Patterns of host genome-wide gene transcript abundance in the peripheral blood of patients with acute dengue hemorrhagic fever

MAJOR ARTICLE

Patterns of Host Genome–Wide Gene Transcript Abundance in the Peripheral Blood of Patients with Acute Dengue Hemorrhagic Fever

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Responses by peripheral blood leukocytes may contribute to the pathogenesis of dengue hemorrhagic fever (DHF). We used DNA microarrays to reveal transcriptional patterns in the blood of 14 adults with DHF. Acute DHF was defined by an abundance of transcripts from cell cycle– and endoplasmic reticulum (ER)–related genes, suggesting a proliferative response accompanied by ER stress. Transcript-abundance levels for immunoresponse-associated genes, including cell surface markers, immunoglobulin, and innate response elements, were also elevated. Twenty-four genes were identified for which transcript abundance distinguished patients with dengue shock syndrome (DSS) from those without DSS. All the gene transcripts associated with DSS, many of which are induced by type I interferons, were less abundant in patients with DSS than in those without DSS. To our knowledge, these data provide the first snapshot of gene-expression patterns in peripheral blood during acute dengue and suggest that DSS is associated with attenuation of selected aspects of the innate host response.

Dengue viruses cause a spectrum of disease, ranging in severity from the self-limiting illness known as "dengue fever" (DF) to more-severe outcomes such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). DHF and DSS are characterized by excessive systemic vascular leak, thrombocytopenia, and, frequently, hemorrhagic manifestations. DF and DHF/DSS are the most important arboviral diseases of humans, with over half of the world's population living in areas of risk.

Received 31 August 2006; accepted 10 November 2006; electronically published 5 March 2007.

Potential conflicts of interest: none reported.

Financial support: Wellcome Trust (to C.P.S. and J.F.); Horn Foundation (grant to D.A.R.).

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The Journal of Infectious Diseases 2007; 195:1097–107

0022-1899/2007/19508-0005$15.00
DOI: 10.1086/512162

Collectively, previous studies have suggested that events in the peripheral blood, such as dengue viral replication, cytokine expression, and cellular activation/proliferation, are associated with disease severity and outcome (reviewed in [1]). Viral load in vivo appears to be important because it is significantly greater in patients with DHF/DSS than in those with DF [2, 3]. DHF/DSS has also been associated with a robust host inflammatory immune response; significantly greater plasma concentrations of inflammatory cytokines [4–6] and activated lymphocytes are found in patients with DHF/DSS than in patients with DF [7, 8]. Most cases of DHF/DSS occur, paradoxically, in individuals with serological evidence of a prior dengue virus infection [9–11]. Antibody-dependent enhancement of viral replication is the most widely accepted explanation for the association between DHF and preexisting antibody. However, there remains considerable uncertainty as to how the virus-host interaction results in vascular leak, the most important clinical characteristic of DHF.

Analysis of host genome–wide transcript abundance patterns has revealed mechanisms of pathogenesis that

Table 1. Primers, probes, and polymerase chain reaciton conditions for the detection of dengue viruses.

The table is available in its entirety in the online edition of the *Journal of Infectious Diseases.*

would not otherwise be apparent. For example, this kind of analysis has elucidated the roles of the NF-κB and type II interferon (IFN) response systems and suggested the importance of a cell "proliferation" response in a primate model of smallpox [12], as well as implicated neutrophil activity in acute malaria [13]. The ability to identify regulatory gene modules from genome-wide data reinforces the value of transcript abundance patterns, even for processes that are subject to posttranscriptional regulation [14]. The purpose of the current study was to better understand the molecular features of dengue pathogenesis and the determinants of disease outcome in the naturally infected, intact human host. Our work revealed cogent biological patterns that distinguished the acute stage of dengue infection from the convalescent stage and from healthy donors.

PATIENTS AND METHODS

Patients and clinical investigations. Blood samples for microarray analysis were obtained from adult patients with dengue at the Hospital for Tropical Disease, Ho Chi Minh City, Vietnam. World Health Organization (WHO) classification criteria [15] were applied to each case after review of study notes. All blood samples were collected between 9 AM and 11 AM on the indicated study days, beginning on the first morning after admission (study day 1). Convalescent samples were obtained after hospital discharge (usually 1 month). Platelet counts and hematocrit values were recorded at least twice per day during hospitalization. Hemoconcentration was determined by comparing the maximum hematocrit recorded during hospitalization with the value recorded at convalescence. Written, informed consent was obtained from each patient. The study protocol was approved by the Scientific and Ethical Committee of the Hospital for Tropical Diseases and by the Oxford University Tropical Research Ethical Committee.

Serological testing and polymerase chain reaction (PCR) testing for dengue. Serological testing of paired plasma samples (collected $\geqslant$3 days apart) was performed using a dengue capture IgM and IgG ELISA (Panbio). In this study, the term "secondary infection" is used to describe the nature of the anamnestic serological response and does not imply that this was necessarily the second dengue or flavivirus infection experienced by the patient. An internally controlled, serotype-specific real-time reserve-transcription (RT) PCR assay was used to measure dengue viral loads in plasma. All primer sequences, probes, and PCR conditions are summarized in table 1; the dengue-specific primers/probes were adapted from Laue et al. [16].

RNA sample collection. Whole blood (2.5 mL) was collected directly from the patient into PAXgene RNA tubes (Qiagen). RNA extraction was performed using Paxgene blood RNA kits (Qiagen). Purified RNA was stored at -80°C.

Table 2. Clinical summary of patients with secondary dengue (DEN) infections.

