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Dengue Virus Nonstructural Protein NS5 Induces Interleukin-8 Transcription and Secretion

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Elevated circulating levels of chemokines have been reported in patients with dengue fever and are proposed to contribute to the pathogenesis of dengue disease. To establish in vitro models for chemokine induction by dengue 2 virus (DEN2V), we studied a variety of human cell lines and primary cells. DEN2V infection of HepG2 and primary dendritic cells induced the production of interleukin-8 (IL-8), RANTES, MIP-1 α , and MIP-1 β , whereas only IL-8 and RANTES were induced following dengue virus infection of HEK293 cells. Chemokine secretion was accompanied by an increase in steady-state mRNA levels. No chemokine induction was observed in HEK293 cells treated with poly(I:C) or alpha interferon, suggesting a direct effect of virus infection. To determine the mechanism(s) involved in the induction of chemokine production by DEN2V, individual dengue virus genes were cloned into plasmids and expressed in HEK293 cells. Transfection of a plasmid expressing NS5 or a dengue virus replicon induced IL-8 gene expression and secretion. RANTES expression was not induced under these conditions, however. Reporter assays showed that IL-8 induction by NS5 was principally through CAAT/enhancer binding protein, whereas DEN2V infection also induced NF- κ B. These results indicate a role for the dengue virus NS5 protein in the induction of IL-8 by DEN2V infection. Recruitment and activation of potential target cells to sites of DEN2V replication by virus-induced chemokine production may contribute to viral replication as well as to the inflammatory components of dengue virus disease.

Dengue virus is a member of the family *Flaviviridae* and has a single-stranded RNA genome of positive-strand polarity. Dengue virus RNA has a type I cap at the 5' terminus and a single open reading frame flanked by untranslated regions. The open reading frame encodes a polyprotein precursor which is co- and posttranslationally processed into three structural proteins (C, prM, and E) and at least seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (39).

The four serotypes of dengue virus (1, 2, 3, and 4) are transmitted to humans by *Aedes aegypti* mosquitoes and cause dengue, an important viral disease in tropical countries. The spectrum of dengue illness ranges from a flu-like disease termed dengue fever to dengue hemorrhagic fever, a fulminant illness that can progress to dengue shock syndrome and death. The clinical features of severe dengue disease include hemorrhagic diathesis, liver involvement, and plasma leakage, the latter being the major determinant of disease severity. Cytokine production and T cell activation appear to be important in dengue hemorrhagic fever pathogenesis (60). Accumulating evidence indicates that intrinsic properties of the infecting strain of dengue virus can contribute to the severity of disease (8, 37, 56, 57).

Chemokines are a family of small, basic, structurally related chemoattractant cytokines that are expressed upon activation by various cell types, including T cells, monocytes, and endothelial cells. Chemokines promote the release of granule proteins by granulocytes, promote Th1- or Th2-dependent im-

mune responses, and activate immune cells, including T cells, NK cells, and monocytes (59). Chemokines have been shown to play an important role in viral pathogenesis and immunity. Viruses have found many ways to subvert the chemokine system, including virally encoded chemokine/chemokine receptors, altering the expression of chemokine/chemokine receptors, and blocking chemokine receptor signaling pathways (43, 45). On the other hand, viruses can exploit the chemokine system to enhance viral replication and dissemination of the virus into neighboring cells (21, 30, 69).

In vitro infection of human myeloid or endothelial cells with dengue virus has been reported to induce secretion of various chemokines, including MIP-1 α , MIP-1 β , and interleukin-8 (IL-8) (1, 3, 5, 32, 42, 62). Patients with acute dengue were reported to have elevated levels of chemokines in blood or pleural fluid (55) and chemokine gene expression in peripheral blood mononuclear cells (PBMC) (62). These have been thought to contribute to inflammation and disease pathogenesis, however, the mechanism of chemokine induction by dengue virus has not been defined.

To study chemokine induction by dengue virus proteins, we transfected susceptible cells with vectors expressing dengue 2 virus (DEN2V) genes. We found that IL-8 secretion and transcription could be induced by expression of the DEN2V NS5 protein. The effect of the NS5 protein was due to activation of CAAT/enhancer binding protein (c/EBP) activity and, to a lesser extent, NF- κ B activity and activating protein 1 (AP-1). These results suggest a novel mechanism by which DEN2V induces chemokine production from infected cells.

MATERIALS AND METHODS

Cells and cell lines. HepG2 (American Type Culture Collection), a human hepatocarcinoma cell line, was maintained in minimal essential medium supplemented with 10% fetal bovine serum, 1.0 mM sodium pyruvate, 0.1 mM nones-

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sential amino acids, penicillin (100 U/ml), and streptomycin (100 µg/ml). HEK293 (American Type Culture Collection) and HEK293A (Invitrogen) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, penicillin (100 U/ml), and streptomycin (100 µg/ml).

Dendritic cells. PBMC were obtained by centrifugation using Accuspin tubes (Sigma). Monocytes were isolated by positive selection using magnetic cell sorting (MACS) according to the manufacturer's protocol (Miltenyi Biotec). The CD14 positive cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, rGM-CSF (800u/ml) and rIL-4 (500u/ml) for 5 to 7 days, changing half the media and adding cytokines every other day. Dendritic cells (DC) were stained to assess purity with lineage markers (CD3, CD14, CD16, CD56, and CD20) (fluorescein isothiocyanate), CD1a (phycoerythrin), and HLA-DR (peridinin chlorophyll protein) and analyzed by flow cytometry. Dendritic cells were >85% pure.

Antibodies and cytokines. Fluorescein isothiocyanate-conjugated dengue virus complex-specific monoclonal antibody (clone M8051125) was obtained from Fitzgerald Industries International, Inc. DEN2V-specific monoclonal antibody was derived from a hybridoma cell line, 3H5-1 (American Type Culture Collection).

