Dengue Virus Infection of Human Endothelial Cells Leads to Chemokine Production, Complement Activation, and Apoptosis

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Dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) are serious clinical conditions that occur almost exclusively in response to secondary infection by dengue virus (DV) (1, 2). It remains a major health problem in South East Asia, Central America, and the Pacific region, representing one of the major causes of child death in several countries (3). Cardinal signs of DHF/DSS include hemorrhage, abrupt onset of vascular leakage, and shock, accompanied by severe thrombocytopenia and massive complement activation (4, 5). Typically, survivors of DHF/DSS show rapid recovery with minimal sequelae (4). The pathogenesis of shock and leakage is poorly understood. Vascular endothelia in serosal tissues appear to be preferentially affected by some critical pathogenetic mechanism, since ascites and pleural effusion clearly account for most of the plasma leakage in DSS (6). As recently proposed by Innis, “the lack of structural damage, the short lived nature of the plasma leakage syndrome, and the remarkably rapid recovery of children with DSS all suggest that altered permeability is effected by a soluble mediator” (6). Deregulated cytokine responses (7–9) and complement activation by non-neutralizing Abs (10) have been implicated in the vascular leakage syndrome. Animal models of DSS do not exist, and biopsies cannot be taken from the pleura and peritoneum of patients because of the unacceptable accompanying risk. As a consequence, tissue samples relevant to studies of dengue shock are rarely available. In the face of this difficulty, all evolving concepts for the pathogenesis of DHF/DSS have been based on experimental in vitro observations. This pertains also to the question regarding the identity of the primary target cells, and their response to infection. The majority of earlier studies concentrated on monocyte/macrophages as possible targets, because uptake of virus-Ab complexes via Fc Rs could most easily explain the well-established phenomenon of Ab-dependent enhancement that was originally described by Halstead and O’Rourke (9). However, even under optimal conditions, DV infection of monocytes is rather inefficient in vitro, and the cytokine responses in infected cells cannot readily explain the leakage syndrome. Endothelial cells have also been considered as targets, and productive in vitro infection of these cells has been reported (11, 12). In the present study, we sought to characterize the infection of endothelial cells (EC) by DV in more detail, to investigate the response of the cells to infection, and to uncover a possible link between EC infection and complement activation. We report that DV infection of human EC provokes production of RANTES and IL-8, in the virtual absence of production of several other proinflammatory cytokines. A directed search revealed that plasma and pleural fluids (PF) of patients suffering from DV/DSS had remarkably high levels of IL-8. Furthermore, we report that cross-seroreactive Abs to DV activate complement on infected EC. Independent of this activation, DV-infected cells die within a few days via programmed cell death. Together, these findings lead to the formulation of a hypothesis on the pathogenesis of DSS that envisages a selective permeability increase in the serosal vasculature due to the selective binding of cationic chemokines acting in concert with complement anaphylatoxins possibly produced locally on DV-infected cells. Vascular permeability increases culminate with the apoptotic...
breakdown of the endothelial barrier, leading to the fulminant but transient leakage syndrome that is the hallmark of DHF/DSS.

Materials and Methods

Cell culture

The human umbilical cord vein endothelial cell line (ECV304), obtained from American Type Culture Collection (ATCC CRL-19988), Manassas, VA, was grown in medium 199 (Life Technologies, Paisley, Scotland) supplemented with 10% FCS (Life Technologies), 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin, and 100 µg/ml streptomycin (Life Technologies) at 37°C in humidified air containing 5% CO₂. C6/36, a cell line from Aedes albopictus, and PscCloneD, a swine fibroblast cell line, were cultured at 28°C and 37°C, respectively, in L-15 medium (Life Technologies) containing 10% tryptose phosphate broth (TPB) (Sigma, St. Louis, MO), 4 mg/ml gentamycin (Sigma) and stored at −70°C until used. Virus was titrated in plaque formation assays on PscCloneD cells. Monolayers of cells were trypsinized and resuspended in L-15 containing 3% FCS and 10% TPB and plated at 1 x 10⁵ cells/well in a volume of 0.5 ml in 24-well plates (Nunc, Roskilde, Denmark). Subsequently, dilutions of virus supernatant were added and the mixtures were incubated at 37°C for about 2–3 h; then 0.5 ml of L-15 containing 3% FCS, 10% TPB, and 2% (v/v) carboxymethylcellulose (Sigma) was added to each well. After 5 days of incubation at 37°C, the plates were visualized by staining with a dye solution composed of 0.1% (v/v) naphthale black 10B (Serva, Entwicklungs labor, Heidelberg, Germany), 1.36% (v/v) sodium acetate (Carl Roth, Karlsruhe, Germany), and 0.6% (v/v) glacial acetic acid (Roth). Virus concentrations are given as plaque-forming units/milliliter.