Study	Day of illness	Platelet nadir, cells/μL	Hemoconcentration,[a] %	Shock[b]	Spontaneous bleeding	Disease grade[a]	Serotype (viral load, cDNA copies/mL) at admission[c]
BC324	5	40,000	23	Yes	Epistaxis and petecchiae	4	…
BC329	4	28,700	47	Yes	Epistaxis	3	…
BC330	6	37,200	21	Yes	Cutaneous bleeding	3	DEN-2 (5448)
BC337	4	5200	20	Yes	GI bleeding	3	…
BC353	6	16,900	44	Yes	Petecchiae and GI bleeding	3	DEN-2 (3485)
BC355	5	18,100	32	Yes	GI and vaginal bleeding	3	…
BC325	4	32,000	12	No	Gum bleeding	Indeterminate	…
BC327	4	33,200	30	No	None	1	…
BC328	5	36,000	20	No	Petecchiae	1	…
BC333	5	32,100	21	No	Epistaxis and cutaneous bleeding	2	…
BC334	5	38,000	27	No	Gastrointestinal bleeding	2	DEN-1 (1943)
BC338	3	18,700	11	No	Gum bleeding and petecchiae	2	DEN-2 (6562)
BC341	4	30,000	2	No	Petecchiae	Indeterminate	DEN-2 (245,625)
BC354	4	30,000	24	No	Petecchiae	2	DEN-1 (1,717,500)

NOTE. GI, gastrointestinal.

[a] World Health Organization (WHO) classification criteria.

[b] Dengue shock syndrome as defined by WHO criteria (pulse pressure $\leqslant$20 mm Hg).

[c] Measured by fluorogenic reverse-transcription polymerase chain reaciton assay.

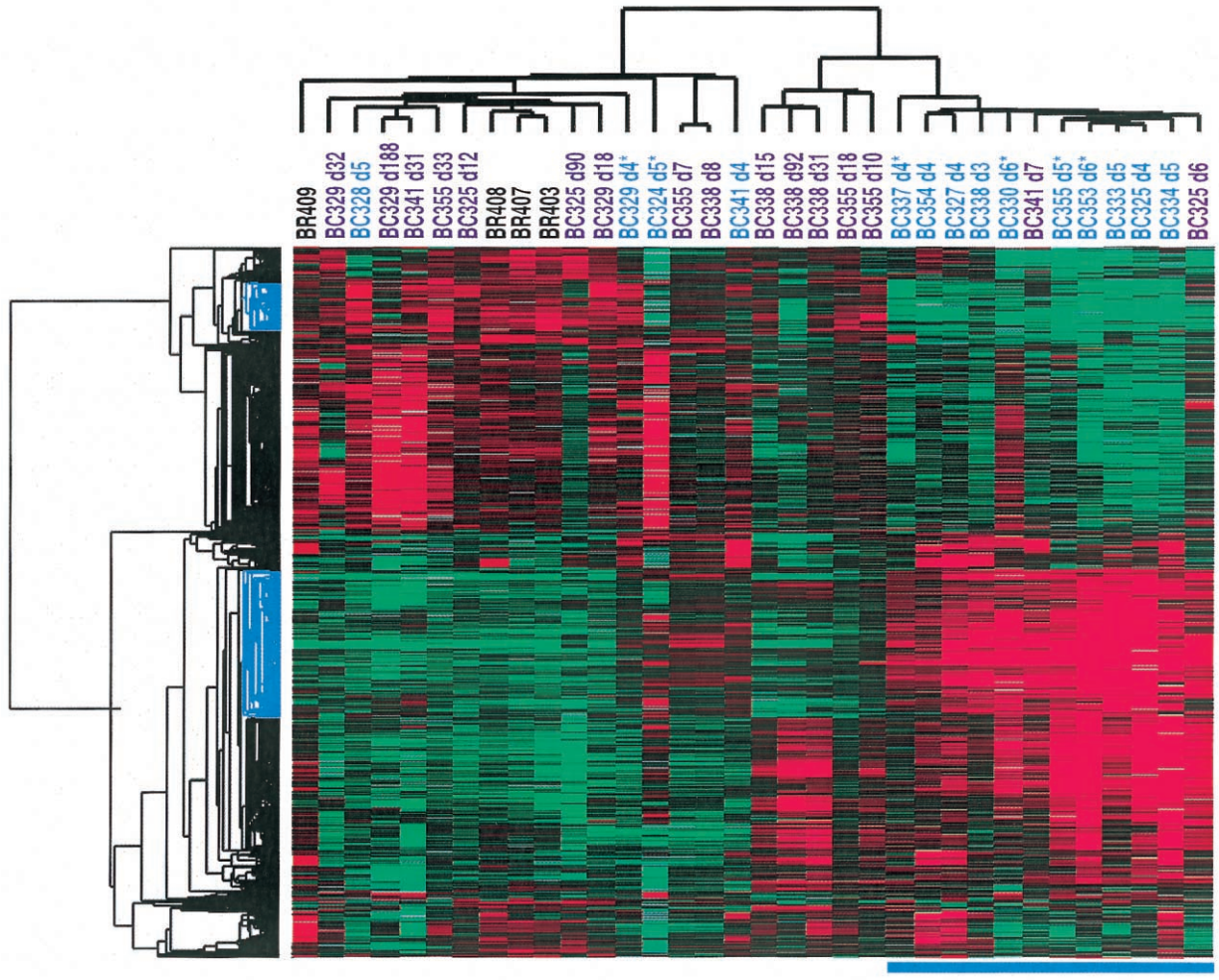

Figure 1. Unsupervised hierarchical clustering of 34 whole blood RNA samples from 14 patients with dengue and 4 healthy donors. Each row represents the relative level of expression for a single gene; each column shows the expression level for a single sample. The red and green colors indicate high and low expression, respectively. Samples collected from patients with dengue on study day 1 are shown in blue, samples collected after day 1 are shown in purple, and samples from healthy donors are shown in black. The blue horizontal bar indicates 10 early samples with similar gene expression patterns, and gene clusters associated with segregation of these samples are displayed in blue. Sample nos. are as shown in table 2, reported day of illness at the time of sampling is preceded by a "d," and asterisks identify those patients who received a diagnosis of dengue shock syndrome on study day 1.