Infection with dengue virus. Cells were infected with DEN2V virus strain New Guinea C strain as previously described (35) at a multiplicity of infection of 1 for 2 h at 37°C. Cells were stained for dengue virus antigen by indirect immunofluorescence on various days postinfection, using 3H5 as the primary antibody and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G antibody (Sigma Chemical Co., St. Louis, MO) as the secondary antibody. For experiments using direct immunofluorescence, dengue virus complex-fluorescein isothiocyanate conjugate antibody was used. The percentage of infected cells was assessed using flow cytometry.

PBMC samples from patients with acute dengue virus infections. Serial PBMC samples were studied from 26 children who participated in a prospective study of acute dengue virus infection (16 with dengue fever and 10 with dengue hemorrhagic fever) in Thailand (27). Study procedures complied with all relevant federal guidelines and institutional policies. Patients were enrolled in the study if they presented within 72 h of the onset of fever. Blood samples were collected daily until 1 day after defervescence. Convalescent samples were taken 8 to 10 days after enrollment. Fever days were assigned retrospectively as described, where fever day 0 refers to the day of defervescence. As described previously, PBMC (approximately 2×10^6 cells) isolated from whole blood using Histopaque (Sigma) were resuspended in 1 ml of buffer (4 M guanidine isothiocyanate, 40 mM Tris-HCl, pH 6.4, 17 mM EDTA, pH 8.0, 1% Triton X-100), frozen at -80°C, and shipped on dry ice. Total RNA was prepared using the RNeasy kit (QIAGEN, Valencia, CA) substituting this lysing buffer for buffer RLT (62).

Virus inactivation. DEN2V was inactivated by UV light. The UV light source was a Phillips TUV 30W G30T8 UV-C light bulb. The virus was exposed at a distance of 15 cm for 30 min at room temperature. In addition, DEN2V was heat inactivated at 56°C for 30 min.

RNA isolation. Total RNA was prepared using the RNeasy kit (QIAGEN) following the manufacturer's instructions. For reverse transcription (RT)-PCR, RNA was treated with DNase according to the manufacturer's protocol (QIAGEN). Viral RNA was isolated from supernatants of DEN2V-infected cells using the QIAmp viral RNA kit (QIAGEN) following the manufacturer's protocol.

RNase protection assay. The human chemokine multiprobe template set (hCK-5) was used to make the radiolabeled probe according to the manufacturer's protocol (Riboquant, Pharmingen). The RNase protection assay was performed according to the manufacturer's protocol (Torrey Pines Biolabs, Inc.). Briefly, equal amounts of total cellular RNA were hybridized to ³²P-labeled riboprobe cocktail hCK-5. The hybridized RNA was digested by RNase A. The precipitated RNA was electrophoresed on a denaturing acrylamide gel and the bands were detected by autoradiography. The specific chemokine bands were identified on the basis of their individual migration patterns in comparison with the undigested probes. The bands were quantified by densitometric analysis using Image Quant densitometric software. Glyceraldehyde-3-phosphate dehydrogenase and L32 were used as internal controls.

ELISA. MIP-1α, MIP-1β, MCP-1, IL-8, and RANTES protein concentrations were determined in cell culture supernatants using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D Systems).

Quantitative RT-PCR. For quantitation of IL-8 mRNA, 200 nanograms of total cellular RNA was used to synthesize cDNA using Omniscript reverse transcriptase and oligo(dT) primers following the manufacturer's protocol (QIAGEN). PCR was performed in triplicate using TaqMan IL-8 and β-actin primers

and probes (Applied Biosystems) and the Gene Amp 5700 Sequence Detection System. For quantitation of mRNA, a standard curve of control DNA was generated and samples were normalized to the endogenous β-actin control. The ratios of infected or transfected cells versus uninfected or untransfected cells, respectively, were calculated.

For DEN2V quantitation from supernatants of virally infected cells, cDNA was synthesized from isolated viral RNA using Multiscribe reverse transcriptase (Applied Biosystems) and the reverse DEN2V primer following the manufacturer's instructions. PCR was performed using the Taqman Universal PCR master mix and DEN2V forward and reverse primers and a DEN2V-specific probe and the GeneAmp 7300 sequence detection system (Applied Biosystems) as described previously (20). Quantitation of viral RNA was performed using a standard curve of DEN2V viral RNA to determine copies of viral genome in supernatants of virally infected cells.

Plasmids. The replicon which contains the DEN2V NGC genome with the prM and E genes deleted was kindly provided by Andrew Dayton (52). Four luciferase reporter constructs containing the IL-8 promoter were used; one contained the wild-type binding sites for NF-κB, AP-1, and c/EBP, and the other three contained mutant binding sites for NF-κB (GGAATTCCT to TAACTT TCCT from -80 to -71), AP-1 (TGACTCA to TATCTCA from -126 to -120), or c/EBP (CAGTTGCAAATCGT to AGCTTGCAAATCGT from -94 to -81); all were generously provided by Naofumi Mukaida (Kanazawa University, Japan) (50). In addition, luciferase reporter constructs containing tandem repeat binding sites for NF-κB, AP-1, and c/EBP were used (Stratagene). Plasmids expressing signaling molecules Mal, MEK1, or TBK1 and the interferon-stimulated regulatory element (ISRE) of the *IFIT2* gene encoding ISG54 were previously described (10, 11). The pcDNA3.1 plasmid was obtained from Invitrogen.