Infection of endothelial cells

Monolayers of EC were trypsinized and resuspended in growth medium. About 2–3 x 10⁴ or 8 x 10⁴ ECV304 cells were seeded into each well of 24-well tissue-culture plates or 8-well glass chamber slide (Nunc), respectively. Primary cells were seeded at a density of 1–2 x 10⁵/well in 96-well tissue-culture plates. After overnight incubation, virus culture fluid or heat-inactivated virus suspension (80°C, 20 min) was added to confluent monolayers of cells at the multiplicity of infection (MOI) of 0.1 and incubated at 37°C for 2 h. The virus supernatant was then removed and fresh growth medium was added to each well. Culture media and infected cells were harvested at various times after infection for further experiments.

Flow cytometry analysis

At 24, 48, and 72 h after infection, DV-infected and control cells were harvested from 24-well plates. Flow-cytometric assessment of percentage of dead cells was done after pooling populations of cells in suspension and trypsinized adherent cells of a given sample. Propidium iodide (Sigma) was added to give a final concentration of 0.2 µg/ml and samples were analyzed by a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). For flow-cytometric determination of DV infection, the harvested cell layers were washed twice with medium 199 and then fixed with 2% formaldehyde (Roth) in PBS for 1 h at room temperature (RT). After two washing steps with PBS, the fixed cells were permeabilized with 0.1% Triton X-100 in PBS. Permeabilized cells were incubated with dengue hyperimmune mouse ascitic fluid, generously provided by Dr. A. Nisalak (Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand), at a final dilution of 1:400 for 1 h or RT. The cells were then washed twice with permeabilization solution and incubated with dichlorotriazinyl amino flourescein (DTAF)-conjugated F(ab)₂ fragment of goat anti-mouse IgG (Di- anova, Hamburg, Germany) at a final concentration of 3.75 µg/ml for 30 min at RT in the dark. The cells were washed once with an excess volume of permeabilization solution and analyzed by flow cytometry.

Chemokine and cytokine quantitation

Supernatants from DV-infected, mock-infected, and heat-inactivated virus-treated EC were quantitated for cytokine and chemokine production. RANTES and monocyte chemotactic protein-1 (MCP-1) were determined using ELISA kits from Biosource International, Camarillo, CA. ELISA kits for IL-8 were obtained from Innogenetics, Zwinjendrecht, The Netherlands; for IL-1β from Immunotech/Coulter, Hamburg, Germany; for IL-1α from Endogen, Biozol Diagnostica Vertrieb, Eching, Germany; for IL-7, and granulocyte-macrophage-CSF (GM-CSF) from R&D Systems, Wiesbaden, Germany; for IL-15 from Cytoscreen, Laboserv Diagnostica, Giessen, Germany; and for TNF-α from Medgenix Diagnostic SA, Fleurus, Belgium.

Plasma and pleural fluid samples from DSS patients

EDTA plasma and PF samples obtained from six children dying of DSS were also assayed for ELISA for the chemokines MCP-1, RANTES, and IL-8. Diagnosis of DSS was based on the clinical criteria established by the World Health Organization (13); all patients were suffering from grade IV DSS. Bacteria were not found in smears and routine cultures of these samples. Albumin ratios for all plasma/PF pairs were above 0.5, indicating that these PF represented exudates. IL-8, MCP-1, and RANTES were determined using the same ELISA kits as described above. Albumin was quantitated at our clinical chemistry department.

RNA extraction and RT-PCR

Total RNA preparations from untreated cells or cells treated with inactivated or active virus were obtained by the method described by Chomczynski and Sacchi with minor modifications (14). RNA was quantitated as spectrophotometry (Pharmacia, LKB Biochrome, Little Chalfont, U.K.) at 260 nm. Approximately 1 µg of total RNA was used for the first-strand synthesis with oligo(dT) primer, AMV-RT, and reaction buffers as described by the manufacturer (reverse transcription system, Promega, Madison, WI). Subsequently, the newly synthesized first-strand cDNA was subjected to 20 rounds of PCR amplification of 95°C for 40 s, 62°C for 1 min, and 72°C for 3 min. Reaction mixtures contained primers at 1.5 µM each, MgCl₂ (1.5 mM), dNTP (0.2 mM each), and 1 U of Taq polymerase (Life Technologies) in a total reaction volume of 50 µl. The amplification primers were 5'-ATGATCCCTTCTTGCGCGTGGC-3' (forward) and 5'-TTCAGCCCTCCCTAAACCTTCTC-3' (reverse) for IL-8; 5'-TGCTTCCCATATTTCTCCTG-3’ (forward) and 5’-CTACTGTTTCGACCTGAAGC-3’ (reverse) for MCP-1; 5'-CGGAGTCACAGGGATTGTTCT-3’ (forward) and 5’-AGCGCTTCCTCATGTTGTAAGCGCT-3’ (reverse) for MCP-1; 5'-GCACTTGAGTAGCTCTTTCCG-3’ (forward) and 5’-CGCC-3’ for RANTES. Total RNA preparations from untreated cells or cells treated with inactivated virus suspension (80°C, 20 min) were added to confluent monolayers of cells at the MOI of 0.1 and incubated at 37°C for 2 h. The virus supernatant was then removed and fresh growth medium was added to each well. Culture media and infected cells were harvested at various times after infection for further experiments.