Sample preparation and cDNA microarray hybridization. RNA transcripts in the samples and a standard reference RNA (Universal Human Reference RNA; Stratagene) were amplified using Message-Amp (Ambion). Sample and reference were then reverse transcribed, labeled with fluorescent dyes (Cy5 and Cy3, respectively), and hybridized to cDNA microarrays as described elsewhere [17] (available at: http://cmgm.stanford.edu/pbrown/protocols). The microarrays contained 37,632 spots derived from cDNA clones representing ~18,000 unique genes, the majority of which were cloned from lymphocytic cells [18]. Images were obtained using a Genepix 4000B microarray scanner and analyzed with Genepix 5.0 (Axon Instruments). Fluoresence ratios were log transformed (base 2), and a total intensity normalization scheme was applied to each array. A local intensity estimation method was used to confirm the absence of significant systematic bias in the log ratios [19]. The data are available in the Stanford Microarray Database (http://smd.stanford.edu).

Microarray data filtering and analysis. Data were filtered to include only clones that met the following criteria for at least 80% of the samples tested: signal intensity 2.5-fold above background in either the Cy5 (sample) or Cy3 (reference) channel and a regression correlation for the 2 channels of at least 0.6 across each measured element. Data for each array were mean centered, and data for each clone were then median centered across all observations. Selected data were organized using a hierarchical clustering algorithm based on a Pearson's correlation

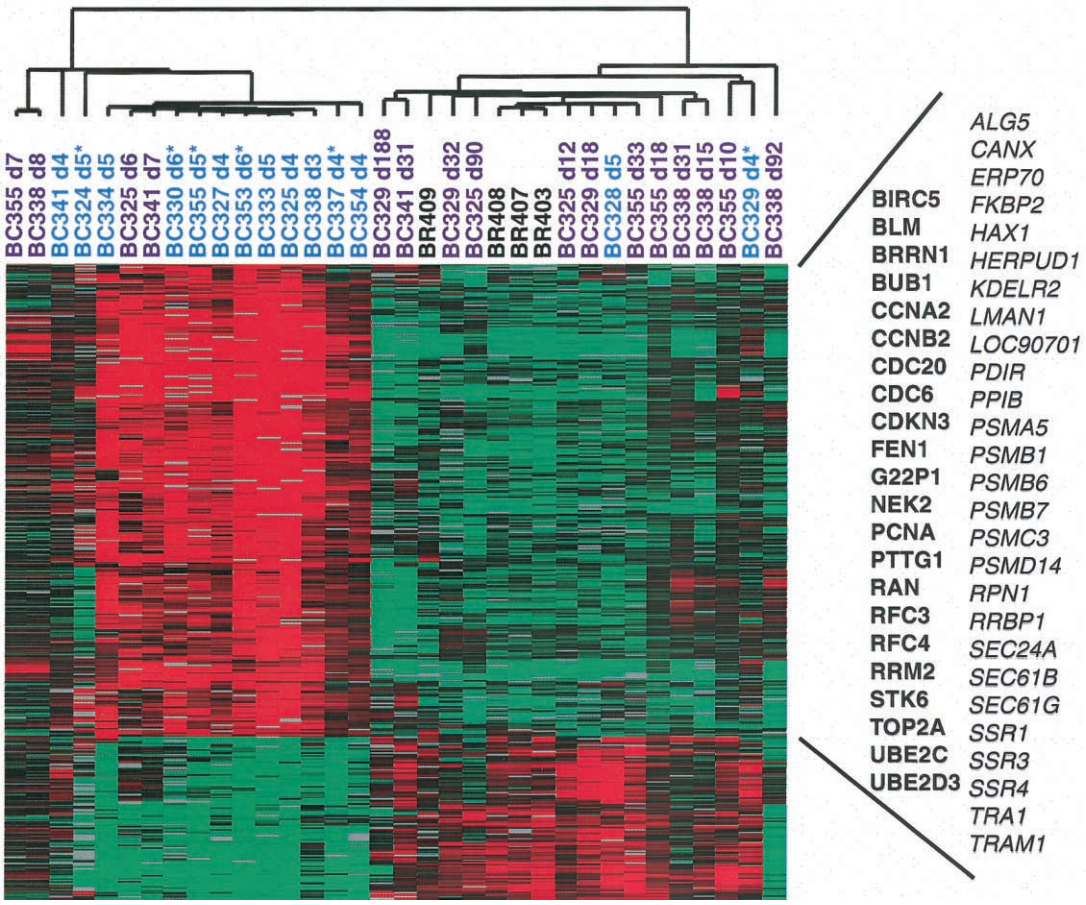

Figure 2. Reclustering of samples from figure 1 using the genes associated with the "early sample" cluster in figure 1. Colors are as in figure 1. Gene ontology terms overrepresented in the cluster of genes expressed at higher levels in the early samples are represented by the associated genes, and Unigene symbols are used to indicate each gene (available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene). Genes whose products are associated with the endoplasmic reticulum are in italics, and genes whose products are associated with the mitotic cell cycle are in bold. Sample nos. are as shown in table 2, reported day of illness at time of sampling is preceded by a "d," and asterisks identify those patients who received a diagnosis of dengue shock syndrome on study day 1.

metric, with average linkage clustering [17], and were visualized using Treeview (http://rana.lbl.gov/EisenSoftware.htm).