Primers were designed for PCR amplification and cloning of each of the DEN2V genes (Table 1). Each 5' and 3' primer contained *attB1* and *attB2* sequences, respectively, for homologous recombination into the Gateway entry vector pDONR201 (Invitrogen). The prM, E, and NS1 forward primers included the putative leader sequences (9, 15, 22, 46).

PCR was performed using rTth DNA polymerase (Applied Biosystems) and DEN2V infectious clone, which was created using DEN2V NGC as the template (kindly provided by Barry Falgout, Food and Drug Administration) (53). The PCR product was cloned into pDONR201 by homologous recombination using Gateway Technology following the manufacturer's instructions. Mammalian expression vectors were made by homologous recombination of each dengue virus gene into the pDEST40 vector or pDEST47 as described in the Gateway Technology manual (Invitrogen). The identities of the clones were confirmed by DNA sequencing. All plasmids were isolated with an Endofree maxiplus kit (QIAGEN).

Transfections. HEK293A cells were transfected using Effectene (QIAGEN) following the manufacturer's instructions. Briefly, HEK293A cells were seeded onto six-well plates at 2×10^5 cells per well 24 h before transfection. To transfect, 0.6 µg of dengue virus protein-expressing plasmid and 0.2 µg of *lacZ*-expressing plasmid were diluted in EC buffer (QIAGEN) for each condition. In addition, 6.4 µl of Enhancer (QIAGEN) was added to the mixture and incubated for 2 min at room temperature. The mixture was spun down; 8 µl of Effectene (QIAGEN) was added and incubated for 5 min at room temperature. Growth medium was added to the mixture and the complex was added to the cells. Transfection efficiency was approximately 30% as assessed by β-galactosidase staining following the manufacturer's protocol (Invitrogen).

Luciferase reporter gene assay. HEK293A cells maintained in Dulbecco's modified Eagle's medium-10% fetal bovine serum at 37°C were plated in a 96-well plate at 2×10^4 cells/ml. After 24 h, the cells were transfected with reporter plasmids and/or expression plasmids using Genejuice according to the manufacturer's protocol (Novagen). Twenty-four hours (for plasmids) and 72 or 96 h (for virus) later, cells transfected with expression plasmids were lysed, and the luciferase activity was determined. A *Renilla reniformis* luciferase reporter under the control of the herpes simplex virus thymidine kinase promoter, pRL-TK, was used as an internal control to normalize reporter gene activity. Luciferase activities were determined by a luminometer using the dual-luciferase reporter assay according to the instructions of the manufacturer (Promega Co.). All conditions were tested in triplicate. At least two independent experiments were performed for each assay.

RESULTS

Induction of chemokine secretion and gene expression by DEN2V. It is unclear which cells are primary targets of dengue virus infection in vivo. In vivo studies suggest monocytes and

TABLE 1. Primers used for cloning of dengue virus genes

| Primer ^a | Sequence (5' → 3') ^b |
|---------------------|--|
| C (forward) | GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGAATAACCAACGAAAAAGGCGAG |
| C (reverse) | GGGGACCACTTTGTACAAGAAAGCTGGGTCCGCCATCACTGTTGGAATCAGC |
| prM (forward) | GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGAGAACTGCAGGCATGATCATTATGCTGATTC |
| prM (reverse) | GGGGACCACTTTGTACAAGAAAGCTGGGTCCATGTGTCATTGAAGGAGCGACAGCTG |
| E (forward) | GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGAGAGCCCTGATTTTCATCTTACTGACAG |
| E (reverse) | GGGGACCACTTTGTACAAGAAAGCTGGGTCC <u>TAGGCCTGCACCATAACTCCCAAATAC</u> |
| NS1 (forward) | GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGAATTCACGCAGCACCTCACTG |
| NS1 (reverse) | GGGGACCACTTTGTACAAGAAAGCTGGGTCCGGCTGTGACCAAGGAGTTGAC |
| NS2A (forward) | GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGGACATGGGCAGATTGACAACCTTTTCA |
| NS2A (reverse) | GGGGACCACTTTGTACAAGAAAGCTGGGTCCCTTTTCTTGTGGTCTTGAAGGGTTG |
| NS2B (forward) | GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGAGCTGGCCACTAATGAGGCTATCA |
| NS2B (reverse) | GGGGACCACTTTGTACAAGAAAGCTGGGTCCCGTTGTTTCTTCACTTCCACAG |
| NS3 (forward) | GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGGCTGGGAGGGGCATCATGAAAA |
| NS3 (reverse) | GGGGACCACTTTGTACAAGAAAGCTGGGTCCCTTCTCCAGCTGCAAATCCTTGAATT |
| NS4A (forward) | GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGTCCCTGACCCTGAACCTAATCACA |
| NS4A (reverse) | GGGGACCACTTTGTACAAGAAAGCTGGGTCTCTTTTCTGAGCTTCTCTGGTTGCTTTTG |
| NS4B (forward) | GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGGACATGGGCAGGGGCATCATGAAAA |
| NS4B (reverse) | GGGGACCACTTTGTACAAGAAAGCTGGGTCCCTTCTCGTGTGGTGTGTTCTTTCAT |
| NS5 (forward) | GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGGAACTGGCAACATAGGAGAGAC |
| NS5 (reverse) | GGGGACCACTTTGTACAAGAAAGCTGGGTCCACAGGACTCCTGCCTCTT |

^a Primer names indicate the gene amplified for cloning.

^b Bold sequence is the dengue virus sequence, and underlined sequences are added stop codons.

lymphocytes are important targets, but these cell types are not highly susceptible to dengue virus infection in vitro (24, 64, 70). Dengue virus antigen has also been found in hepatocytes and endothelium from biopsies of dengue hemorrhagic fever patients (6, 16, 24, 58). In vitro, dengue virus can productively infect many cell types. To find a cell line(s) that would facilitate in vitro studies of dengue virus-induced chemokine induction, we assessed chemokine induction by DEN2V infection in various cell lines, including myelomonocytic cell lines U937, K562, and Thp-1, hepatoma cell line HepG2, endothelial cell line ECV304, and embryonic kidney cell line HEK293.