Reporter plasmids pIL8 (-420+102)LUC and pRANTES (-935+73)LUC were constructed by standard procedures. Fragments of the human IL-8 promoter region comprising position -420 to +102 and of the human RANTES promoter region between -935 and +73 were generated from human genomic DNA by PCR amplification with primers 5’-GGATCCTAGGGTGCCGGATCTTACACACCACACACACACAGGAGAAGTTG-3’ (IL-8, forward) and 5’-TGAAGTCAGCTATGTTTACACACACCTCGGTTGACATCTGGAAGAAGTTG-3’ (IL-8, reverse) or 5’-TCCAAGGCAGCAGCTCTTCTCCTCGCCT-3’ (RANTES, forward) and 5’-CGGAGTCAACAGGGATTGTTCT-3’ (reverse) for MCP-1; 5’-CGAGTCAACAGGGATTGTTCT-3’ (forward) and 5’-AGCGCTTCCTCATGTTGTAAGCGCT-3’ (reverse) for MCP-1; 5'-GCAGATCAACAGGGATTGTTCT-3’ (forward) and 5’-AGCGCTTCCTCATGTTGTAAGCGCT-3’ (reverse) for MCP-1; 5’-GCACTTGAGTAGCTCTTTCCG-3’ (forward) and 5’-CGCC-3’ for RANTES. Total RNA preparations from untreated cells or cells treated with inactivated virus suspension (80°C, 20 min) were added to confluent monolayers of cells at the MOI of 0.1 and incubated at 37°C for 2 h. The virus supernatant was then removed and fresh growth medium was added to each well. Culture media and infected cells were harvested at various times after infection for further experiments.

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treated with virus at a MOI of 0.1 or with an equal amount of inactivated virus. Luciferase assays with cellular lysates were performed 48 h after treatment using assay reagents from Promega and a Bioulmat LB9500 instrument from Berthold (Wildbad, Germany). Calculations of the degree of reporter gene induction were based on the relative numbers of viable cells in the samples, as assessed by ATP determinations.

**Bioluminescence assay for determination of cellular ATP**

DV and mock-infected cells cultured in 24-well plates for various times after infection were harvested for the determination of intracellular ATP by chemiluminescence measurements with luciferase (Boehringer Mannheim, Mannheim, Germany) as described previously (16). Intracellular ATP of virus-infected cells is given as percentage of luminescence relative to that of control cells.

**Determination of DNA strand breaks by TdT-mediated dUTP nick-end labeling (TUNEL)**

TUNEL assays were performed on DV-infected and control cultures in 8-well glass chamber slides. At 24, 48, and 72 h after infection, cells were fixed with 2% paraformaldehyde (Merck) in PBS for 30 min at RT. The fixed cells were processed for the detection of free 3′OH termini due to DNA strand breaks using the TUNEL kit (Boehringer Mannheim) according to the manufacturer’s protocol. In some experiments, mouse anti-human TNF-α neutralizing Ab (R & D Systems, Wiesbaden, Germany) at a concentration of 5 or 10 μg/ml was added 2 h after virus infection and the cells were processed for the determination of apoptosis as described above.

**Detection of nuclear translocation of nuclear factor-κB (NF-κB)**

Confluent ECV304 cells in eight-well glass chamber slides were fixed after dengue or mock infection with cold 70% ethanol for 20 min on ice, washed twice with PBS, and double stained with uninduced culture fluid of mAbs specific for dengue nonstructural protein-1 (NS1) and rabbit Abs against NF-κB p65 (Santa Cruz Biotechnology) at the final concentration of 5 μg/ml for 1 h at RT. Cells were washed three times with PBS and then incubated with a mixture of DTAF-conjugated F(ab′)2 fragment of goat anti-mouse IgG plus Cy3-conjugated donkey anti-rabbit IgG (Dianova) at a concentration of 3.75 μg/ml each for 30 min at RT. After final washes, the cells were covered with mounting fluid and visualized under a fluorescence microscope (Axiopt, Zeiss, Oberkochen, Germany).

**Electrophoretic mobility shift assays (EMSA)**

Whole cell lysates of untreated or virus-infected ECV304 cells were prepared by four cycles of freezing and thawing in a lystate buffer composed of glycerol, 20% (v/v) KCl, 0.4 M Tris-HCl buffer, 20 mM DTT, 2 mM PMSF, 10 μg/ml aprotinin, 1% (v/v) glycerol, 20% (v/v) KCl, 0.4 M Tris-HCl buffer, 20 mM DTT, 2 mM PMSF, 10 μg/ml aprotinin. Complementary synthetic oligonucleotides (CTCTGCCTCTCGGAAGTAGCTCCCTTGG-3′, 5′-GAAGTCCAGGGGACTTTCCGAGA-3′) comprising the NF-κB site (bold letters) of the murine Ig κ-chain gene were annealed, and overhangs (italics) were radioactively labeled with [32P]dATP using Klenow polymerase. Labeled, double-stranded oligonucleotides were purified by nondenaturing PAGE. Binding reactions were performed by incubating 10 μg of protein for 30 min at 4°C with 5 μg of dsDNA, 30,000 cpm of labeled probe were added per reaction mix. After an additional incubation for 15 min at RT, samples were loaded onto 4% nondenaturing PAGE. Complexes were revealed by autoradiography of the vacuum-dried gel for 6–16 h using a reflection screen. In competition experiments, a 200-fold excess of unlabeled double-stranded oligonucleotide was added to the binding reaction. Polyclonal Ab directed against either the fragment of goat anti-mouse IgG at a final concentration of 3.75 μg/ml in the dark for 30 min on ice. The cells were washed twice, covered with mounting fluid, and analyzed by fluorescence microscopy (Axiopt, Zeiss). Medium 199 containing 10 mM NaCl (Merck) was used as a diluent and washing solution during the experiment.