The EASE software program was used to identify gene ontology (GO) terms represented in the filtered data set [20]. To identify terms enriched in specific subsets of the data, EASE scores were calculated; EASE scores represent an adjustment to the Fisher exact probability that penalizes terms containing only a few genes. A conservative Bonferroni adjustment for multiple testing was used to generate the reported *P* values. Enrichment of gene clusters for genes found in other gene expression data sets was determined using the hypergeometric distribution.

The association of each gene's expression pattern with clinical or immunological parameters across the 34 samples was defined using Pearson's correlation coefficient. After calculation of the correlation coefficient, the specified clinical data were randomly permuted, and the correlation coefficient was recalculated. This permutation scheme was performed 1000 times; *P* values were calculated as the fraction of times the permuted data resulted in a correlation coefficient greater than that observed with the actual data. The negative logarithm (base 10) of the *P* values was assigned a positive or negative value corresponding to the direction of the original correlation coefficient. The resulting significance curves were plotted as a moving average with a window size of 15.

Flow cytometry and cytokine measurements. Fluorochrome-conjugated monoclonal antibodies were used to stain whole blood, and all analyses were performed on a FACSCalibur flow cytometer (Becton Dickinson). Absolute counts of lymphocyte subsets and granulocytes were determined using Trucount tubes (Becton Dickinson). Plasma concentrations of interleukin (IL)–6, IL-1β, tumor necrosis factor (TNF)–α, IL-8, IL-12p70, and IL-10 were measured by cytometric bead array

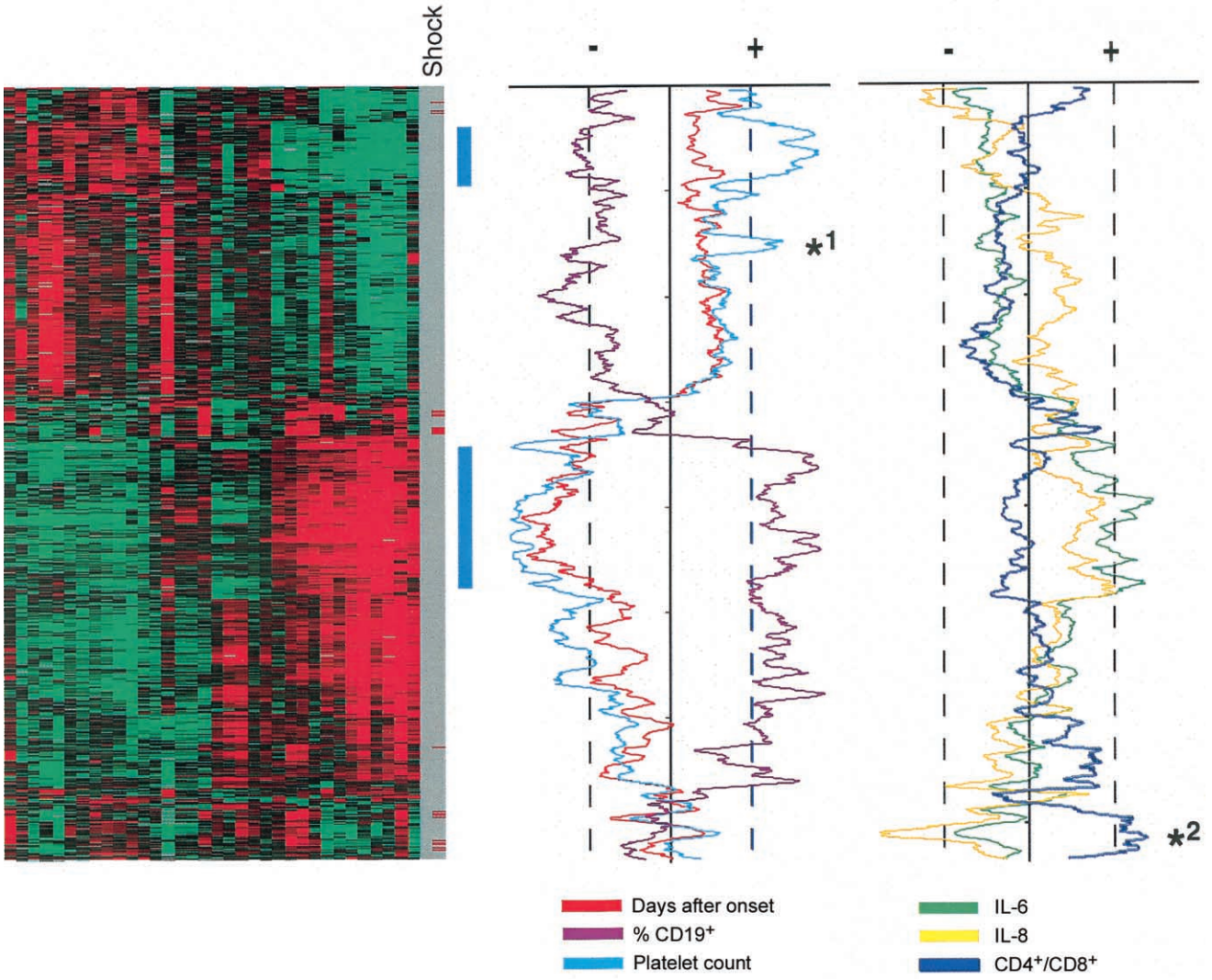

Figure 3. Association of clinical and laboratory parameters with gene expression. For panel A, sample nos. and genes are as shown in figure 1. The gray column on the right highlights genes *(red)* that were identified as being associated with shock at presentation. Vertical blue bars mark the same gene clusters as in figure 2. For panel B, Pearson correlation coefficients were used to determine the significance and direction of association of each gene's expression pattern with a given clinical or laboratory parameter (see Patients and Methods); the resulting log-transformed *P* values are plotted as a moving average, with a window size of 15. Stronger associations are placed farther from the axis, and whether the correlation was positive (+) or negative (−) is indicated. The dotted line corresponds to $P < .01$. Asterisks mark associations of gene expression with a given parameter (see Results).

assay (Becton Dickinson). IFN-α concentrations in plasma were measured using a commercial ELISA (Biosource International).