Of the cell lines tested, HepG2 and HEK293 cells were most susceptible to DEN2V infection and both produced chemokines in response to DEN2V infection (Table 2). DEN2V-infected HepG2 cells secreted IL-8, RANTES, MIP-1 α , and MIP-1 β . Only IL-8 and RANTES were expressed in DEN2V-infected HEK293 cells, indicating a unique chemokine profile for each cell type. The secretion level of chemokines in both cell lines peaked late in infection, between days 3 and 5. In comparison, DEN2V RNA levels in culture supernatants of

HepG2 and HEK293 cells reached maximum values (~10⁷ genome copies/ml) by 48 h postinfection (data not shown).

To assess whether protein expression of each chemokine is regulated at the transcriptional level or during translation, chemokine mRNA levels in HepG2 cells were analyzed by RNase protection assay (Fig. 1). DEN2V infection induced the expression of IL-8, RANTES, MIP-1 α , MIP-1 β , and IP10 mRNAs. The level of mRNA peaked between days 3 through 5, which was similar to the kinetics of protein expression.

Chemokine production by dengue virus in primary human cells infected in vitro and by PBMC in vivo. Chemokine induction has been shown in primary monocytes following dengue virus infection (3, 5). Since dendritic cells have also been proposed as an important target for dengue virus infection in vivo, we isolated monocytes from PBMC and cultured them for 7 days with IL-4 and granulocyte-macrophage colony-stimulating factor to generate myeloid dendritic cells (17, 70). The dendritic cells were infected with DEN2V at a multiplicity of infection of 1. Culture supernatants were collected 24 h postinfection and chemokine levels were determined by ELISA.

TABLE 2. Chemokine production from DEN2V-infected HepG2 and HEK293 cell lines^a

| Cells | Chemokine | Production (pg/ml) | | | | | | | | | |
|--------|---------------------|--------------------|--------|-------|--------|-------|--------|-------|--------|-----------------|--------|
| | | Day 1 | | Day 2 | | Day 3 | | Day 4 | | Day 5 | |
| | | DEN2V | Medium | DEN2V | Medium | DEN2V | Medium | DEN2V | Medium | DEN2V | Medium |
| HepG2 | IL-8 ^b | 11 | 3 | 44 | 0 | 97 | 0 | 164 | 5 | 168 | 0 |
| | RANTES ^b | 4 | 2 | 73 | 0 | 427 | 0 | 1,350 | 1 | 1,721 | 3 |
| | MIP-1 α | 48 | 4 | 480 | 5 | 873 | 5 | 819 | 7 | 669 | 5 |
| | MIP-1 β | 6 | 8 | 22 | 7 | 67 | 6 | 284 | 9 | 623 | 8 |
| HEK293 | IL-8 ^b | 0 | 0 | 0 | 0 | 18 | 0 | 69 | 0 | 138 | 0 |
| | RANTES | 0 | 2 | 6 | 1 | 64 | 1 | 110 | 0 | ND ^c | ND |

^a Cell lines were infected with DEN2V NGC at a multiplicity of 1. Both HEK293 and HepG2 cell lines were infected 25 to 30% with DEN2V NGC by day 1 as assessed by flow cytometry (data not shown).

^b Results are representative of three experiments.

^c ND, not determined.

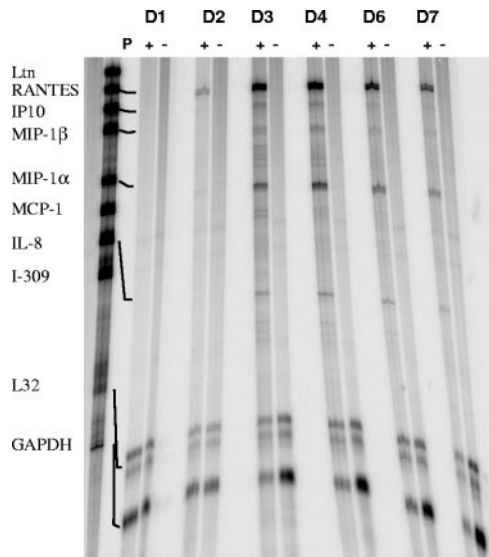


FIG. 1. Chemokine mRNA expression from HepG2 cell line infected with DEN2V. HepG2 cells were infected with DEN2V at a multiplicity of infection of 1. Total RNA was isolated for each time point after viral infection. Detection and quantification of the indicated human chemokine mRNAs were analyzed by RNase protection assay as outlined in Materials and Methods. P, probe lane; D, day; +, infection with DEN2V; and -, mock infection.

IL-8, RANTES, MIP-1 α , MIP-1 β , and MCP-1 levels were significantly higher in cultures of infected dendritic cells (Table 3). The results indicate that chemokine induction by DEN2V is also detected in primary cells, which are relevant to in vivo infection.

To determine whether IL-8 gene expression also occurs in vivo during dengue virus infections, we analyzed IL-8 mRNA levels in PBMC collected from 26 subjects with acute dengue virus infections using quantitative RT-PCR. All subjects had dengue virus viremia detected by RT-PCR at the time of entry into the study (63). IL-8 mRNA levels were numerically expressed as ΔC_t values relative to β -actin mRNA levels. As shown in Fig. 2, IL-8 mRNA was detected in PBMC from all subjects tested. Mean IL-8 mRNA levels were highest later in infection, towards the end of the febrile period (fever days -1 to 0), especially in subjects with dengue hemorrhagic fever.

