided by C. Mathiot) (data not shown).

The level of NS1 circulating in the bloodstream of dengue virus type 1-infected patients was estimated to range from 0.01 to 50 μg/ml by interpretation of ODs within the linear part of the standard curve. In contrast to previous results (35), we detected NS1 equally well in sera from patients with primary and secondary infections (Table 2). However, patients from both categories solely developed classical DF, and our assay may not quantitatively take into account NS1 involved in immune complexes that may be present at higher levels in secondary infections. It would be valuable to determine the ratio of the different forms of NS1 in humans experiencing dengue of different clinical grades in order to evaluate specific contributions of free or complexed NS1 to the disease outcome.

Up to now, no particular marker has been unambiguously related to the severity of the disease. Controversial results have accumulated over the last few decades about the role of antibodies in dengue pathogenesis (2, 10, 28). The presence of immune complexes in plasma has been demonstrated in a large number of acute-phase sera, but depending on the study, it correlated or did not correlate with the severity of the disease (22, 25). A role was also suggested for anti-E cross-reactive antibodies to plasminogen (18) and anti-NS1 cross-reactive antibodies to human blood clotting factors (7). Nevertheless, the correlation between the antibody specificity and the clinical outcome remains unclear, and recent studies have shown that sera from DF and dengue hemorrhagic fever patients have similar anti-NS1 antibody responses (29). The fact that NS1 circulates to high levels in the bloodstream of patients during the acute phase of the disease suggests that the protein may contribute per se to the pathophysiology of dengue virus infections.

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