RESULTS

Patient population. Study subjects were Vietnamese adults with secondary dengue virus infections. Key clinical and virological parameters in the 14 subjects are described in table 2. At study entry, the mean length of illness history was 4.6 days. Six patients received a diagnosis of DSS on admission, and a blood sample for microarray analysis was collected during the period of cardiovascular instability. DSS was defined according to WHO criteria (pulse pressure $\leqslant$20 mm Hg) and was supported by the clinical finding of poor peripheral perfusion. Patients with DSS had lower platelet nadirs and, on the basis of changes in hemoconcentration, significantly greater vascular leak than patients without DSS ($P = .04$, Student's *t* test) (table 2). All patients survived. There were no significant differences in age or sex of patients with shock (mean [SD] age, 17 [1.3] years; 33% male) versus those without shock (mean [SD] age, 19 [2.4] years; 50% male).

Gene transcript abundance during acute infection. Serial whole blood samples from patients with dengue were collected

Table 3. Genes with transcript abundance that correlates with $CD4^+$:$CD8^+$ lymphocyte ratio.

The table is available in its entirety in the online edition of the *Journal of Infectious Diseases.*

for microarray analysis. For comparative purposes, samples were also collected from 4 healthy adult donors (age range, 22–25 years). Whole blood RNA samples ($n = 34$) were hybridized to microarrays containing 37,632 elements. Analysis was restricted to those genes whose transcript abundance differed >3-fold from the median level in at least 3 of the 34 samples. The resulting 1203 genes (1474 array elements) and samples were organized using hierarchical clustering (figure 1).

Unsupervised organization of the samples based on gene transcript abundance patterns resulted in segregation of samples obtained early during disease from those taken later; 10 of the 14 samples collected on the day of enrollment and 12 of the 17 samples collected before illness (day 5) were grouped together (figure 1, horizontal blue bar) and separated from samples collected later during the disease course and from the samples from healthy donors. Two sets of genes were associated with this early-late separation of samples; the first set consisted of 230 genes (307 elements) expressed at higher levels in the early samples, and the second set consisted of 100 genes (107 elements) expressed at higher levels in the late samples and in healthy donors (figure 1). To confirm the association of these 2 gene sets with the distinction between early and late samples, we used these genes to recluster the samples; 15 of 17 samples collected before day 5 were grouped together (figure 2). One of the 2 samples from day 5 was also in this group, whereas all later samples and samples from the healthy donors were segregated with a separate branch of the dendrogram (figure 2).

To identify biological themes associated with these 2 gene clusters, we extracted GO terms using EASE [20]. The GO is a hierarchical structured vocabulary that describes gene products in terms of their biological process, cellular component, and molecular function [21]. Most of the genes expressed at higher levels in the early samples are predicted to encode intracellular proteins; among these, genes whose protein products are found at the endoplasmic reticulum (ER) were particularly enriched ($P < .0001$) (figure 2, gene symbols on the right in italics). Many of these gene products, including peptidylprolyl isomerase B (cyclophilin B; *PPIB*); signal sequence receptors α, γ, and δ (*SSR1, SSR3,* and *SSR4*); and translocation-associated membrane protein 1 *(TRAM1)*, participate in the processing and folding of nascent proteins or the transport of proteins across the ER.

Another GO category enriched among early disease-associated genes refers to progression of the mitotic cell cycle and includes genes that encode cyclins, cyclin-dependent kinases, and topoisomerase 2A, suggesting that cell division or related processes are involved in the early host response to dengue ($P < .01$) (figure 2; gene symbols on the left in bold). In addition, transcript levels for genes associated with proapoptotic effects were also elevated in acute samples, including proapoptotic caspase adaptor protein *(PACAP);* apoptosis-inducing factor, mitochondrion-associated, 2 *(AMID);* BCL2-antagonist killer 1 *(BAK1);* programmed cell death 5 *(PDCD5);* and serine/threonine kinase 17a *(STK17A)*. Other genes for which transcripts were more abundant in early patient samples encode CD38, CD59, and immunoglobulin light and J chain subunits.

Genes with less-abundant transcript levels in early samples were not associated with the same degree of enrichment of GO terms. However, "oxygen transport" was overrepresented by the presence of 4 different hemoglobin genes: *HBA1, HBB, HBE1,* and *HBG2*. This finding may reflect a deficit in reticulocyte numbers during acute infection. This conclusion is supported by the overrepresentation of genes whose expression in whole blood samples was previously associated with the level of reticulocytes ($P < .001$, hypergeometric) [22].

Gene expression patterns and the host response. Clinical and laboratory parameters that correlated with transcript levels for coexpressed sets of genes were identified. Six parameters showed particularly strong associations ($P < .01$) with subsets of the genes presented in figure 1: platelet count, $CD4^+$:$CD8^+$ lymphocyte ratio, plasma IL-6 and IL-8 concentrations, and relative abundance (percentage) of $CD19^+$ B cells (figure 3). Age and sex were not significantly associated with transcript abundance patterns.

Transcript abundance patterns for a cluster of 31 genes were associated with platelet count, independent of phase of illness (figure 3, asterisk 1). This cluster included the canonical platelet-associated genes *SPARC* and *PDGFB*, as well as others

Table 4. $CD138^+CD38^+$ plasmablasts/plasma cells as a percentage of $CD19^+CD20^-$ B cells in peripheral blood of control subjects and in children (mean age, 11 years) with acute secondary dengue at hospital admission and discharge.

