Dengue virus (DENV) antibody-dependent enhancement of infection upregulates the production of anti-inflammatory cytokines, but suppresses anti-DENV free radical and pro-inflammatory cytokine production, in THP-1 cells

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The immunopathogenesis of dengue haemorrhagic fever and dengue shock syndrome is thought to be mediated by a variety of host factors. Enhancing antibodies are one of the key regulating molecules. These antibodies, via antibody-dependent enhancement (ADE) of infection, are able to facilitate dengue virus (DENV) growth in Fc-bearing host cells. The mechanism of ADE-enhanced DENV production is believed to be mediated through increasing the infected-cell mass. In the present work, the effect of ADE infection was explored further, focusing on the post-entry events of ADE infection. It was hypothesized that the higher virus production in ADE infection compared with DENV infection may be due to the ability of this infection pathway to suppress key antiviral molecules. Therefore, the influence of ADE infection on pro- and anti-inflammatory cytokines, including interleukin-12 (IL-12), gamma interferon (IFN-γ), tumour necrosis factor alpha (TNF-α), IL-6 and IL-10, was investigated and it was found that DENV infection via the Fc receptor-mediated pathway was able to suppress the transcription and translation of IL-12, IFN-γ and TNF-α. In contrast, infection via this route facilitated expression and synthesis of the anti-inflammatory cytokines IL-6 and IL-10. Moreover, this study demonstrates that the ADE infection pathway also suppresses an innate anti-DENV mediator, nitric oxide radicals, by disrupting the transcription of the iNOS gene transcription factor, IRF-1, and blocking the activation of STAT-1. In conclusion, ADE infection not only facilitates the entry process, but also modifies innate and adaptive intracellular antiviral mechanisms, resulting in unrestricted DENV replication in THP-1 cells.

INTRODUCTION
Dengue virus (DENV) has become a mosquito-borne virus of immense global health importance, as the disease caused by DENV is now endemic in more than 100 countries, particularly in Asia and Latin America, and about 50 million cases of DENV infection are estimated to occur annually worldwide (Ligon, 2005). DENVs, members of the family Flaviviridae, have positive, single-stranded RNA and belong to four related serotypes, known as DENV-1, -2, -3 and -4. DENV induces a wide range of clinical manifestations and the majority of infected patients experience uncomplicated dengue fever (DF), which is an acute febrile illness typically lasting 3–7 days. Dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) are plasma-leakage syndromes that represent life-threatening manifestations of DENV infection. DHF/DSS can be distinguished from DF by three primary criteria: namely, defects in vascular permeability, haemostatic abnormalities with marked thrombocytopenia and bleeding diathesis (da Fonseca & Fonseca, 2002; Rothman, 2004).

Determinants that predispose infected patients to develop DHF have been partially identified (Lei et al., 2001). Various types of cell, such as monocytes and macrophages, dendritic cells, mast cells and hepatocytes, are able to support the replication of DENV (Jindadamrongwech et al., 2004; Kliks et al., 1988; Tassaneetrithep et al., 2003; Wu et al., 2000). After DENV infection, these cells produce cytokines and chemokines, which correlate with disease severity (Chaturvedi et al., 2000). In addition, virus variation, viral
load and antibody-dependent enhancement (ADE) of infection all have been suggested to contribute to the progression and severity of dengue disease (Rothman, 2003). ADE infection has been reported in various virus systems and has been shown to contribute to disease severity and to cellular tropism switching (Kliks et al., 1989; Morens & Halstead, 1990; Trischmann et al., 1995). During natural DENV infection, ADE is postulated to contribute by increasing the number of infected cells, resulting in high viral production. Unfortunately, the molecular mechanism by which ADE facilitates DENV production remains unclear. In an in vitro study, ADE infection involves the entry of virus–antibody complexes into monocytic cells via the Fc receptor, resulting in a significantly enhanced virus titre (Klimstra et al., 2003; Rulli et al., 2003; Sullivan, 2001). However, a study on Ross River virus found that ADE-facilitated infection and virus production were not simply due to an initial enhanced infectivity, but showed clearly that ADE infection mediates suppression of intracellular production of antiviral mediators, such as tumour necrosis factor alpha (TNF-α), nitric oxide synthase 2 and interferon (IFN)-regulatory factor 1 (IRF-1) (Mahalingam & Lidbury, 2002). Whether a similar phenomenon occurs in the DENV system required further study.

