Dengue virus induced hemorrhage in a nonhuman primate model

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Abstract

Lack of a dengue hemorrhagic animal model recapitulating human dengue virus infection has been a significant impediment in advancing our understanding of the early events involved in the pathogenesis of dengue disease. In efforts to address this issue, a group of rhesus macaques (RM) were intravenously infected with dengue virus serotype 2 (strain 16681) at 1X10^7 PFU/animal. A classic dengue hemorrhage developed 3-5 days post infection (pi) in 6 out of 6 animals. Blood chemistry appeared to be normal with exception of creatine phosphokinase (CPK) which peaked at 7 days pi. A modest thrombocytopenia and noticeable neutropenia concomitant with slight decrease of hemoglobin and hematocrit were registered. In addition, the concentration of D-Dimer was elevated significantly. Viremia peaked at 3 to 5 days pi followed by an inverse relationship between T and B lymphocytes and a bimodal pattern for platelet-monocytes and platelet-neutrophil aggregates. Dengue virus containing platelets engulfed by monocytes was noted at 8 or 9 days pi. Thus, RM inoculated intravenously with a high dose of dengue virus produced dengue hemorrhage which may provide a unique platform to define the early events in dengue virus infection and help identify which blood components contribute to the pathogenesis of dengue disease.
Introduction

Dengue is one of the most important mosquito-borne viral diseases affecting humans, with over half of the world’s population at risk. Previously, dengue infections occurred primarily as epidemics in tropical and subtropical countries. But over time, increasing globalization has contributed to the geographic spread of dengue vectors, including Aedes aegypti and Aedes albopictus mosquitoes, leading to a steady penetration of dengue virus infection in just about every corner of the world \(^1,^2\). A wide spectrum of clinical manifestations has been noted which range from asymptomatic, mild febrile illness (dengue fever, DF) to dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS), a life-threatening illness. There are 4 serotypes of the dengue virus (DENV 1-4) and each serotype is capable of inducing DHF/DSS upon infection. The pathological hallmarks that determine disease severity and distinguish DHF from DF and other viral hemorrhagic fevers are plasma/vascular leakage resulting from increased vascular permeability and abnormal hemostasis. However, little is known about the mechanisms leading to DF and DHF/DSS. Although DHF/DSS has been reported to occur at a higher frequency following secondary infection with a heterologous dengue serotype, numerous reports have also documented DHF in primary dengue virus infections \(^3-^9\) and dengue viral loads appear to correlate with severity of dengue disease \(^10\), suggesting that the level of virus replication may dictate the occurrence of clinical disease. Currently, there are no effective vaccines or therapeutic drugs available to prevent or treat dengue viral infection.

A central problem in understanding the pathogenesis of dengue virus infection is the paucity of small animal models of human dengue virus infection \(^11,^12\). Each of the small animal models that have been described so far, while clearly informative, possess inherent limitations and do not faithfully mimic human dengue virus illness. The development of neurovirulence not typically observed in dengue infected humans in one such small animal model of dengue infection highlights the limitations of using such animal models \(^13,^14\). Thus, the development of a reliable animal model of DHF that recapitulates the clinical sequelae of human dengue virus
infection would provide a powerful tool to begin to examine some of the fundamental issues that have remained unresolved with regards to the mechanisms of dengue virus induced pathogenesis. The availability of such a model also provides a tool for the optimal screening of dengue virus directed anti-viral drugs and more importantly as a model for the evaluation of effective prophylactic and/or therapeutic dengue virus vaccines. Moreover, the availability of such a model might provide a consensus regarding the initial lineage of the host cell that serves as the target of initial infection and replication, an issue that remains a subject of debate in spite of all these years of dengue virus research.

The Asian rhesus macaque has been accepted to be a valid nonhuman primate (NHP) model to study select aspects of dengue viral infection and disease. The subcutaneous and/or intramuscular experimental inoculation of rhesus monkeys with dengue virus has been reasoned to mimic the route of natural mosquito infection; however infection of such monkeys via these routes results in viral loads that are several orders of magnitude below human viral loads in patient experiencing DHF/DSS and likely because of this modest replication, clinical sequelae typical of human dengue virus infection have never been observed in macaques. While the reasons for this remain unclear, perhaps the failure may in fact be due to the route of infection. This view is supported by studies conducted almost a half a century ago that documented evidence for the direct deposit of dengue virus into the capillary by the Aedes aegypti mosquito during the engorgement period. Interestingly, Ashburn and Craig in an attempt to identify the etiology of dengue demonstrate that intravenous injection of unfiltered dengue blood into healthy men is capable of producing signs and symptoms typical of human dengue virus infection. Results of the studies reported herein in fact substantiate this hypothesis. Thus, “intravenous” experimental infection with a high virus inoculum (1x10^7 PFU/animal) of a group of adult rhesus macaques with dengue virus serotype 2 resulted in readily visible hemorrhage on day 3-5 post infection, which is one of the cardinal features of human DHF. We submit that while these findings are preliminary, they do for the first time
provide a model that can readily be tested and verified. The systematic evaluation of the
dynamics of the clinical, immunological, and virological manifestations of “intravenous” dengue
virus infection in these rhesus macaques is the subject of this communication.
Materials and Methods

Nonhuman primates (Rhesus macaques, Macaca mulatta), Virus, and Cells.