IL-8 induction requires replication-competent virus and is not due to IFN- α . To assess whether the IL-8 expression in HepG2 cells was due to DEN2V antigen or replicating virus, DEN2V was heat inactivated or UV inactivated. The virus was added to HepG2 cells at a multiplicity of infection of 1 for 2 h at 37°C and supernatants were collected daily for analysis of

TABLE 3. DEN2V induces chemokines in dendritic cells

| Chemokine | Production (pg/ml) | | |
|----------------|--------------------|-------|--------|
| | DEN2V | C6/36 | Medium |
| IL-8 | 17,600 | 220 | 400 |
| RANTES | 12,700 | 11 | 6 |
| MCP-1 | 2,100 | 440 | 520 |
| MIP-1 α | 8,980 | 70 | 70 |
| MIP-1 β | 17,300 | 300 | 180 |

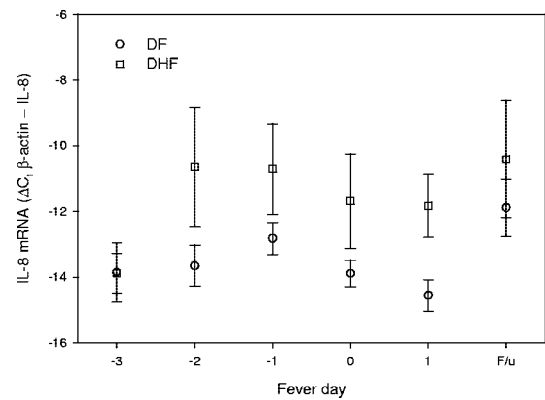


FIG. 2. IL-8 gene expression in PBMC of patients with acute dengue virus infection. Total RNA was isolated from serial PBMC samples obtained from 16 subjects with dengue fever (DF) and 10 subjects with dengue hemorrhagic fever (DHF). IL-8 and β -actin mRNA levels were measured by quantitative RT-PCR using TaqMan primers and probes. IL-8 mRNA levels are expressed as the difference in C_t between β -actin and IL-8. Values represent the mean \pm standard error of the mean. F/u, follow-up visit, approximately 1 week after defervescence.

IL-8 expression by ELISA. Viral progeny was measured in the supernatants of HepG2 cells. Supernatants from DEN2V-infected HepG2 cells contained approximately 10^6 PFU/ml by day 3, whereas infectious DEN2V was not detected in the supernatants from HepG2 cells treated with UV or heat inactivated virus (data not shown). As seen in Fig. 3, IL-8 secretion was induced only in cells infected with live DEN2V.

We considered the possibility that induction of IL-8 in cells infected with DEN2V was due directly or indirectly through induction of alpha interferon (IFN- α) production. Neither poly(I:C) nor IFN- α treatment of cells induced IL-8 expression. However, phorbol myristate acetate and ionomycin induced IL-8 secretion by 3 h poststimulation in HepG2 cells and by 8 h in HEK293 cells (data not shown).

Expression of NS5 is sufficient to induce expression of IL-8 but not RANTES. To define further the mechanism for induction of chemokine production by DEN2V infection, we studied the effects of expression of individual DEN2V proteins or a DEN2V replicon. The plasmids were transfected into HEK293A cells and supernatants were collected on day 2. As shown in Fig. 4A, the replicon induced IL-8 protein expression sixfold over the *lacZ* control plasmid. NS5 also induced expres-

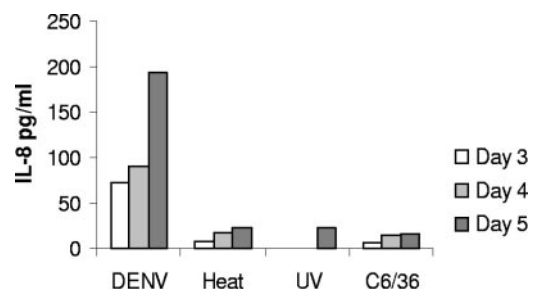


FIG. 3. IL-8 expression in HepG2 cells requires replication competent DEN2V. UV- and heat-inactivated virus, untreated virus, and C6/36 supernatants were added to HepG2 cells for 5 days. Supernatants were analyzed for IL-8 protein by ELISA.

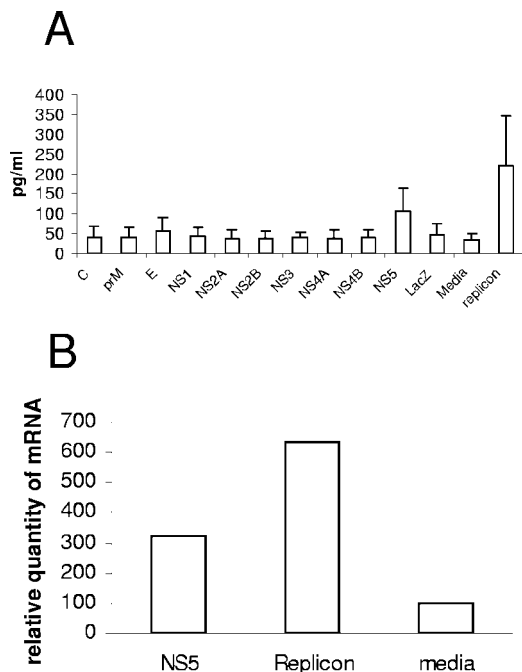


FIG. 4. NS5 and DEN2V replicon induce IL-8 expression from HEK293A cells. (A) Plasmids expressing DEN2V proteins or replicon were transfected into HEK293A cells. Supernatants were harvested day 2 posttransfection and analyzed for IL-8 protein by ELISA. (B) IL-8 mRNA levels from HEK293A cells transfected with NS5 plasmid, replicon, or untransfected cells were assessed by Taqman PCR on day 2 posttransfection. Results are normalized to untransfected cells (= 100).

sion of IL-8, with levels threefold over the *lacZ* control plasmid. However, there was no RANTES secretion in cells transfected with either NS5 or the replicon (data not shown). The levels of IL-8 mRNA expression measured by RT-PCR correlated with IL-8 protein secretion from cells transfected with the plasmid expressing NS5 or the replicon (Fig. 4B). These results show that IL-8 expression can be induced by the NS5 protein.