**Detection of C5b-9 complex formation**

Cells grown in 24-well plates were used for complement-activation experiments at 24 or 48 h postinfection. Infected cells were washed twice with medium 199, and 0.2 ml of medium 199 containing complement (1:5) or anti-dengue Ab (1:100) or both was added and incubated for 1 h at 37°C. In some experiments, 10 mM EDTA and 10 mM MgCl2 (Sigma) were used for inhibition of the classical pathway of complement activation. Heat-inactivated serum was used as control. Thereafter, cells were washed twice with PBS. In pilot experiments we used an indirect immunofluorescence assay to detect C5b-9 complexes on cell surfaces and propidium iodide exclusion to assess membrane integrity of the treated cells. Upon microscopic inspection, C5b-9, formed on intact membranes, could be detected by this technique but gave very low fluorescence signals. Therefore, we used a sensitive capture ELISA technique for determination of C5b-9 formation. In this assay, cells were again first treated with Ab and complement. After deposition of C5b-9 complexes on the cell surface, cells were lysed by applying 0.2 ml of lysing buffer (1% Triton X-100 (Roth) in PBS) for a few minutes. Lysates were clarified by centrifugation at 10,000 × g for 5 min to remove cell debris and frozen at −20°C until used. The actual assay for the detection of C5b-9 complexes was performed according to the protocol of Hugo et al. (18) except that swine anti-rabbit IgG conjugated with horseradish peroxidase (Dako, Glostrup, Denmark) at a final concentration of 0.24 μg/ml was used instead of the biotinylated anti-rabbit IgG in the last step and tetramethylbenzidine (Medgenix Diagnostics, Fleurus, Belgium) was used as a chromogen. The absorbency was read at 450 nm in an ELISA reader (EAR 400, SLT Labinstruments, Salzburg, Austria).

**Results**

**Infection of human endothelial cells by dengue virus**

DV-infected cells express viral Ags in their cytoplasm (19–21). Hyperimmune mouse asctic fluid was used to detect dengue viral proteins, and the number of infected ECV304 cells was quantitated using flow cytometry. The analysis of green fluorescence-positive cells was performed with 5000 events from each sample. About 63.5% of the cells at 24 h after infection at MOI of 0.1 expressed DV Ags and almost all cells were infected after 48 and 72 h of incubation (94 and 98.5%, respectively) (Fig. 1). Approximately 25% of untransformed EC were infected at 72 h as assessed by

**FIGURE 1.** Kinetics of infection of ECV304 cells by DV. DV-infected or control cells were harvested at 24, 48, and 72 h after infection. The cells were fixed, permeabilized and treated with dengue hyperimmune mouse ascitic fluid. After staining with fluorescent secondary Abs, the cells were analyzed by flow cytometry. A set of histograms derived from one representative out of three experiments is shown. The solid line indicates the result with uninfected cells. Similarly, controls with nonimmune asctic fluid yielded negative results with both infected and uninfected cells (not shown).
FACS or immunofluorescence microscopy (not shown). For DV-infected ECV304 cells, we also determined the release of infectious virions into culture supernatants by plaque-forming assays. The peak titer, obtained 2 days after infection of 2–3 $\times 10^5$ ECV cells at an MOI of 0.1 was 19 $\times 10^5$ PFU/ml (SD $\pm 2 \times 10^5$, $n = 4$).

Induction of RANTES and IL-8 in dengue-infected endothelial cells

To analyze cytokine production in response to DV, we collected supernatants of infected ECV304 cells after 24, 48, and 72 h from parallel cultures, and screened them by ELISA. No virus-mediated induction was found for MCP-1, IL-1β, IL-1α, IL-7, IL-15, and GM-CSF, and only minimal induction of TNF-α was noted (data not shown). In contrast, a marked time-dependent increase of RANTES and IL-8 became detectable in supernatants of DV-infected ECV304 cells (Fig. 2). Likewise, untransformed EC from umbilical vein or dermal microvascular EC, cultured in media with 2% FCS, produced significant amounts of RANTES only upon DV infection (Fig. 3). IL-8 levels were increased twofold in supernatants of HUVEC 96 h after infection as compared with controls (not shown). On a per infected cell basis, ECV304 cells produced roughly five times more RANTES than did primary cells.