We inoculated a total of 6 rhesus macaques (Table 1) of Indian origin with one ml of dengue serotype 2 (16681 strain, Vero grown, kindly provided by Dr. Gubler, University of Hawaii Asia-Pacific Institute of Tropical Medicine and Infectious Diseases) containing 10^7 PFU/ml intravenously while one control animal received control Vero cell supernatant fluid. Virus titrations were carried out by plaque assay in Vero cells. All experimental protocols and procedures were conducted following approval by the Emory IACUC and all animals were housed at the Yerkes National Primate Research Center (YNPRC) of Emory University and cared for in conformance to the guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council and the Health and Human Services guidelines "Guide for the Care and Use of Laboratory Animals". There were basically 2 types of monkeys used (Table 1): Older ovariectomized females (12-14 years of age, n=4) and young adult males (~3 year old).

Complete Blood count and Coagulation parameter assays

Samples were collected under ketamine anesthesia: Blood was collected by venipuncture using 3.2% citrate as an anti-coagulant and bone marrow was aspirated from the iliac crest and supplemented with heparin. Complete blood counts were performed with each blood collection by the Yerkes Clinical Pathology Laboratory and complete serum chemistries were analyzed on the samples by Antech (Smyrna, GA). Citrated plasma samples were submitted to the Emory University Hospital Laboratories for analyses of the coagulation parameters.

Quantitation of viral load with Real-time RT-PCR.

Plasma viral load was determined according to a method described previously. Briefly, RNA was extracted from 140 µl of plasma using QIAmp Viral RNA Mini kit (Qiagen, Valencia, CA). The dengue 2 fluorogenic probe and its flanking primers were prepared as described by
Houng et al. and custom synthesized by Operon. The viral RNA copy number was determined using a real-time one step qRT-PCR assay using the Taqman RT kit (PE Applied Biosystem Inc.) and Bio-Rad iCycler system using a standard control for virus quantitation by the qRT-PCR assay similar to the one previously described. The limit of detection is about 100 copies of RNA equivalent viral genome per ml in this assay.

**FACS Analysis and Immunohistochemistry**

Whole blood was stained with a panel of cell surface markers conjugated with various fluorochromes, lysis of red blood cells, and subjected to multi-color FACS analysis according to a protocol standardized in our lab. Two panels of antibodies were used; one was for T cells, B cells, and NK cells subset phenotyping which included CD16/NKG2A/CD14/CD45/CD4/CD8/CD56/CD3/CD20, and the second panel was for platelet-leukocyte aggregation and included CD41/CD61/CD62P/CD14/CD45. The frequency of leukocyte subpopulations that aggregate with platelet was identified by gating on CD41+ CD61+ CD62P+. In addition, the characterization of monocytes and neutrophils populations was performed as described by Lafont et al. Multi-color flow cytometric analysis was performed on a LSRII flow cytometer (BDB) using BD FACSDiva software (BDB). All data were analyzed by FlowJo software (Tree Star, Ashland, OR). Immunohistochemistry was performed according the standard protocol.

**Enzyme-linked immunosorbent assay (ELISA)**

Serum titers of anti-dengue IgM and IgG was determined by antibody-capture ELISA as described elsewhere. The increase of antibody titers was expressed as a percent of the O.D. values obtained on samples from the same monkey on day 0 (prior to infection). The following formula was used to calculate the percent of the antibody increase in each monkey.

\[
\text{Percent of antibody increase} \, (\%) = \frac{(\text{daily OD readings} - \text{OD reading of day 0})}{\text{OD reading of day 0}} \times 100
\]

**Statistical analyses:**
Data analysis was performed using GraphPad software (Prism 5, www.graphpad.com). The t test was employed for the comparisons of specific populations of subphenotype of lymphocytes between lymphocytes and the sub-lymphocyte populations. P values <0.05 were considered statistically significant.

Results

The need to conduct detailed multiple analyses during the acute infection period coupled with the constraints placed on the volume of blood that can be collected from individual animals within a fixed period of time, dictated that select studies be performed on samples from individual animals only. The data presented therefore are representative of staggered sample collections from individual animals or when available an average from combined data in the case when a complete set of the parameters were available.

Hemorrhage in dengue infected rhesus monkeys.

For these studies we infected 6 rhesus monkeys with a high dose of dengue virus intravenously, and unexpectedly succeeded for the very first time, in inducing coagulopathy reminiscent of dengue hemorrhagic symptoms previously documented in human cases of DHF in 6 of the 6 infected animals (Fig. 1 and Suppl. Fig. 1). By day 3-5 all 6 monkeys exhibited petechiae (small red spot) and mild to extensive subcutaneous hematoma consistent with coagulopathy which lasted for about 10 days before symptoms resolved by day 14 post infection (pi). Fig.1 shows representative gross morphological images that appear to be similar to the hemorrhagic manifestations observed in dengue infected human patients. In one animal, a rash characterized by a general morbilliform eruption with petechiae and islands of sparing—white islands in a sea of red (Fig. 1, RM#4), a general pattern thought to be an immune response to the dengue virus 27-29, was observed. Clinical symptoms were in general more extensive and apparent in the older females than the young adult males, suggesting potential relative differences in susceptibility secondary to gender and/or age, an aspect that will require address
in future studies. Of note, the animals did not show any other apparent clinical symptoms such as fever, inappetence or lethargy.