NS5 and replicon do not activate ISRE or RANTES. The RANTES promoter contains binding elements for transcription factors NF- κ B, and interferon-regulated factor 3 (IRF-3) (13, 41). IRF-3 has been shown to bind to the ISRE domain in the RANTES promoter to induce RANTES expression during viral infection (41). We wanted to analyze the ability of NS5, replicon, and DEN2V to activate the interferon-stimulated regulatory element (ISRE) as well as RANTES and IL-8 promoters (Fig. 5). The IKK-related kinase TBK1 has recently been shown to phosphorylate and activate IRF-3; overexpression of TBK1 induced transcription from both the ISRE and RANTES promoters (positive control), consistent with published reports (10, 61). Similarly, overexpression of Mal, the TLR4 adaptor molecule, induced transcription from the IL-8 promoter (positive control), as previously reported (11, 14). Neither NS5 nor the replicon activated the ISRE reporter or induced the RANTES promoter, whereas DEN2V infection activated ISRE 13-fold and RANTES fivefold 72 h postinfection.

Transcription factors induced by NS5 and DEN2V. The transcription factors involved in IL-8 mRNA induction include

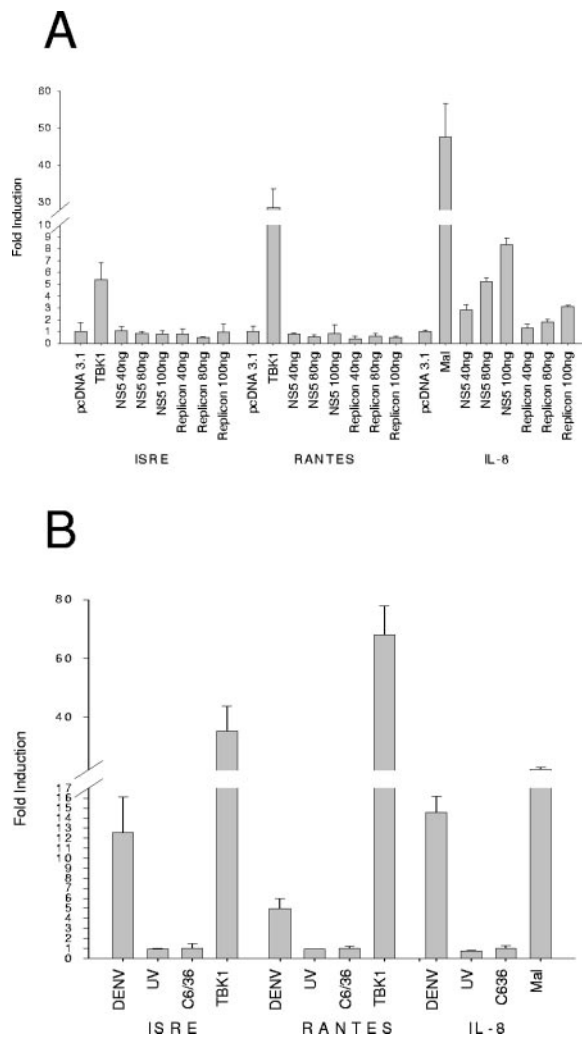


FIG. 5. NS5 and DEN2V replicon do not activate the ISRE or RANTES promoter. HEK293A cells were transfected with a vector containing a reporter gene for ISRE, RANTES, or IL-8 and (A) co-transfected with an expression vector for NS5 or replicon or (B) infected 24 h later with DEN2V. After 24 h (NS5 and replicon) or 72 h (DEN2V), luciferase reporter gene activity was measured and data were normalized to transfection efficiency with *Renilla reniformis* luciferase.

c/EBP, AP-1, and NF- κ B (19). To identify the transcription factors that are induced by NS5 and DEN2V, we used promoters that contain multiple copies of binding sites for c/EBP, AP-1, or NF- κ B. Mal or MEKK1 (mitogen-activated protein kinase kinase), as positive controls, induced transcription from the NF- κ B and AP-1 reporter constructs, as previously reported (7, 11). NS5 induced c/EBP-driven transcription sevenfold, AP-1 fivefold, and NF- κ B less than twofold over the control plasmid (Fig. 6A). DEN2V infection induced c/EBP 1.5-fold, AP-1 twofold, and NF- κ B threefold over the control (Fig. 6B). These results suggest activation of c/EBP, AP-1, and NF- κ B by DEN2V infection and NS5 expression.