The prime target cells of DENV both in vitro and in vivo are well recognized as professional nitric oxide producers. Nitric oxide is one of the most versatile players in the immune defence system; it inhibits viral genome synthesis, blocks viral protease activity via nitrosylation and promotes viral clearance (Akaike & Maeda, 2000; Benz et al., 2002). Nitric oxide production has been reported in response to DENV infection (Lin et al., 2002; Valero et al., 2002) and our group showed recently that this free radical is a potent inhibitor of DENV replication via the specific inhibition of NS5 activity (Charnsilpa et al., 2005; Takhampunya et al., 2006). Cytokines are believed to be involved in the pathogenesis of DENV infection (Chaturvedi et al., 2000) and the overproduction of pro- and anti-inflammatory cytokines, such as TNF-α, interleukin-6 (IL-6), IL-10, MIF and IL-8, potential predictors of disease severity and clinical outcome in dengue patients (Chen et al., 2006; Nguyen et al., 2004; Rahgupathy et al., 1998). Therefore, in the present study, the effect of ADE infection on pro- and anti-inflammatory cytokine production and on intracellular free radical production was investigated. From our data, antibody-facilitated DENV entry into THP-1 cells upregulated IL-10 and IL-6 production strongly, but suppressed nitric oxide radical production significantly and also downregulated IL-12 and IFN-γ synthesis. The significance of the nitric oxide radicals on the viral burden in dengue patients was investigated preliminarily. Finally, the molecular process of nitric oxide suppression during ADE infection was illustrated.

METHODS

Clinical samples. Blood samples were collected from DENV-infected patients who were enrolled in this project after having given informed consent at the Queen Sirikit National Institute of Child Health, Bangkok, Thailand. The investigation protocol was approved by the Committee on Human Rights Related to Human Experimentation, Mahidol University, Bangkok, Thailand. The enrolled patients had an age range between 5 and 10 years. Blood was obtained on the first day of admission (fever day), the defervescent day, and 30 days after admission (convalescent day). Plasma was separated immediately and kept frozen at −80°C. The patients’ disease severity was graded as DF or DHF according to WHO criteria (Gubler, 1998). DENV serotyping was done by RT-PCR (Lanciotti et al., 1992). All cases were classified as having either primary or secondary infection by haemagglutination inhibition (HI) titre and IgM ELISA (Kuno et al., 1991).

Chemicals and antibody. S-Nitroso-N-acetylpenicillamine (SNAP), an exogenous nitric oxide donor, was purchased from Molecular Probes. L-N6-(1-Iminoethyl)lysine (L-NIL), a selective inhibitor of inducible nitric oxide synthase, was obtained from Sigma. HB-46 is a mAb against a type-specific determinant on DENV-2. Its hybridoma culture was purchased from the ATCC.

Enhancing antibody and dengue-non-immune serum. Convalescent serum from a patient infected with DENV serotype 3 (DENV-3) was used in all DENV-ADE infection experiments. This serum, when used at a dilution between 1:10,000 and 1:100,000, exhibited enhancing activities to DENV serotype 2 (DENV-2) strain 16681 infection using a peripheral blood mononuclear cell (PBMC) culture system.

Dengue-non-immune serum was obtained from a donor whose serum was negative by HI test and plaque-neutralization test for all four serotypes of DENV and Japanese encephalitis virus.

Virus and cell culture

Virus. DENV-2 16681 was used in the study. Virus was propagated in C6/36 cells and kept at −80°C. The titre of stock virus was determined by plaque assay on LLC-MK2 cells as described by Butrapet et al. (2000). For UV-irradiated virus, DENV-2 was exposed to a 30 W UV lamp at a distance of 55 cm for 5 min.

Low-passage isolates of DENV-2 were obtained from the Virology Department, Armed Forces Research Institute of Medical Science, Bangkok, Thailand. Viruses from patients were first amplified in Toxorhynchites splendens mosquitoes and were subsequently amplified twice in C6/36 cells. Some of these isolates were sensitive to 50 μM SNAP treatment, as described elsewhere (Charnsilpa et al., 2005).

Cell culture. THP-1 cells were obtained from the ATCC. The cells were cultured in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum (Gibco) at 37°C in a 5% CO2 atmosphere.