The laboratory evaluations included blood chemistries, measurements of proteins involved in the coagulation system, and complete blood counts. A modest increase in the plasma levels of the liver enzymes aspartate aminotransferase (AST or SGOT) and alanine aminotransferase (ALT or SGPT), or the myocardial infarction indicator enzyme, creatine phosphokinases (CPK) was noted in samples obtained on day 7 after infection. Plasma glucose levels also experienced an increase by day 7 pi, while phosphorus showed a late decline by day 14 (Table 2A).

Coagulation parameters are summarized in Table 2B: The data from these studies revealed that while there was a marked increase in the plasma values of D-Dimer noted in samples obtained on day 7 pi, whereas AT, TAT and protein S, showed marked elevations starting on day 1 post infection. In contrast, there did not appear to be any detectable change from baseline values in the levels of soluble fibrin monomers, protein C, PT and APTT in the samples examined from these monkeys (Table 2B). Results of the evaluations of blood parameters are summarized in Table 3. As seen, while there was a modest decrease in platelet count and a noticeable decrease in total WBC during the first week of infection, values for both of these parameters remained within the normal ranges and returned to pre-infection levels during the second week pi. In addition, there appeared to be a consistent reduction of hematocrit values and a slight decrease in the levels of hemoglobin on all samples collected following infection. In contrast, there was no detectable change in the values for total RBC count in these same samples.

Viremia is one of the major clinical manifestations of dengue virus infection. The dengue viral RNA in the plasma of infected monkeys was quantified utilizing real-time RT-PCR. The monkeys were infected in 2 separate studies with slightly differing monitoring schedules. In the initial study which included 3 monkeys (Fig 2A), blood specimens for viral load determination
were collected prior to and then on alternate days pi. Plasma viral loads rose to $10^5$ copies/ml by
day 1 pi and then reached a peak at $\sim 10^5$ copies/ml on day 3 followed by a gradual decline with
values dipping below the detectable level by day 14 p.i. In order to derive an even more detailed
view of the kinetics of virus replication, daily instead of alternate day blood specimens were
collected from the animals from the second series of animals. The trend of viral RNA kinetics
appeared to be similar to that noted for samples from the first study except that a transient but
consistent one log drop in the level of plasma viral RNA was noted in samples collected on day
2 p.i. from their day 1 values (Fig. 2B). The viral loads did however peak by day 3-4 in all
animals and the viremia was completely controlled by 2 weeks pi. The viral titers were also
evaluated using an FFU assay (Suppl. Fig. 2) which showed that the level of viremia peaked on
day 3 pi with a gradual decline to undetectable levels on day 10 pi. The lower FFU titer
compared to that noted by qRT-PCR assays was expected due to differences in the sensitivity
of the 2 assays.

Serum IgM and IgG specific for dengue viral antigens were measured by ELISA. As
expected, a typical profile of dengue virus specific IgM and IgG antibodies was noted in these
infected monkeys (Fig. 3, A and B, respectively).

**Leukocyte subpopulation in dengue hemorrhagic monkeys**

Subpopulation of leukocytes. A transient drop of the WBC was observed as early as day
1 with a nadir on day 7 and then returning to normal levels by day 10 as determined by values
of complete blood count (Table 3). These changes, however, were within the normal reference
range for rhesus macaques. The analysis of the leukocyte subsets based upon absolute counts
showed that a decrease in the absolute number of neutrophils most likely accounted for WBC
decrease. There did not appear to be any detectable change in the absolute number of
monocytes during the acute infection period (Fig. 4, monocytes) and a modest increase was
noted in the absolute number of lymphocytes toward the end of the acute infection period (Fig.
4). The transient neutropenia (Fig.4) noted in samples from the dengue virus infected monkeys
are similar to those documented to occur during natural infection in dengue patients.

Subpopulation of lymphocytes. Since logistic issues make it difficult to ascertain the kinetic effects of dengue virus infection on changes in lymphoid cell subsets during the acute viremia period in humans, the experimentally infected NHP model provides a valuable tool to begin to address this issue. Flow cytometric analysis of sequential PBMC samples from the experimentally dengue virus infected rhesus macaques prior to and following acute infection was therefore analyzed in detail. The lymphoid cells were identified as those that expressed high levels of CD45 accompanied by low side scatter (SSC) (Suppl. Fig. 3).