Group	Admission	Discharge
Secondary dengue[a]	10.9 ± 15.4[b]	16 ± 14[c]
OFI[d]	1.1 ± 0.61	5.5 ± 10.08
Healthy adults[e]	0.30 ± 0.26	NA
Healthy children[f]	0.71 ± 0.93	NA

NOTE. Data are the mean ± SD percentage of $CD138^+CD38^+$ cells in the $CD19^+CD20^-$ B cell population in whole blood. NA, not available; OFI, other febrile illness.

[a] $n = 24$.

[b] Dengue vs. OFI at admission ($P = .006$, Mann-Whitney *U* test).

[c] Dengue vs. OFI at discharge ($P = .02$, Mann-Whitney *U* test).

[d] $n = 8$.

[e] $n = 11$.

[f] Children who had recovered from a dengue virus infection 1 month previously ($n = 22$).

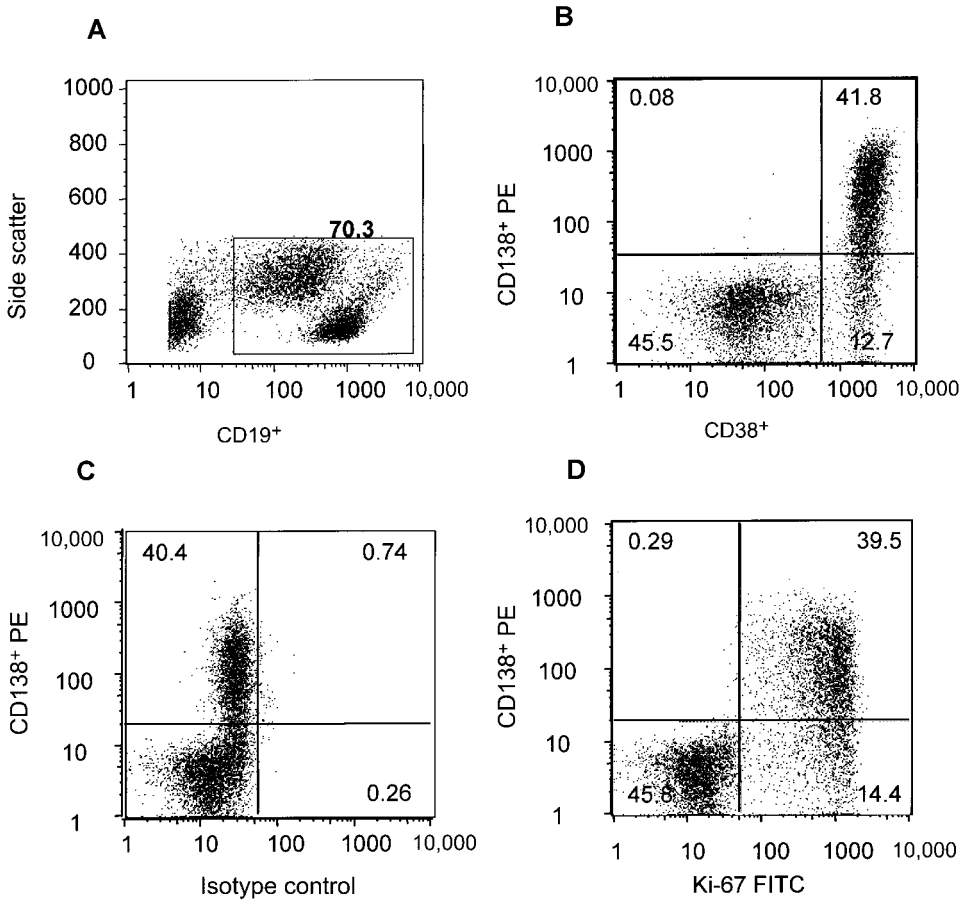

Figure 4. Flow cytometry results derived from staining whole blood collected from a child with dengue shock syndrome (DSS; day 5 of illness). $CD19^+$ B cells, which comprised 2 populations distinguished by granularity *(A),* were gated, and the percentage of $CD138^+CD38^+$ B cells among the $CD19^+$ population was determined *(B).* Virtually all $CD19^+CD138^+$ B cells expressed the nuclear-associated antigen Ki-67, indicating they were in cell cycle (*C;* isotype control *[D]*, anti–Ki-67). FITC, fluorescein isothiocyanate; PE, phycoerythrim.

(*OSMR*, *NR2F1*, *EDG2*, *RBM9*, and *CRADD*) that have been found to be expressed in purified platelet populations [23].

Expression of a set of 56 genes (80 array elements) was significantly correlated with the $CD4^+$:$CD8^+$ lymphocyte ratio (figure 3, asterisk 2) and was highly enriched for the GO term "immune response" ($P < .0001$). This set included many genes involved in the innate immune response and in regulating development of the adaptive immune response; the full list is presented in table 3.

Of the cytokines measured, IL-6 and IL-8 correlated ($r = .70$) with each other and also with the same sets of genes. Thus, plasma IL-6 concentrations were strongly associated with the set of genes more highly expressed early during infection (cell cycle and ER associated) and with $CD19^+$ cell abundance (figure 3). Plasma concentrations of TNF-α, IL-10, IL-1β, and IL-12p70 were not associated with transcript abundance patterns, nor were there significant differences in cytokine concentrations between patients with DHF and shock and those without shock (data not shown).