Role of nitric oxide radicals in DENV-2-infected THP-1 cells. THP-1 cells (1 × 105) were cultured and pretreated with 100 μM L-NIL at 37°C for 30 min or pretreated with medium alone. The cells were washed three times with PBS before being infected with low-passage isolates of DENV from patients at an m.o.i. of 1. After 1.5 h incubation at 37°C, the infected cells were washed and cultured further in growth medium containing 100 μM L-NIL or growth medium without L-NIL. Supernatants at days 0–5 were then subjected to detection of viral genome production, using real-time RT-PCR, and nitric oxide production. Uninfected cultures were used as a negative-control experiment.

ADE infection in THP-1 cells. ADE of DENV-2 16681 infection (DENV-ADE infection) was performed by using THP-1 cells and a 1:10,000 dilution of heat-inactivated enhancing antibody or a 1:10,000 dilution of dengue-non-immune serum. One hundred
microlitres of DENV-2 strain 16681 (1 × 10^6 p.f.u. ml^−1) was mixed with an equal volume of a 1 : 10 000 dilution of serum and incubated at 4 °C for 30 min. At the end of the incubation, the virus/antibody mixture was inoculated into 1 × 10^6 THP-1 cells. Therefore, an m.o.i. of 0.1 was used. After 1.5 h incubation at 37 °C, the infected cells were washed and cultured further in growth medium. The supernatants and infected cells were harvested every 24 h for 5 days. Supernatants were then subjected to detection of viral genome production (by using real-time RT-PCR), nitric oxide production, IL-6, IL-10, IL-12, TNF-α and IFN-γ production. Harvested cells were used for the detection of phosphorylated STAT-1 and of IL-6, IL-10 and IRF-1 gene expression.

In addition to DENV-ADE infection, the following control infection/treatments were performed: anti-DENV antibody alone (an enhancing antibody), non-infectious DENV-ADE infection (a complex of UV-irradiated DENV and ADE) and UV-irradiated (non-infectious) DENV alone.

Detection of DENV infectivity by immunofluorescence. THP-1 cultures were infected with either DENV-2 16681 or DENV-2 16681 with ADE serum as mentioned above. Infected cells were harvested at 7, 12 and 24 h post-infection, fixed with cold acetone and then stained with an anti-DENV-2 mAb (HB-46; ATCC) to determine the number of infected cells by using a fluorescence microscope.

Viral RNA copy-number titration by fluorogenic real-time RT-PCR. RNA was extracted from plasma and culture supernatants by using a NucleoSpin RNA virus kit (Macherey-Nagel). The purified RNA was then subjected to RT-PCR using QuantiTect Probe RT-PCR (Qiagen) as described by Houng et al. (2000). RT-PCR amplification, data collection and analysis were performed by using a Rotor-Gene 3000 (Corbett Research). The RNA copy number was calculated by using dengue serotype-specific copy standards kindly supplied by Dr Huo-Shui H. Houng, Walter Reed Army Institute of Research, Silver Spring, MD, USA.

Quantitative detection of nitric oxide production. The amount of nitric oxide in plasma was determined by using a nitric oxide colorimetric kit (Cayman Chemical Company) that detects the stable products of nitric oxide, NO_3^- and NO_2^- . The experiment was performed according to the manufacturer’s instructions.

Determination of gene expression by semiquantitative RT-PCR. The levels of gene expression in DENV- and DENV-ADE-infected THP-1 were semiquantified by RT-PCR as described previously (Ubol et al., 1998). Briefly, harvested cells were lysed, RNA was purified and then subjected to first-strand cDNA synthesis before being amplified further by PCR. Specific primers for IL-6, IL-10, IRF-1 and β-actin genes were as follows: IL-6: sense, 5'-GCTGGACTGCCCTGCCCCAGT-3'; antisense, 5'-CTGTTCTGCTCGCCTGAGCG-3'; and β-actin: sense, 5'-AGCTATCCCAGAGCCCCAGAT-3'; antisense, 5'-TAAAACGCAGCTCAGT-3'. The PCR products were electrophoresed and the density of each band was semiquantified by using a densitometer. The levels of IL-6, IL-10 and IRF-1 gene expression were presented as a percentage of that of β-actin, an internal control for gene expression.