There were 3 basic observations that highlight the changes consistently observed in each of the experimentally dengue virus infected rhesus macaques. Thus, while there was no detectable difference in the total number of lymphoid cells during the first week pi (Fig. 4A) there appeared to be an increase (p<0.005) in the frequency of T cells (Fig. 4B) with a concomitant decrease in the frequency of total B cells (Fig. 4B) (p=0.0006). Of interest also was the observation of a decrease in the frequency of NK cells on day 1 pi (Fig. 4B) followed by a marked sustained increase thereafter. These changes were also apparent when the values for each of these subsets were calculated as absolute numbers (data not shown). The values for T cells, B cells and NK cells each returned to baseline values by 14 days pi (Fig. 4B). Further analysis of changes in the absolute number of T cell subsets revealed that the increase in total T cells was secondary predominantly to an increase in the CD4+ T cell subset with only a modest increase in the absolute number of CD8+ T cells and a decrease in CD4/CD8 double positive subsets (DP, Fig. 4B). This rapid decrease in DP cells was seen consistently in each of the monkeys and it is not clear whether this is a signature for dengue virus infection uniquely seen in NHPs or is also potentially induced in dengue infected patients following acute infection.

Platelet-leukocyte aggregation. Thrombocytopenia although often subclinical, is one of the clinical hallmarks in dengue virus infected patients which may contribute to the coagulopathies observed in our animals (Fig. 1). The etiology of this phenomenon remains ill-
defined. One explanation for this observation is that the decrease is primarily due to platelet-leukocyte aggregation which has been documented in a number of physiological and pathological states and has been implicated to contribute to inflammatory processes.

Flow cytometric analysis using platelet specific monoclonal antibodies were used to investigate the formation of platelet-leukocyte aggregation during the acute dengue virus infection. The gating strategy of citrate whole blood samples utilized is illustrated in Fig. 5. Briefly, the appropriate cocktail of various fluorochrome labeled monoclonal antibodies was added to an aliquot of whole blood. Antibodies against the platelet specific surface markers CD41 and CD61 in addition to markers that identify unique lymphoid cell subsets were utilized in efforts to determine the identity of lymphoid cell subsets which demonstrated platelet adhesion. The platelet activated surface glycoprotein p-selectin (CD62p), was utilized as a marker of platelet aggregation. As seen in Fig. 6A, a significant amount of platelet aggregation was noted with monocytes as previously noted on PBMC samples from human patients during acute dengue virus infection. Platelet-neutrophils aggregates were also detected albeit at lower frequencies (Fig. 6B). Interestingly, platelet-leukocyte aggregates showed 2 consistent separate peaks pi (day 1-3 and day 7 pi), for reasons that remain to be elucidated. The lower percentage of the platelet-neutrophils aggregates compared to that of platelet-monocytes aggregates may be a reflection of higher numbers of neutrophils circulating in the blood, thus in absolute numbers, neutrophil-platelet aggregates markedly surpass the numbers of monocyte-platelet aggregates.

Blood film smears performed in parallel confirmed the occurrence of platelet-leukocyte aggregates (Fig. 7 and Supp. Fig. 4). Platelet-monocyte aggregates were observed on blood smears obtained from monkeys on the 8th and the 9th day pi (Fig. 7, A and B, respectively). Immunofluorescence staining with dengue specific antibody (clone 3H5) also revealed that some of these platelets were positive for dengue antigen (Fig. 7C). These results are consistent with previous reports that have documented the presence of dengue antigen positive leukocytes (or monocytes) in samples from patients examined towards the end of the acute infection period.
coincident with the disappearance of virus from the plasma. It is reasonable to assume that the presence of dengue viral antigens within monocytes in samples obtained towards the end of the acute infection period may be secondary to the process of phagocytosis. Interestingly, a recent report also suggests a prominent role of monocytes and/or macrophages in the control of dengue virus in infected mice. However, the contribution of platelet-leukocyte aggregation in promoting the phagocytic activity of monocytes requires further study.

In addition, attempts to identify the phenotype of the cells that harbor dengue antigen were performed (Suppl. Fig. 4A). Dengue antigen appeared associated with a cell that expressed a cell surface marker normally expressed by platelets. These dengue antigen positive “platelets” were localized within or on the membrane of a cell with an unknown phenotype, though likely to be either belonging to the neutrophil or monocyte lineages as seen by Wright's Giemsa staining. However, the true identity of the phenotype of these cells remains to be verified.