B lymphocyte–associated gene expression patterns. The cell cycle/ER gene cluster was strongly positively correlated with $CD19^+$ B lymphocytes (figure 3). To understand better the association of $CD19^+$ B cells with gene expression patterns during early dengue, we performed ex vivo flow cytometry studies on peripheral blood from 5 adults with DHF. Acutely, up to 18% of the $CD19^+$ B cell population had a plasmablast/plasma cell phenotype ($CD20^-CD19^+CD38^+CD138^+$) (table 4). For reasons of ease of patient recruitment, we performed further studies in children with suspected DHF at the same clinical center. Children with acute DHF also had elevated numbers of circulating $CD20^-CD19^+CD38^+CD138^+$ B plasmablasts/plasma cells (table 4 and figure 4). Most $CD19^+CD38^+CD138^+$ B cells also expressed the cell cycle–associated nuclear antigen Ki-67, indicating they were in cell cycle (figure 4). $CD19^+CD38^+CD138^+$ B plasmablasts/plasma cells were more abundant in children with acute DHF than in children with other febrile illnesses (not dengue) or in children who had recovered from DHF 1 month previously (table 4). Thus, highly activated, proliferating

Table 5. Genes associated with dengue shock syndrome in Vietnamese adults.

Gene symbol	Gene name	Reference[a]	Summary of function[b]
ANXA1	Annexin A1		Ca^{2+}-dependent phospholipid binding protein; potential anti-inflammatory activity via inhibition of phospholipase A2
ANXA3	Annexin A3		Inhibition of phospholipase A2; may also have role in anticoagulation
AQP9	Aquaporin 9		Role in immunological response and bactericidal activity
CD14	CD14 antigen	[28]	Expressed on monocytes/macrophages; LPS binding protein and apoptotic cells
CTSL	Cathepsin L	[12, 28]	Plays a major role in intracellular protein catabolism
FLJ31033	Hypothetical protein FLJ31033	[28]	Unknown
G1P2	Interferon, alpha-inducible protein (clone IFI-15K)	[12, 24]	Unknown
GBP1	Guanylate binding protein 1	[12, 24]	Specifically binds guanine nucleotides
HP	Haptoglobin	[24]	Unknown
IFIT2	Interferon-induced protein with tetratricopeptide repeats 2	[12]	Unknown
IL13	Interleukin 13		Up-regulates CD23 and MHC II expression, down-regulates macrophage activity
IL1RN	Interleukin 1 receptor antagonist		Inhibits the activities of IL-1α and IL-1β, modulates IL–1 related immune responses
IMAGE:2072531		[12]	Unknown
IMAGE:2121258			Unknown
LANCL1	LanC lantibiotic synthetase component C–like 1		Involved in the biosynthesis of antimicrobial peptides
LY6E	Lymphocyte antigen 6 complex, locus E	[24]	Unknown
MAT2A	Methionine adenosyltransferase II, alpha		Unknown
MX1	Myxovirus (influenza virus) resistance 1	[12, 24]	In mice, the IFN-inducible Mx protein is responsible for a specific antiviral state against influenza virus infection
MX2	Myxovirus (influenza virus) resistance 2	[12, 24]	Upregulated by IFN-α but does not contain the antiviral activity of a similar myxovirus resistance protein 1
OAS3	2′-5′-oligoadenylate synthetase 3	[12]	Unknown
POLR2B	RNA polymerase II polypeptide B	[24]	The second largest subunit of RNA polymerase II
SERPING1	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	[28]	Inhibits activated C1r and C1s of the first complement component and thus regulates complement activation
SLIC1	Selectin ligand interactor cytoplasmic–1		Unknown
TNFAIP6	Tumor necrosis factor, alpha-induced protein 6		Encodes a secretory protein that forms a stable complex with IαI and thus enhances the serine protease–inhibitory activity of IαI

NOTE. C1, complement component 1; IαI, inter-α inhibitor; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MHC, major histocombatibility complex; Mx, myxovirus.

[a] Indicating IFN induced.

[b] Adapted from SOURCE database (http://source.stanford.edu).

The figure is available in its entirety in the online edition of the *Journal of Infectious Diseases*.

Figure 5. Oligoadenylate synthetase 3 and cathepsin L mRNA levels in patients with and without shock. The figure and legend (which includes a description of the methods) are available in their entirety in the online edition of the *Journal of Infectious Diseases*.

plasmablasts/plasma cells are prominent in acute DHF and may make a significant contribution to the gene expression patterns linked to mitosis and immunoglobulin synthesis.

Gene expression patterns associated with DSS. Significance analysis of microarrays was used to identify elements on the arrays whose transcript levels were associated with DSS at the time point DSS was diagnosed. Transcripts for 24 genes were less abundant in adults ($n = 6$) with DSS (median false discovery rate, 1 gene [3.6%]) than in those with non-DSS dengue ($n = 8$). A number of these genes are canonical IFN-stimulated genes (ISGs), including *MX1, MX2, ISG15 (G1P2), IFIT-2,* and *OAS3.* As a group, these DSS-related genes are enriched for genes identified as IFN-regulated in other studies ($P < 10^{-6}$, hypergeometric) [12, 24]. Additional genes in this group may be IFN-regulated; another study has found that 15 of the 24 were induced in stimulated peripheral blood mononuclear cells in vitro by 1 or more type I IFNs (S. Waddell and D. A. Relman, unpublished data). Some of the genes not induced by IFN also have known roles in the immune response; *ANXA1, ANXA3, IL13,* haptoglobin, and *TNFAIP6* all have roles in moderating inflammatory responses [25–27]. These genes and their reported functions are listed in table 5. In addition, RT-PCR was used to measure levels of 2 of the shock-associated transcripts, cathepsin L (CTSL) and oligoadenylate synthetase 3 (OAS3). The RT-PCR results were strongly correlated with the array measurements of the study day 0 samples ($r^2 = 0.91$ and $r^2 = 0.93$, for CTSL and OAS3, respectively) and were significantly associated with shock status ($P < .01$ and $P < .02$, respectively). Convalescent samples from the same patients showed no differences in levels of the same transcripts associated with day 0 shock status (figure 5).