Detection of cytokine production by ELISA. The levels of IL-6, IL-10, IL-12, IFN-γ and TNF-α production in the supernatants of infected THP-1 cell cultures were measured by using a Quantikine ELISA kit (R&D Systems, Inc.). Briefly, 200 μl standard or sample was pipetted into wells precoated with polyclonal antibodies specific to these cytokines. The antibody–cytokine interaction was detected by using enzyme-linked polyclonal antibodies specific for these cytokines and the substrate. The intensity of the colour was measured at 450 nm.

Detection of activated STAT-1 by using immunoblotting. DENV- and DENV-ADE-infected THP-1 cells were lysed in buffer containing 20 mM Tris, 100 mM NaCl and 1 % NP-40. The lysates were electrophoresed through 8 % SDS/polyacrylamide gels and then electrotransferred to nitrocellulose membrane (Schleicher & Schuell). The membranes were blocked with 10 % blocking reagent for 1 h before incubation overnight with a goat polyclonal antibody against phosphorylated STAT-1 or a mouse polyclonal antibody against β-actin (Santa Cruz). Bands were detected by using appropriate secondary antibodies and an enhanced chemiluminescence kit (Roche Diagnostics) as described by Utaisincharoen et al. (2004).

Statistical analysis. Values were expressed as means ± SD of three independent observations. The significance of difference was tested by Student’s t-test, one-way ANOVA or Pearson’s correlation. P values of < 0.05 were considered significant.

RESULTS

Intracellular free radicals suppress replication of DENV in THP-1 cells

Our group has recently reported the strong inhibitory effect of exogenous nitric oxide radicals on DENV replication in vitro (Charnsilpa et al., 2005; Takhampunya et al., 2006). In the present work, the role of nitric oxide radicals on in vitro DENV replication was examined further. THP-1 cells were infected with low-passage DENV-2 at an m.o.i. of 1 in the presence and absence of 100 μM L-NIL, a specific inhibitor of iNOS. The amount of virus production was monitored via RNA copy number. The level of nitric oxide in the culture medium was measured as described in Methods. As demonstrated in Fig. 1(a, b), L-NIL suppressed nitric oxide production significantly in infected THP-1 cells. The concentration of L-NIL used had no effect on cell viability based on trypsin blue exclusion (data not shown). DENV RNA genome production was increased in the presence of L-NIL, suggesting that endogenous nitric oxide suppresses progeny production of low-passage viruses isolated from dengue patients.

Level of plasma nitric oxide correlates inversely with the amount of circulating virus

To investigate whether nitric oxide radicals have any role in natural DENV infection, the relationship between plasma nitric oxide, viral load and severity of infection was studied. The level of nitric oxide synthesized during DENV infection was determined in 60 patients (11 primary DF, six primary DHF, 20 secondary DF and 23 secondary DHF). The mean levels of plasma nitric oxide were compared among these groups. As shown in Table 1, levels of plasma nitric oxide of primary DF, primary DHF and secondary DF patients were not significantly different on the fever, defervescence or convalescent days. However, a difference was found for the secondary DHF group, in which the level of plasma nitric oxide was significantly lower on the fever day than on the defervescence day. The results suggested that anti-DENV free
radical suppression occurred in the severe form of secondary infection.

To investigate the significance of nitric oxide radicals on virus production in the severe form of secondary dengue infection, the relationship between nitric oxide level and corresponding viral load was observed in primary DHF and secondary DHF plasma. In the secondary DHF group, the highest level of viraemia was found on the fever day, which was the day when the lowest level of plasma nitric oxide was detected. The level of nitric oxide increased gradually on the defervescent and convalescent days, when the level of DENV genomic RNA in plasma decreased significantly (Fig. 2c, d).

An inverse correlation between plasma nitric oxide and DENV RNA copy number (Pearson’s correlation: $r^2 = -0.998$, $P \leq 0.036$) suggested that nitric oxide radicals may be one of the host factors that determine production of DENV from infected cells in vivo. Suppression of nitric oxide production was found in secondary DHF patients, but not in primary DHF, flavivirus-non-immune DHF patients (Fig. 2a, b). These data suggested that ADE might play a role in nitric oxide suppression.