Discussion

NHPs have been used to investigate several aspects of dengue virus infection. These studies have included those involved with the effect of natural and experimental infection, studies of the immune response of animals infected with dengue viruses, and the evaluation of a number of candidate dengue viral vaccine formulations. The generation of dengue virus specific antibody and the kinetics of dengue viremia in these monkeys have been shown to be essentially similar to that seen in human dengue virus infection. The Dengue virus infected NHPs have therefore been viewed as an acceptable animal model to study the compendium of virological and immunological aspects of experimental dengue virus infection. The only major exception to the use of the NHP model has been the failure of the dengue virus infected animals to develop any detectable signs of disease including manifestations of DHF and DSS that is characteristic of a defined frequency of human dengue virus infection.
Using high dose IV route, we were surprised to observe that all three monkeys during the first series of studies developed visible signs of cutaneous hemorrhage recapitulating one of the clinical manifestations characteristic of dengue virus infection of humans. The age matched rhesus macaque that was inoculated with mock cell culture supernatant fluid from the same cell line utilized for producing the dengue virus stock used for the present studies failed to show any detectable signs of cutaneous hemorrhage. It is important to note that only a single monkey was used as a control for this first set of studies. However, within the same context, it is also important to note that the subcutaneous dengue hemorrhage seen during the first set of studies was also noted in the second set of rhesus monkeys using the same dose and route of IV infection. Coagulopathy was manifest by extensive subcutaneous bleeding, petechiae and a marked delay in blood clotting time in venous blood being collected during this time period. These initial hemorrhagic manifestations were similar to those of dengue patients and appeared starting on days 3-5 post infection. Although petechiae could be found in several parts of the body, in general, the presence of petechiae was observed initially on the rear thigh, and sequentially around the abdomen, shoulder, and chest as a function of time. However, these petechiae were similar in each of these locations and faded away over time, which is somewhat similar to human dengue virus infection. In addition, upon pressing, the red spot of the petechiae did not disappear, branch, or fade away, which is typical of dengue petechiae in dengue patients. Petechiae are the most common clinical manifestations in dengue patients in dengue endemic countries, such as Thailand, and can occur on any part of the body in dengue patients and can worsen upon trauma, such as a bump with an object, a blood draw, or tourniquet test. Although these coagulopathies were noted in each of the 6 dengue infected rhesus monkeys, the animals fully recovered by about 14 days pi and cleared their symptoms shortly thereafter.

Although epidemiological data suggest that DHF/DSS occurs predominantly following secondary infection with a heterologous dengue serotype, numerous reports have also
documented DHF following primary dengue virus infection 3-6. Such primary infection induced DHF/DSS has been documented in dengue naïve travelers who visit dengue endemic regions 7-9 suggesting that DHF/DSS may be primarily linked to permissiveness of viral replication and the levels of viral load which tend to be magnified during secondary exposure of humans to a different serotype reasoned to be due to a phenomenon termed antibody mediated enhancement 45. Of note, the viremia detected in our monkeys, while high, was still about 1-2 log below the viremia noted in patients with DHF/DSS 10 which may account for the relatively benign overall disease course in the monkeys. Perhaps, a threshold viral load is required to induce fulminant DHF/DSS, while the lower viral load in our monkeys is sufficient to induce mild hemorrhage but not high enough to induce the more severe form of dengue DHF/DSS. Future manipulations of the model are expected to address the link between viremia and disease in vivo. Thus, this non-human primate model for dengue virus infection may not only provide a valuable tool for the detailed study of the various pathophysiological effects of dengue virus infection but will also provide a comprehensive analysis of host-virus interactions, with the potential to lead to the identification of the cellular and molecular mechanisms that lead to DHF/DSS and the lineage of cells that serve as the primary target of infection and virus replication. In addition, such a model also provides an important model for the testing and evaluation of potential dengue virus vaccines specially those that have the added benefit of protecting patients from the development of DHF/DSS.

Although alterations of blood immune cell subsets including a transient CD4/CD8 ratio inversion in dengue patients have been noted 34,46,47, we did not find such a change in our dengue infected animals. The reason for the difference might be due to decreased disease severity that we observed in our animals. In addition, an increase in CD19+ B cells was also observed in human dengue virus infection 48. However, we did not observe any increase in B cells when using CD20 as a B cell marker. It is possible that it is due to differences in the type of infection since most human patients that have been studied are due to secondary dengue virus
infection whereas our animals were studied during primary infection. The secondary infection in humans might induce a rapid expansion of memory B cells that are specific for dengue virus and thus result in an increase in CD19+ B cells.

In our sequential and systematic phenotypic analyses, we frequently observed platelet-leukocyte aggregation, in particular in association with monocytes and to some extent, with neutrophils. The flow cytometry based observation of platelet-monocyte aggregation was further strengthened by immunofluorescence assays, in which monocytes appeared to engulf platelets containing dengue antigen (Fig. 7 and Suppl. Fig. 4). Attempting to identify the cell lineage that was positive for intracellular expression of dengue antigen by FACS, IFA or IHC with proper isotype antibody control was inconclusive, partly due to high fluorescence background. Additionally, alteration of dengue antigens or epitopes engulfed by the phagocytic cells may result in the failure for the antibody to react. Thus, there is a need for a suitable dengue antigen staining method for the detection of the viral antigen by standard flow cytometry and IFA or IHC. This may partially explain why even up to date, the identity of the cell lineage(s) that harbor dengue antigens in the circulating blood of patients are still an enigma. Nevertheless, the combination of single staining and Wright’s Giemsa staining supports the results noted by FACS analysis.

Interestingly, the importance of monocytes/macrophages in the control of dengue virus infection has been recently emphasized. Since dengue is a disease of timing, studies reported so far have involved specimens collected from dengue patients primarily collected after the onset of clinical manifestations. While there is a considerable amount of variability in the kinetics of the various pathological manifestations of dengue virus infection among individuals, it is likely that the studies reported involved samples representing the peak of dengue viremia. This may partially explain why Durbin et al. observed quite a few immune cells with a variety of cell surface markers that were positive for dengue viral antigens PrM or NS3. Thus, data collected and analyzed from sequential and systematic specimens in a suitable dengue animal
model may provide a more objective analysis of the early events during acute dengue virus infection.