Transcriptional up-regulation of RANTES and IL-8 during dengue infection of endothelial cells

RT-PCR for IL-8 and RANTES with total RNA from uninfected and control cells revealed increased steady state levels of specific mRNAs in infected cells (Fig. 4). We therefore investigated whether IL-8- and RANTES-mRNA accumulation could be attributed to transcriptional up-regulation of their promoters. Transient transfections with promoter/reporter hybrid constructs were performed. A 4.9-fold induction of transfected pIL-8($-420/+102$)LUC was observed 2 days after infection with DV of ECV304 cells and a 10- to 20-fold activation with pRANTES ($-935/+73$) (Fig. 5).

Chemokines in samples from DSS patients

The results of the in vitro assays prompted us to analyze PF and plasma samples of DSS patients for the presence of IL-8, MCP-1, and RANTES. ELISAs were performed twice on each sample with virtually identical results. As shown in Table I, IL-8 in plasma and PF of DSS patients were all markedly increased over the healthy donor controls; the difference exceeded 2 logs in some cases. Combined treatment of cell-rich plasma from healthy donors with insulin, 50 mg/ml at 37°C for 1 h (to activate complement), and 0.1% Triton X-100 for an additional 30 min at RT (to liberate any cell-bound IL-8) led to increased plasma IL-8 levels of up to 627 pg/ml. MCP-1 levels in the patients’ samples were also markedly elevated. Presumably due to the activation of platelets during sampling, RANTES was detectable in all plasma samples of DSS patients and healthy donors, with concentrations ranging between

![FIGURE 2. Selective production of chemokines by DV-infected ECV304 cells. Culture media were collected from untreated cells (med), from cells treated with heat-inactivated DV (intact.DV), or from DV-infected cells (DV) at 24, 48, and 72 h after infection, and assayed by ELISA. Results represent the accumulative amounts of IL-8 (A), RANTES (B), and MCP-1 (C) divided by the number of seeded cells. Data are displayed as the mean ± SD from three independent experiments.](image)

![FIGURE 3. DV infection induces RANTES in primary endothelial cells. Human umbilical vein endothelial cells or human dermal microvascular endothelial cells were cultured in media of low serum content in the presence of DV or of inactivated DV (CO). Supernatants were assayed for RANTES by ELISA. The data represent the mean ± SD from three independent experiments.](image)

![FIGURE 4. Selective induction of IL-8 and RANTES mRNA in DV-infected ECV304 cells. RT-PCR was performed on total RNA isolated 24 h after initiation of cultures from untreated cells ($\phi$), cells treated with inactivated DV (i.DV), or DV (DV). The constitutively expressed GAPDH gene served as a control. The same results were reproduced in two further experiments.](image)
DNA strand breaks. As a control, cells were treated with TNF- and the TUNEL method was next used to detect intrachromosomal endogenous endonucleases is another hallmark of apoptosis (22), they succumbed to programmed cell death. Nicking of the DNA by morphology of dying cells in DV-infected cultures suggested that approximately 50% ATP reduction after 48 h and to cell death of ECV304 after infection with DV

Said to be dependent on TNF- and for p65 was performed (Fig. 8). Nuclei of untreated cells are essentially negative for p65, some cytoplasmic staining is seen (Fig. 7A) or with anti-TNF Abs (Fig. 7B) contained less than 1% apoptic cells. Treatment with TNF-α plus cycloheximide (10 μg/ml) for 16 h caused apoptosis in about 5–10% of cells (Fig. 7C), and this effect was inhibited by anti-TNF Abs (Fig. 7D). DV infection led to apoptosis of 15–25% of the cells after 24 h (not shown) and 50–60% of the cells after 48 h (E), and anti-TNF Abs were unable to suppress this effect (Fig. 7F). Thus, apoptosis of DV-infected cells appeared not to be dependent on TNF-α secreted by ECV304. Apoptosis of hepatoma cells following DV infection has been reported recently (23).

NF-κB activation following DV infection of ECV304 cells

The crucial role of transcription factor NF-κB for the control of immune responses, including the production of chemokines, is well documented and NF-κB has recently also been recognized to be a central player in the regulation of cell death (24). We therefore investigated whether DV-infected ECV304 cells activate NF-κB. First, double-immunofluorescence staining for viral Ag and NF-κB p65 was performed (Fig. 8). Nuclei of untreated cells are essentially negative for p65, some cytoplasmic staining is seen (Fig. 8A). Positive staining with Ab to the viral Ag NS1 proved that virtually all cells were infected (Fig. 8B). Double staining for NS1 and for p65 revealed that p65 was translocated to the nuclei in some of the cells and to different degrees. Most of the cells exhibiting intense nuclear staining for NF-κB and complete translocation were characterized by a strong and focal perinuclear staining for NS1 and appeared condensed (Fig. 8C). Orange to yellow staining resulted from double exposure with the two fluorescent dyes. Thus, NF-κB p65 activation occurred in infected cells.