To further assess the transcription factors important for induction of IL-8, IL-8 reporter constructs containing mutations in the binding site for c/EBP (mtc/EBP), AP-1 (mtAP-1), or NF- κ B (mtNF- κ B) were tested (50). As shown in Fig. 7A, a

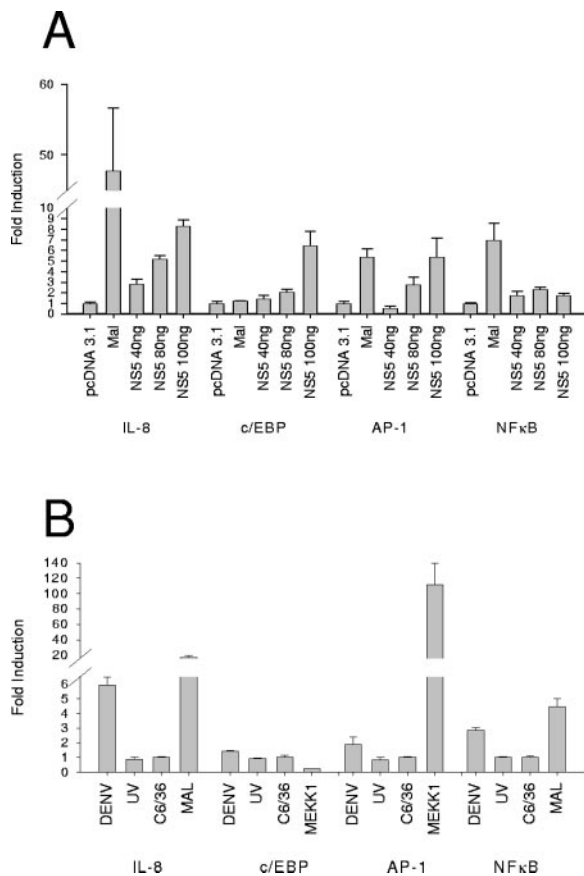


FIG. 6. Transcription factors activated by NS5 and DEN2V. HEK293A cells were cotransfected with vector encoding a reporter gene for IL-8, c/EBP, AP-1, or NF- κ B and (A) an expression vector for NS5 or (B) infected after 24 h with DEN2V. After 24 h (NS5) or 96 h (DEN2V), luciferase gene activity was measured and data are normalized to transfection efficiency with *R. reniformis* luciferase. There is a statistically significant difference ($P < 0.01$) between DEN2V- and C6/36-treated cells for the IL-8, c/EBP, AP-1, and NF- κ B reporters.

mutation in the c/EBP binding site within the IL-8 promoter reduced activation by NS5 twofold compared with the wild-type IL-8 promoter. A mutation in the NF- κ B binding site had a modest effect on IL-8 transcription (1.5-fold reduction) compared to the wild-type IL-8 promoter. However, a mutation in the AP-1 binding site enhanced NS5-driven transcription from the IL-8 promoter by almost twofold. These results suggest that c/EBP is a dominant component in the induction of the IL-8 promoter by NS5. In contrast, AP-1 did not have a predominant effect on IL-8 induction by NS5 and may even be inhibitory.

Transcription from the mtc/EBP, mtAP-1, and mtNF- κ B IL-8 promoters was reduced twofold compared to the wild-type IL-8 promoter by day 4 postinfection with DEN2V (Fig. 7B). These results suggest that c/EBP, AP-1, and, in addition, NF- κ B are important for IL-8 induction during DEN2V infection. This suggests an additional mechanism for IL-8 induction by DEN2V infection that includes NF- κ B activation.

DISCUSSION

Our results demonstrate that DEN2V infection of diverse human cell lines can induce the production of multiple che-

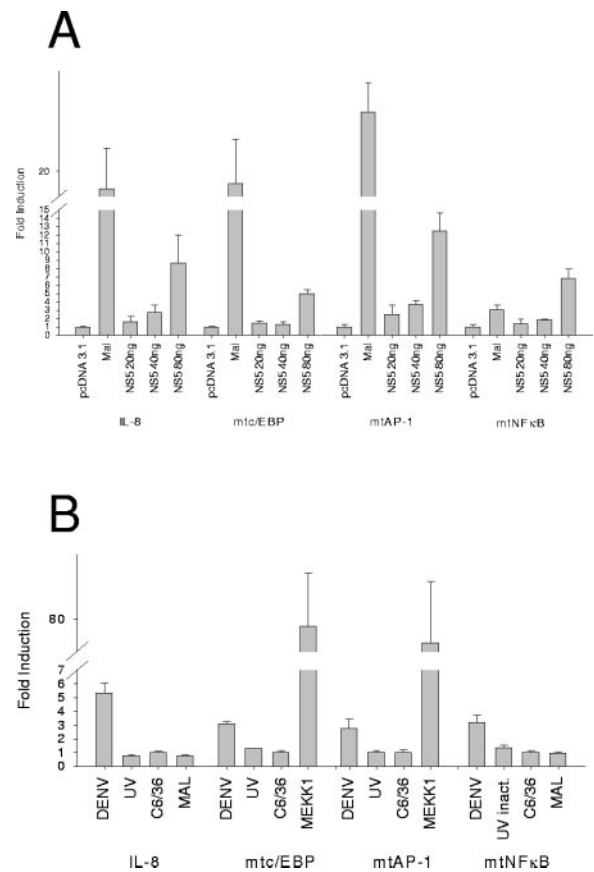


FIG. 7. Effect of NS5 expression or DEN2V infection on transcription from luciferase-mutated IL-8 promoters. HEK293A cells were cotransfected with vector encoding a reporter gene for IL-8, mtc/EBP, mtAP-1, and mtNF- κ B and (A) an expression vector for NS5 or (B) infected after 24 h with DEN2V. After 24 h (NS5) or 96 h (DEN2V), luciferase gene activity was measured and data are normalized to transfection efficiency with *R. reniformis* luciferase. There is a statistically significant difference ($P < 0.01$) between DEN2V- and C6/36-treated cells for the IL-8, mtc/EBP, mtAP-1, and mtNF- κ B reporters. mtc/EBP, mtAP-1, and mtNF- κ B represent the 133-luc plasmid with point mutations in the NF-IL-6, AP-1, and NF- κ B binding sites, respectively.

mokines in vitro, which is similar to previous results (1, 3, 5, 32, 62). Each cell line had a unique chemokine induction profile. These differences may be explained by differences in the expression of transcription factors in each cell type (23, 44). Dendritic cells expressed IL-8, RANTES, MCP-1, MIP-1 α , and MIP-1 β as early as 24 h postinfection. This could reflect the role that dendritic cells play as sentinels of the immune system (44). In contrast, the chemokines analyzed in this study peaked late, 3 to 5 days after DEN2V infection, for all the cell lines we tested. A similar profile was found in primary monocytes/macrophages (5). Possible explanations for the delay in chemokine expression could be a need for the virus to reach a threshold of viral load to induce chemokines, a need for transcription factors involved in chemokine induction to be induced, or a time delay in protein translocation into the nucleus.