One of the major clinical and pathological features that differentiate DHF from DF is plasma leakage which is reasoned to be a consequence of increased vascular permeability.\cite{49} Disseminated intravascular coagulation (DIC) is not only a very prominent feature that occurs in patients with DSS but DIC has also been noted in some cases of DHF.\cite{49} Obviously, an imbalance or dysregulation between the prothombotic proteins and the natural anticoagulant pathway(s) may contribute to the tendency to develop hemorrhage in select patients following dengue infection. This view is supported by the data of aberrant levels of a number of plasma coagulation factors in DHF patients due either to intravascular consumption or impaired synthesis by the liver.\cite{50,51} The precise mechanisms involved in the induction of hemorrhage in DHF patients, however, are believed to be multiple. It is of interest to note that in the studies reported herein, no detectable changes were noted in the PT and PTT levels, while levels of Protein C appeared to fluctuate but did not show a distinct pattern of change. However, distinct elevations were noted in the levels of D-dimer, an accepted marker for DIC,\cite{52} as well as those of AT, TAT and Protein S. In addition, as compared with human DHF/DSS, only mild levels of thrombocytopenia were noted in the dengue virus infected rhesus monkeys in the present study. One possible explanation for this discrepancy is that the changes in the aforementioned coagulation proteins in conjunction with the other changes are more typical of liver function perturbations rather than procoagulant DIC. The other could be due to the fact that the peripheral blood of rhesus macaques as a species consists of three or four times more platelets than humans. Thus, a 10-20% drop in platelet count may not be noted as being significant in dengue virus infected monkeys but, nonetheless significant in terms of absolute numbers. Furthermore, the kinetics by which these changes occur is an important issue to keep in mind since the clinical changes seen in human dengue virus illness are notably a function of time post infection. Thus, to accurately define the mechanisms that lead to DHF with specimens collected
at, during, or after the clinical symptoms develop may not be an easy task to achieve.

Consequently, descriptive reports on the coagulation parameters are somewhat inconsistent\textsuperscript{53-57}. The NHP model of dengue virus infection in which cutaneous hemorrhage is consistently observed, may perhaps for the first time provide a valuable model for the investigation of the bleeding mechanisms that are a cardinal feature of DHF in patients.
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Author contributions and Conflict of Interest Statements

Contribution: Nattawat Onlamoon designed and performed the FACS experiments and analyses; Sansanee Noisakran discussed the experimental strategy and performed FACS experiments and analyses; Hui-Mien Hsiao performed the immunohistochemistry staining, real-time PCR and ELISA; Alexander Duncan discussed the experimental strategy, performed the coagulation parameter assays, and edited the manuscript; Francois Villinger discussed the experimental strategy, directed the monkey experiments and edited the manuscript; Aftab A. Ansari discussed the experimental strategy, assisted in approval of IACUC protocol, and edited the manuscript, and Guey Chuen Perng designed, defined and discussed the experiment strategy, wrote the IACUC protocol, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.
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Table 1. Animal specifications.

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</tbody>
</table>

<sup>a</sup>All monkeys used in this study were Indian rhesus macaque monkeys. The monkeys were designated as RM# in the text. However each bore a specific tattoo marked shown in parentheses.

<sup>b</sup>Sacrificed on day 7 p.i.

<sup>c</sup>Mock infection with media from Vero cells cultured for the dengue virus serotype 2 stock. This animal was used as a control.

<sup>d</sup>Uninfected rhesus monkey for the deriving values of the normal levels of coagulation parameters.
Table 2A. Blood Chemistry.

<table>
<thead>
<tr>
<th></th>
<th>RM#1</th>
<th>RM#2</th>
<th>RM#3</th>
<th>Ref. range&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>74</td>
<td>65</td>
<td>99</td>
<td>102</td>
</tr>
<tr>
<td>Protein (g/dl)</td>
<td>7.2</td>
<td>6.8</td>
<td>6.4</td>
<td>6.3</td>
</tr>
<tr>
<td>SGPT (U/l)</td>
<td>39</td>
<td>39</td>
<td>114</td>
<td>51</td>
</tr>
<tr>
<td>SGOT (U/l)</td>
<td>32</td>
<td>32</td>
<td>43</td>
<td>27</td>
</tr>
<tr>
<td>Phosphorus (g/dl)</td>
<td>4.3</td>
<td>4.8</td>
<td>4</td>
<td>2.7</td>
</tr>
<tr>
<td>CPK (U/l)</td>
<td>386</td>
<td>410</td>
<td>3768</td>
<td>276</td>
</tr>
</tbody>
</table>

<sup>a</sup>The reference ranges are based on values previously published by Matsuzawa et al<sup>58</sup>.
Table 2B. Coagulation parameters.