The time course of NF-κB activation was assessed by EMSA. (Fig. 9A); extracts from cultures infected for 24 and for 48 h showed increased binding activity, at least in part due to increased p65 binding. No induction was seen at 12 h or earlier. While competition with a 200-fold molar excess of unlabeled oligonucleotides showed that complexes with either extracts from uninfected or DV-infected cells were specific for the probe, immunosupershift experiments clearly demonstrated that binding of both NF-κB p65 and of p50 was markedly activated in virus-treated cells (Fig. 9B). C1 disappeared after preincubation with anti-p65 and thus probably represents a p65 homodimer while C2 was reduced after either preincubation with anti-p50 or anti-p65 and thus may be a p50/p65 heterodimer. Preincubation with both Abs (lane 6) led to a decrease of C2, SC1, and SC2, suggesting that these complexes comprise p50 and p65, the major activating form of NF-κB.

Table I. Chemokines in body fluids of DSS patients

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Pathogenesis of Dengue Shock Syndrome

FIGURE 5. Up-regulation of the IL-8 and the RANTES promoter in transiently transfected ECV304 cells following infection with DV. Promoter/reporter hybrid plasmids pIL8(-429/+102)LUC or pRANTES(-935/+73)LUC were transiently transfected into ECV304 cells using Lipofectin Life Technologies. Luciferase activity was measured in cell lysates from parallel cultures that were either treated with DV for 48 h (DV) or with PMA at 50 ng/ml for 16 h (PMA) as a positive control or left untreated (med.). The data shown represent the mean of three independent experiments ± SD, each done in triplicate, and are reported as the fold activation over the untreated control.

750 and 1500 pg/ml. Plasma RANTES determinations were considered uninformative. In contrast, RANTES concentrations in PF of DSS patients 1 through 6 were: 34, 1018, 530, 13, 12, and 60 pg/ml, respectively. Thus, the two samples with the highest amounts of IL-8 also displayed the highest levels of RANTES.

Cell death of ECV304 after infection with DV

On microscopic inspection of infected ECV304 or primary cells we regularly noted rounding, detachment, and nuclear condensation of many cells after 48–72 h. Measurements of intracellular ATP and propidium iodide-uptake were performed. DV led to approximately 50% ATP reduction after 48 h and to 73% reduction by 72 h postinfection (Fig. 6). The number of propidium iodide-positive cells increased from 7% at 24 h to almost 40% at 72 h. The morphology of dying cells in DV-infected cultures suggested that they succumbed to programmed cell death. Nicking of the DNA by endogenous endonucleases is another hallmark of apoptosis (22), and the TUNEL method was next used to detect intrachromosomal DNA strand breaks. As a control, cells were treated with TNF-α, and anti-TNF Abs were employed to detect cytokine-dependent apoptosis. In the experiment of Fig. 7, controls incubated without (Fig. 7A) or with anti-TNF Abs (Fig. 7B) contained less than 1% NF-κB p65

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The crucial role of transcription factor NF-κB for the control of immune responses, including the production of chemokines, is well documented and NF-κB has recently also been recognized to be a central player in the regulation of cell death (24). We therefore investigated whether DV-infected ECV304 cells activate NF-κB. First, double-immunofluorescence staining for viral Ag and NF-κB p65 was performed (Fig. 8). Nuclei of untreated cells are essentially negative for p65, some cytoplasmic staining is seen (Fig. 8A). Positive staining with Ab to the viral Ag NS1 proved that virtually all cells were infected (Fig. 8B). Double staining for NS1 and for p65 revealed that p65 was translocated to the nuclei in some of the cells and to different degrees. Most of the cells exhibiting intense nuclear staining for NF-κB and complete translocation were characterized by a strong and focal perinuclear staining for NS1 and appeared condensed (Fig. 8C). Orange to yellow staining resulted from double exposure with the two fluorescent dyes. Thus, NF-κB p65 activation occurred in infected cells.

The time course of NF-κB activation was assessed by EMSA. (Fig. 9A); extracts from cultures infected for 24 and for 48 h showed increased binding activity, at least in part due to increased p65 binding. No induction was seen at 12 h or earlier. While competition with a 200-fold molar excess of unlabeled oligonucleotides showed that complexes with either extracts from uninfected or DV-infected cells were specific for the probe, immunosupershift experiments clearly demonstrated that binding of both NF-κB p65 and of p50 was markedly activated in virus-treated cells (Fig. 9B). C1 disappeared after preincubation with anti-p65 and thus probably represents a p65 homodimer while C2 was reduced after either preincubation with anti-p50 or anti-p65 and thus may be a p50/p65 heterodimer. Preincubation with both Abs (lane 6) led to a decrease of C2, SC1, and SC2, suggesting that these complexes comprise p50 and p65, the major activating form of NF-κB.