We have shown an increase in steady-state chemokine mRNAs over time during DEN2V infection of cell lines. Although we have not excluded the possibility that increased chemokine secretion is due to mRNA stabilization, as has been reported

with respiratory syncytial virus and RANTES expression and for adenovirus and IL-8 (33, 38), we have shown that transcription from the IL-8 promoter was induced by DEN2V infection through activation of transcription factors NF- κ B, AP-1, and c/EBP.

Viral infection of a host cell activates signaling pathways that lead to IFN production as a consequence of double-stranded RNA intermediates from viral replication. In this regard the RNA helicase RIG-I has recently been identified as a cytoplasmic double-stranded RNA sensing mechanism. The RIG-I pathway elicits the activation of transcription factors such as NF- κ B and IRF3 that are also involved in chemokine transcription (71). Although we have reported that induction of IL-8 production by DEN2V occurs independently of IFN- α , it will be interesting to determine if RIG-I plays a role in DEN2V detection and signaling.

Our finding of IL-8 induction by NS5 reveals a novel mechanism for DEN2V-induced chemokine production. Dengue virus NS5 is a large multifunctional protein containing an S-adenosylmethionine transferase domain in the N-terminal region (34) and an RNA-dependent RNA polymerase domain in the C-terminal region (49, 66). NS5 was shown to be differentially phosphorylated and located in both the cytosol and the nucleus (28). In the late stage of infection, NS5 becomes hyperphosphorylated and dissociates from NS3, exposing a nuclear localization signal (25, 28). The function of nuclear localized NS5 has not been identified. The kinetics of IL-8 secretion late in infection correlates with movement of NS5 to the nucleus (28). It is possible that nuclear NS5 can bind directly or indirectly to the IL-8 promoter to induce gene expression.

It has been shown that different strains of DEN2V (i.e., American versus Asian genotypes) are associated with various degrees of disease (57). Previous research compared sequences of the Asian genotype of DEN2V, which is associated with dengue hemorrhagic fever, and the American genotype, which appears to be incapable of causing dengue hemorrhagic fever (37). Five of the eight amino acid differences between genotypes within the nonstructural proteins that led to an amino acid change were located in the N terminus of NS5. We constructed the NS5 expression plasmid from the infectious cDNA clone of Polo et al. (53), derived from the DEN2V strain New Guinea C, a member of the Asian genotype. It will be interesting to determine whether sequence differences within NS5 change the ability of the protein to induce IL-8.

High local levels of IL-8 are associated with pleural effusion (2, 51). IL-8 has been shown to be elevated in serum and PBMC of patients with the more severe dengue virus disease dengue hemorrhagic fever (26, 55). A recent paper has shown that IL-8 can alter the cytoskeleton and tight junctions of microvascular endothelium and change the permeability of the endothelial monolayer (65). The general mechanism of IL-8 induction is through the cytokine network that includes IL-1 and tumor necrosis factor alpha (19). During dengue virus infection, tumor necrosis factor alpha and IL-1 β are induced in dengue fever and dengue hemorrhagic fever patients and may induce low levels of IL-8 (4, 18). Previously, it has been reported that dengue hemorrhagic fever patients tend to have a higher viral burden early in infection (40, 68), which may correlate with increased NS5 expression. The increase in NS5 may

elevate levels of IL-8 and contribute to the altered vascular permeability seen in patients with dengue hemorrhagic fever. We found that the mean levels of IL-8 mRNA in PBMC were higher later during dengue virus infection. Although we did not find statistically significant differences in IL-8 mRNA levels between dengue fever and dengue hemorrhagic fever patients, it should be noted that mean viremia titers were not significantly different in the dengue hemorrhagic fever patients compared to dengue fever patients in this sample of subjects (63).

IL-8, a proinflammatory CXC chemokine, is induced in multiple cell types by many stimuli, including viruses (31). IL-8 induces a respiratory burst of neutrophils, release of lytic enzymes, platelet-activating factor, and leukotrienes, which are all inflammatory reactions to rid the host of invading pathogens (2). IL-8 induction has been found to counteract the antiviral effects of IFN- α and enhance viral replication of many viruses, including picornavirus, encephalomyocarditis virus, poliovirus, cytomegalovirus, and human immunodeficiency virus (30, 36, 48, 54).

Hepatitis C virus, a distant relative of dengue virus in the family *Flaviviridae*, has been shown to counteract the type I IFN response. The HCV NS5A and E2 proteins prevent phosphorylation of eIF2 α and the arrest of viral translation by inhibiting double-stranded RNA-dependent protein kinase R activity (12, 67). In addition, the core and NS5A proteins of hepatitis C virus were able to activate the IL-8 promoter (29, 54). Recently, DEN2V NS4B has been shown to inhibit the IFN-induced signal transduction cascade by interfering with STAT1 function (47). Our data shows that the dengue virus NS5 protein can induce expression of IL-8. DEN2V may use this mechanism to counteract the antiviral effects of innate immunity, allowing further dissemination of the virus to neighboring uninfected cells.

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