<table>
<thead>
<tr>
<th></th>
<th>RM#1</th>
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<th></th>
<th></th>
<th>RM#2</th>
<th></th>
<th></th>
<th></th>
<th>RM#3</th>
<th></th>
<th></th>
<th></th>
<th>RM#7</th>
<th></th>
<th></th>
<th>RM#8</th>
<th></th>
<th></th>
<th>Ref. range&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>7</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>14</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>14</td>
<td>0</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>PT (secs)</td>
<td>-</td>
<td>9.9</td>
<td>8.9</td>
<td>9.7</td>
<td>-</td>
<td>10.1</td>
<td>9.1</td>
<td>8.8</td>
<td>10.1</td>
<td>9.6</td>
<td>9.2</td>
<td>9.3</td>
<td>9.7</td>
<td>10.4</td>
<td>10.7±4.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APTT (secs)</td>
<td>45.6</td>
<td>46.5</td>
<td>33.7</td>
<td>33.6</td>
<td>-</td>
<td>40.2</td>
<td>32.5</td>
<td>37.4</td>
<td>59.0</td>
<td>29.6</td>
<td>42.0</td>
<td>44.5</td>
<td>48.5</td>
<td>34.6</td>
<td>25±20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dimer (ng/ml)</td>
<td>-</td>
<td>&lt;150</td>
<td>486</td>
<td>1102</td>
<td>&lt;150</td>
<td>862</td>
<td>1170</td>
<td>1131</td>
<td>-</td>
<td>586</td>
<td>725</td>
<td>882</td>
<td>&lt;150</td>
<td>&lt;150</td>
<td>&lt;260</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>ATIII (%)</td>
<td>-</td>
<td>134</td>
<td>174</td>
<td>128</td>
<td>120</td>
<td>138</td>
<td>124</td>
<td>138</td>
<td>-</td>
<td>140</td>
<td>138</td>
<td>137</td>
<td>125</td>
<td>113</td>
<td>82-133</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibmon (mg/dl)</td>
<td>-</td>
<td>&lt;7</td>
<td>&lt;7</td>
<td>&lt;7</td>
<td>-</td>
<td>40</td>
<td>&lt;7</td>
<td>&lt;7</td>
<td>&lt;7</td>
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<td>&lt;7</td>
<td>&lt;7</td>
<td>&lt;7</td>
<td>&lt;10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAT (µg/ml)</td>
<td>19.0</td>
<td>7.2</td>
<td>&gt;60</td>
<td>16.8</td>
<td>-</td>
<td>&gt;60</td>
<td>&gt;60</td>
<td>&gt;60</td>
<td>14.8</td>
<td>&gt;60</td>
<td>11.6</td>
<td>53.0</td>
<td>7.0</td>
<td>7.6</td>
<td>4-33</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prot C (%)</td>
<td>301</td>
<td>&lt;10</td>
<td>292</td>
<td>276</td>
<td>151</td>
<td>221</td>
<td>&gt;301</td>
<td>&gt;301</td>
<td>299</td>
<td>269</td>
<td>&gt;301</td>
<td>290</td>
<td>140</td>
<td>134</td>
<td>76-208</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prot S (%)</td>
<td>83</td>
<td>166</td>
<td>151</td>
<td>127</td>
<td>86</td>
<td>94</td>
<td>164</td>
<td>175</td>
<td>149</td>
<td>123</td>
<td>101</td>
<td>114</td>
<td>47</td>
<td>48</td>
<td>62-153</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The reference ranges are based upon reported by Matsuzawa et al<sup>58</sup>. Prothrombin (PT), Activated Partial Thromboplastin time (APTT), D-Dimer (Dimer), Antithrombin III (ATIII), Soluble fibrin monomers (sFM), Thombin-Antithrombin complexes (TAT), Protein C and Protein S activities.
Table 3. Laboratory evaluations of blood parameters.

<table>
<thead>
<tr>
<th>Day P.I.</th>
<th>Hemoglobin (g/dl)</th>
<th>Hematocrit (%)</th>
<th>WBC (per µl)</th>
<th>RBC (X10⁶/µl)</th>
<th>Platelet (X1000/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13.90 +/- 1.22</td>
<td>43.57 +/- 4.49</td>
<td>6500 +/- 1833</td>
<td>5.90 +/- 0.50</td>
<td>386 +/- 89</td>
</tr>
<tr>
<td>1</td>
<td>12.98 +/- 1.13</td>
<td>39.88 +/- 3.72</td>
<td>5566 +/- 1268</td>
<td>5.47 +/- 0.48</td>
<td>356 +/- 138</td>
</tr>
<tr>
<td>3</td>
<td>12.98 +/- 1.44</td>
<td>39.74 +/- 4.92</td>
<td>4622 +/- 1692</td>
<td>5.47 +/- 0.60</td>
<td>320 +/- 112</td>
</tr>
<tr>
<td>5</td>
<td>11.80 +/- 0.67</td>
<td>36.70 +/- 2.63</td>
<td>4898 +/- 2124</td>
<td>5.03 +/- 0.38</td>
<td>375 +/- 85</td>
</tr>
<tr>
<td>7</td>
<td>11.46 +/- 0.53</td>
<td>35.50 +/- 2.20</td>
<td>4436 +/- 1050</td>
<td>4.88 +/- 0.37</td>
<td>411 +/- 71</td>
</tr>
<tr>
<td>10</td>
<td>11.90 +/- 0.42</td>
<td>37.20 +/- 1.84</td>
<td>6034 +/- 2007</td>
<td>5.06 +/- 0.36</td>
<td>389 +/- 71</td>
</tr>
<tr>
<td>14</td>
<td>11.96 +/- 1.17</td>
<td>37.14 +/- 4.13</td>
<td>7584 +/- 3100</td>
<td>5.04 +/- 0.47</td>
<td>399 +/- 56</td>
</tr>
<tr>
<td>Reference range</td>
<td>10.5-12.5</td>
<td>35.4-41.4</td>
<td>4200-9200</td>
<td>5.55-6.63</td>
<td>195-339</td>
</tr>
</tbody>
</table>