FIGURE 6. DV infection causes cell death in ECV304 cells. At 24, 48, and 72 h after infection, ECV304 cells (pool of adherent cells and cells in culture fluid) were harvested and stained with propidium iodide prior to analysis by flow cytometry. No significant change in the number of propidium iodide-positive cells was observed in uninfected cultures (not shown). In parallel experiments, DV-infected ECV304 monolayers were analyzed for the level of intracellular ATP. The data shown are representative of two experiments showing the average of six measurements ± SD.
Dengue virus-infected endothelial cells are capable of activating complement

A marked reduction of complement proteins and a concomitant increase in complement fragments is observed in DHF/DSS, and the degree of complement activation correlates with the severity of the disease (5, 25, 26). Therefore, we investigated whether DV-infected EC activate complement in vitro. DV-infected ECV304 cells were incubated in the presence and absence of anti-dengue Abs with human complement for 1 h at 37°C. Ab-dependent deposition of C3dg fragments on the surface of infected cells was observed using a mAb specific for a neoantigen that is exposed in C3bi and C3dg (27) (Fig. 10A). This neoepitope is expressed only after cleavage of substrate-bound complement C3b by factor I, in the presence of factor H, upon binding to a complement-activating surface (28). Positive staining for C3dg was time dependent: staining was not observed when cells that had been infected for only 12 h were analyzed. The latter negative finding at the same time provided an important control. Deposition of C3dg fragments was also not seen when infected ECV304 cells were incubated with anti-dengue Abs and heat-inactivated nonimmune serum (not shown), or with nonimmune serum alone (Fig. 10B). Together, these results indicated that activation of complement by dengue-infected ECV304 cells is Ab dependent.

Activation of complement does not always lead to the generation of membrane attack complexes, C5b-9. Circulating SC5b-9 complexes have been detected particularly in patients with severe disease (P. Malasit, J. Mongkolsapaya, S. Nimmannitya, and S. Bhakdi, unpublished observations). Their origin is not known. Small surfaces, such as soluble immune complexes, are less efficient in terms of C5b-9 complex generation than large activator surfaces, e.g., cells (29). Therefore, in the next experiments, the formation of C5b-9 complexes on control or DV-infected ECV304 cells treated with anti-dengue Abs plus complement were analyzed. DV infection led to C5b-9 formation (Fig. 10C). Activation occurred via both classical and alternative pathways since adding EGTA-MgCl₂ only partially abolished the formation of C5b-9 complexes (data not shown).

Since the formation of C5b-9 complexes on the surface of cells may lead to cell lysis, measurements of cellular ATP were undertaken. No changes were observed after incubation of infected cells with anti-dengue Abs and complement (data not shown). This result indicated that no direct cytotoxic effect occurred after complement activation of dengue-infected ECV304 cells.

Discussion

Short-lived plasma leakage occurring selectively at serosal sites, and massive complement consumption are hallmarks of DSS. The objective of the present study has been to seek for possible explanations and causal links between these phenomena. As in all previous studies on DV pathogenesis, we were restricted to model studies in vitro; however, these led to a directed search and to the novel finding of high IL-8 levels in plasma and PF of DSS patients.

Based on the collective data, several pathways are envisaged that possibly converge to cause the massive but transient leakage syndrome. First is a selective action of cationic chemokines on the vascular endothelium in serosal tissues, which may be mediated via their interaction with heparan sulfate expressed at these sites.
A second mechanism is the activation of complement on the surface of infected cells, and the third is apoptotic cell death.

The cellular source of chemokine production remains an open question. In line with the classic Ab dependent enhancement concept, which envisages non-neutralizing Abs to augment infection of macrophages, these cells may provide a source of these chemokines. However, the present study raises another possibility, i.e., that EC may also be major producers of IL-8 and RANTES. This hypothesis derives from two findings: first, EC are effectively infected by DV, and second, DV-infected EC selectively up-regulate transcription and secretion of IL-8 and RANTES.

That EC can be infected by DV has been shown previously, and we confirm that the virus replicates to high titers in cultured EC, independent of the presence of enhancing Abs. The reason for differential susceptibility of ECV304 and HUVEC remains to be elucidated. Possibly ECV304 express a higher density of DV receptor. That DV-infected EC liberate large amounts of IL-8 and RANTES is a novel finding. Transcriptional up-regulation was demonstrated with the use of reporter gene constructs, and secreted proteins were quantified by ELISA. Quite remarkably, chemokine production occurred selectively, and there was no virus-mediated induction of MCP-1, IL-1β, IL-1α, IL-15, or GM-CSF. The selective induction of cytokine synthesis is now emerging as a common theme that possibly directs pathology in many diseases. The underlying mechanism in the case of DV will be the subject of future studies, but the very fact that the virus selectively induces endothelial production of IL-8 and RANTES harbors potentially significant consequences. It is known that IL-8 binds to heparan sulfate (30) and to EC of serosal tissues (31) that express this proteoglycan (32). It is also known that both IL-8 and RANTES increase vascular permeability via transient recruitment and local activation of neutrophils (33). That the chemokines are indeed produced in quantity during viral infection became evident from IL-8 measurements in plasma and pleural fluids from DSS patients. Very high levels of circulating IL-8 were detected in all cases.

**FIGURE 9.** A, Time course of DV-induced NF-κB activation. At various times after infection with DV, ECV304 cells were harvested and whole cell extracts were prepared. EMSA was performed with equal amounts of protein per lane. Note the increased binding at 24 and 48 h. The data on the right half of panel A were obtained with Ab against p65 in the binding reaction. B, Immunoblot analysis with unlabelled oligonucleotides. Lanes marked with a C contained binding reactions performed with extracts from control cells (treatment with inactivated DV), in lanes marked V, binding reactions with extracts from DV-infected cultures were analyzed. Extracts were obtained 48 h after initiation of cultures. SC1 and SC2 indicate supershifted complexes generated after preincubation of extracts with Abs against p65 or p50, respectively, from complexes C1 and C2. The experiments shown in both panels, A and B, were reproduced once.