One might suspect these DSS-related differences in transcript abundance are attributable to the timing of sample collection. However, there was no significant difference in day of illness between patients with DSS and those without DSS. In addition, the DSS-related genes were not associated with day of illness in our correlation analysis (figure 3). Reanalysis of the data set, restricting samples to those from patients seen on days 4 and 5, yielded a similar and overlapping list of DSS-related genes (data not shown). To explore further the nature of the IFN response in these patients, we measured concentrations of IFN-α in all acute plasma samples. In all patients, concentrations of IFN were below the level of detection of the assay (20 pg/mL).

DISCUSSION

This study revealed 2 previously unrecognized aspects of dengue pathogenesis. First, gene expression profiles in peripheral blood suggested that acute dengue virus infection is associated with a pronounced ER stress response and cellular mitotic activity. B plasmablasts and plasma cells likely make significant contributions to this transcriptional profile. Second, multiple type I IFN–regulated genes were underexpressed in patients with DSS relative to those without DSS, suggesting important differences in the nature of the host immune responses.

The pathophysiological processes that result in the characteristic features of severe dengue disease, such as hemorrhage, vascular leak, and occasionally hypovolemic shock, are poorly understood. Gene expression studies represent an opportunity to identify novel markers linked to immunity and disease pathogenesis and an opportunity to classify patients with dengue in a manner that provides greater biological relevance and clinical utility.

Transcripts from genes participating in cell cycle control and mitosis were abundant in early peripheral blood samples from patients with dengue. These data are consistent with the cellular events known to take place in peripheral blood during acute dengue; dengue epitope–specific $CD8^+$ T cells are found in cell cycle [8], and an atypical lymphocytosis with blast cells is present [29, 30]. In addition, the present study revealed a striking abundance of proliferating B plasmablasts and plasma cells in the peripheral blood. The presence of these B plasmablasts might explain the strong association between relative B cell numbers and the proliferative transcript signature seen in early blood samples. Future studies should include purification of individual lymphocyte subsets to verify this association. We hypothesize that these B plasmablasts/plasma cells represent dengue-specific memory B cells that have been stimulated by cross-reactive dengue antigens in lymphoid tissue during secondary infection. Potentially, these plasmablasts and plasma cells participate in the original antigenic sin phenomena described by Halstead et al. [31]. Original antigenic sin implies that the antibody response during a secondary infection is directed toward a serotype responsible for a previous infection, rather than toward the currently infecting virus. These B cells might also be sites of dengue virus replication and sources of inflammatory cytokines, as suggested by ex vivo [32] and in vitro [33] studies.

Transcripts from genes encoding ER-associated proteins were significantly more abundant during acute dengue disease. The extensive membranous network of the ER provides a unique environment for the synthesis, folding, and modification of secretory and cell surface proteins. Flaviviruses use the ER as

the primary site of envelope glycoprotein biogenesis, genomic replication, and particle assembly [34] and may induce "ER stress" in infected cells [35–37]. The prominence of ER-associated gene expression in acute dengue disease might reflect dengue virus replication in blood mononuclear cells or, more likely, may reflect indirect effects caused by nonspecific cellular activation. We examined a list of genes associated with ER stress and the "unfolded protein response," as defined in nonlymphoid cell types [38], and found them to be overrepresented in this set of acute dengue–associated transcripts (8/230 vs. 18/1203 transcripts; $P < .01$). Genes associated with the regulation of apoptosis were also found among the group whose transcripts were more abundant during early dengue virus infection. This finding suggests increased levels of apoptosis in blood mononuclear cells and is consistent with previous reports of apoptosis in $CD8^+$ T cells in patients with acute dengue [8, 39].

DSS is the most severe clinical presentation of dengue infection. The exact mechanisms driving the profound vascular leak that occurs in patients with DSS are not clear, although host immune factors have been repeatedly nominated to be among the most important (reviewed in [1]). At presentation, all 6 patients with DSS were distinguished from the 8 patients without DSS and healthy controls by the relative underabundance of transcripts for 24 genes. Intriguingly, type 1 ISGs were strongly represented in this cluster of 24 genes. Among these ISGs, *MX1* and *ISG15* have well-described antiviral activities [40, 41], whereas others (*IL13, IL1RN,* and annexin A1) have significant immune regulatory roles [25, 26]. We caution against overinterpretation of these observations, given the small sample size of this study and the possible external confounders (e.g., viral factors and the dynamic nature of vascular leak). Nevertheless, we speculate that impaired in vivo expression of these ISGs in patients with DSS might occur via multiple mechanisms. For example, reduced expression of selected ISGs may be related to intrinsic host genetic differences that affect IFN expression or IFN-mediated signaling pathways in acute disease. Efforts to correlate reduced expression of selected ISGs to low plasma IFN-α concentrations was not successful, because as IFN-α levels were below detectable levels in acute samples. Reduced expression of ISGs might be a virus-mediated phenomenon. For example, the capacity of dengue virus proteins, in particular NS4b, to inhibit the activation of IFN-stimulated response elements is well documented [42, 43]. Understanding whether attenuated IFN responses in vivo are the result—or the cause—of high viral loads remains a challenging but important question.

Acknowledgments

We thank the doctors and nurses at the Hospital for Tropical Diseases for patient recruitment, Judy Yen (Stanford University) for her technical assistance, and Chana Palmer and Alok Saldanha (Stanford University) for the Pearson correlation permutation script.

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