Reference range
Figure legends

Figure 1. Dengue hemorrhage in rhesus monkeys. Rhesus monkeys were intravenously infected with dengue virus as described in the Methods. Hemorrhagic manifestations were captured with digital camera on days 3 (RM#2), 4 (RM#4), and 5 (RM#3) after infection. Different severity of hemorrhage, ranging from petechiae to severe coagulopathy, was seen. A classic clinical hemorrhage was observed in infected animal RM#4. The top and bottom panels indicate that the images of the skin hemorrhage were captured from different part of the body within the same animal.

Figure 2. Viral load in plasma. Blood was drawn at the indicated days, RNA was isolated from plasma and purified viral RNA was quantified by real-time RT-PCR as described in the Methods. The peak of dengue viremia in infected RM was from 3-7 days after infection.

Figure 3. Typical primary IgM and IgG antibody responses. Presence of dengue specific antibodies in the sera was assayed by ELISA as described in the Methods. Variations of IgM response in individual RM were observed. But in general, a typical quick and robust response of IgM antibody as well as a delayed response of IgG antibody was registered.

Figure 4. Profiling of leukocyte subpopulation. Cell surface markers conjugated with proper fluorochrome, which can differentiate the leukocyte subpopulation, were used to stain the fresh-drawn blood and subjected to FACS as described in the Methods. (A) Absolute counts of each leukocyte subpopulation with standard error bar from each animal were presented. A noticeable reduction of monocytes on day 1 after infection was observed, and thereafter a rebounded pattern to normal level was registered. A slight fluctuation with a trend of gradual increase in lymphocytes during acute infection was seen. A consistent and gradual reduction of neutrophils was documented during the acute period, which returned to uninfected level on 14 days after
infection. (B) Percentage of lymphocyte subpopulation with standard error bar from each animal was presented.

**Figure 5. Strategy to profile the aggregation of platelets with neutrophils or monocytes.** Whole blood flow cytometry was performed after samples were stained with specific cell surface markers conjugated with proper fluorochrome as described in the Methods. The strategy to gate the specific platelet-leukocyte aggregation was described. The first step is to differentiate neutrophils from monocytes with CD14 surface marker, which then further identified with makers for platelets, respectively.

**Figure 6. Profiles of platelet-monocyte or-neutrophil leukocyte aggregation.** Kinetics of platelet-leukocyte aggregation were presented as percentage of the gated event. A similar pattern of platelet aggregation with monocytes or neutrophils was observed.

**Figure 7. Engulfment of platelets by monocytes/macrophages.** Blood smears were prepared from dengue virus infected rhesus monkeys and Wright’s Giemsa and immunofluorescence staining were performed. (A and B)Wright’s Giemsa staining revealed that tangled platelets were engulfed by monocytes. (C) Immunofluorescence staining revealed that some of these platelets were positive for dengue viral antigen (3H5, Red). Nuclear was stained with DAPI (Blue) and SYTOX Green.
Figure 1

RM#2  RM#3  RM#4
Figure 2

A.

B.

Plasma Viral RNA (Copies/ml) vs. Days P.I. for different samples.

Day P.I.: 1 to 14

Samples: RM#1, RM#2, RM#3, RM#4, RM#5, RM#6

Log scale is used for both Y-axis and X-axis.
Figure 3

A.

B.
Figure 4A
Figure 4B

%CD3+  %CD20+  %NK

%CD4+  %CD8+  %CD4+CD8+
Figure 5

Neutrophils

Monocytes

CD41+CD61+

CD41-CD61-

52

4.91

5.2

16.6

72

6.2

CD41+CD61+

CD41-CD61-

4.47

38.7

44.2

12.7

44.2

12.7

59.7

15.8

% of Max

% of Max

% of Max

% of Max
Figure 6

A. Monocytes

B. Neutrophils
Dengue virus induced hemorrhage in a nonhuman primate model

Nattawat Onlamoon, Sansanee Noisakran, Hui-Mien Hsiao, Alexander Duncan, Francois Villinger, Aftab A. Ansari and Guey Chuen Perng