**FIGURE 10.** Deposition of C3dg and C5b-9 on the surface of DV-infected cells. A, Detection of C3dg by immunofluorescence. DV-infected cells were incubated with pooled dengue hyperimmune sera plus complement, and C3dg was subsequently detected by the use of a mAb specific for a neoantigen. B, No fluorescence was detected in DV-infected cells treated with complement alone. Representative photomicrographs from one out of two experiments are shown in A and B. C, Detection of cell-bound C5b-9 by ELISA. DV-infected or control cells were incubated with anti-dengue Ab and complement, subsequently washed, and lysed with detergent. Cell lysates were subjected to ELISA for the detection of C5b-9 complexes. The ELISA signal obtained in lysates from controls (C) ranged at background levels whereas positive signals were observed in lysates of infected cells (DV). No C5b-9 complexes were detected when control or DV-infected ECV304 cells were incubated with complement without anti-dengue Abs, in the presence of EGTA/MgCl2, or with heat-inactivated complement (not shown). The data represent the average of four experiments ± SD.
These could not have derived from circulating Granulocytes, because maximal IL-8 levels in control blood samples were two orders of magnitude lower despite Granulocyte-stimulation via complement activation with inulin combined with cell lysis with Triton X-100 to liberate any nonsecreted IL-8. We believe it is reasonable to assume that the high chemokine levels in DSS patients reflected production in infected cells, with EC representing attractive candidates. While MCP-1 levels in supernatants of ECV304 cells were not induced by DV, all plasma and PF samples of DSS patients displayed elevated levels of this chemokine. Levels of MCP-1 in PF from various clinical conditions have previously been shown to correlate with the numbers of monocytes (34). Thus, monocytes might also be an important source of the chemokines in PF of DSS patients. Cytologic studies with PF from DSS patients are in progress now. Of note, high levels of IL-8 have been detected in patients with pleural effusion of other etiology, whereby the values measured in our study markedly exceed those previously published (34). The presence of large amounts of RANTES in two out of six PF from DSS patients complements the findings on IL-8.

Once infection of EC has occurred, cross-reacting, non-neutralizing Abs to DV will activate complement on these cells. This provides a second possible mechanism leading to vascular leakage. Complement attack on virus-infected cells is, of course, not conceptually novel, but its possible involvement in DSS has not received much consideration hitherto. In an early hypothesis, one of us pointed out that such auto-attack would result in local liberation of anaphylatoxins and the generation of C5b-9 complexes (10). Both processes could contribute to disease. Here, the Ab-dependent deposition of activated C3 and C5b-9 on DV-infected cells was demonstrated. Our results indicate that the terminal complexes were not directly cytoidal, and it is known that subcytoidal C5b-9 attack increases vascular permeability of endothelial cell monolayers (35). C5b-9 generation can only occur in conjunction with the liberation of anaphylatoxins. In this context, high levels of C3a and C5a have indeed been detected in PF of DSS patients (P. Malasit, unpublished observation). C3a induces histamine release from mast cells, and thus would indirectly enhance vascular permeability. It is of interest that high histamine concentrations have actually been measured in urine samples of DSS patients (36). In addition to activating phagocytes, C5a induces shedding of heparan sulfate from EC (37). In the context of DV pathogenesis, this may be important because, if chemokines are targeted to serosal EC via binding to heparan sulfate, C5a-induced shedding might represent a counteracting factor. Furthermore, if heparan sulfate represents a binding site for DV on EC, shedding might also serve to limit infection.

The third putative pathway to vascular leakage could derive from apoptosis of infected cells. Massive reduction in cellular ATP, accompanied by positive TUNEL stainings for nicked DNA occurred after 3–4 days of infection. Apoptosis of DV-infected EC would acutely enhance local vascular leakage. Furthermore, apoptosis would be followed by rapid removal of infected cells, which would explain the difficulties in detecting the infectious agent in tissue specimens. Apoptosis of infected EC and complement-induced shedding of the viral receptor heparan sulfate from bystander cells would also nicely explain the abrupt termination of infection.

In summary, there are three major findings in this study that can be accommodated in a coherent hypothesis of the DSS leakage syndrome. First, EC are highly permissive to DV infection and respond by the selective production of chemokines IL-8 and RANTES. These may accumulate at serosal sites, causing local vascular leakage. Second, cross-reacting but non-neutralizing Abs to DV will activate complement on the surface of infected EC, causing liberation of anaphylatoxins and deposition of C5b-9. Of these, C3a may mediate histamine release, and C5a will induce shedding of heparan sulfate. The latter might counteract infection and reduce chemokine binding. Third, infected EC eventually undergo apoptosis. This would augment vascular leakage, and also provide a mechanism for the sudden disappearance of the infectious agent, explaining the short-lived duration of disease and the eradication of clues to the nature of the infected cells in vivo.

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