### Table 1. Level of plasma nitric oxide of different groups of dengue patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>n</th>
<th>Level of nitric oxide (μM) (mean ± SD) on:</th>
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<tr>
<td></td>
<td>Fever day</td>
<td>Defervescent day</td>
</tr>
<tr>
<td>Primary DF</td>
<td>11</td>
<td>6.99 ± 1.84</td>
</tr>
<tr>
<td>Primary DHF</td>
<td>6</td>
<td>6.70 ± 1.88</td>
</tr>
<tr>
<td>Secondary DF</td>
<td>20</td>
<td>7.99 ± 2.08</td>
</tr>
<tr>
<td>Secondary DHF</td>
<td>23</td>
<td>4.25 ± 1.97</td>
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*Significantly different at $P \leq 0.05$.

Suppression of nitric oxide production in DENV-ADE-infected THP-1 cells

Because suppressed nitric oxide radical production was only found in secondary DHF patients, we hypothesized that enhancing antibodies may interfere with nitric oxide production. In order to test our hypothesis, DENV-ADE infection was performed in THP-1 cells and the levels of nitric oxide and viral genome in the culture supernatants were analysed. As shown in Fig. 3(a), diluted convalescent DENV-3 serum at dilutions of 1:5000, 1:10000 and 1:100000 was able to enhance DENV-2 16681 replication. The levels of enhancing activities were increased by 1.55- to 9.77-fold over the baseline infection, with $P$ values of 0.002, 0.001 and 0.001 for 1:5000-, 1:10000- and 1:100000-diluted sera, respectively. The level of nitric oxide production is consistent with our prediction that nitric oxide production in infected THP-1 cells was decreased significantly in DENV-ADE infection, whereas viral genome replication increased (Fig. 3b). Moreover, ADE sera at neutralizing levels (dilutions of 1:100 and 1:500) blocked DENV replication, and the DENV-ADE neutralizing complex stimulated nitric oxide production to
the same level that in uninfected cultures (data not shown). The control infected cells, DENV-infected THP-1 and DENV-non-immune-infected THP-1 cells, synthesized lower levels of DENV genomic RNA, but higher levels of nitric oxide. The THP-1 cultures infected with non-infectious DENV-ADE complex, non-infectious DENV alone or DENV antibody alone had no alteration of nitric oxide production (Fig. 3c). These data indicate that suppression of nitric oxide production requires infection to occur via the ADE pathway.

Analysis of DENV-ADE infectivity

DENV infectivity in DENV-ADE infection and DENV infection was compared at 7, 12 and 24 h post-infection. At 7 and 12 h post-infection, there were no differences in the percentage of cells positive for DENV antigen in both conditions of infection (Table 2). A significant increase in infectivity was found after 24 h, with higher numbers of infected cells in DENV-ADE infection. Therefore, initial DENV infectivity was not different between ADE and non-ADE infection in THP-1 cells, suggesting that post-entry intracellular activities are of greater importance.

Downregulation of STAT-1 and IRF-1 activity in ADE-mediated DENV infection

Expression of the iNOS gene is regulated by at least two transcription factors, STAT-1 and IRF-1 (Utaisincharoen et al., 2004). To investigate whether ADE infection has any role in the phosphorylation of STAT-1 and IRF-1 gene expression, THP-1 cell lysates from DENV infection and DENV-ADE infection were subjected to immunoblotting and RNA analysis by RT-PCR in order to investigate the kinetics of STAT-1 phosphorylation and IRF-1 gene expression, respectively. During DENV infection, phosphorylation of STAT-1 was increased dramatically by 24–48 h. However, in DENV-ADE infection, phosphorylation of STAT-1 was blocked (Fig. 4a). A similar result was also found for the activation of IRF-1 gene expression, in which DENV replication stimulated IRF-1 gene expression, but infection in the presence of enhancing antibodies suppressed IRF-1 gene expression significantly (Fig. 4b).

Fig. 2. Inverse relationships between DENV RNA copy number and nitric oxide plasma of the severe form of secondary DENV-infected patients. Levels of plasma nitric oxide in primary DHF (a) and secondary DHF (c) patients were measured by using a nitric oxide colorimetric kit. Viral genomic numbers in primary DHF (b) and secondary DHF (d) patients were monitored by real-time RT-PCR.
These results suggest that suppression of STAT-1 phosphorylation and IRF-1 gene expression may be the mechanism by which DENV-ADE infection downregulates an innate response, nitric oxide radical production, during DENV infection.

AED infection upregulates production of IL-6 and IL-10, but suppresses IL-12, TNF-α and IFN-γ synthesis

It is well documented that the production of nitric oxide radicals by activated macrophages is elevated in the presence of IL-12 and IFN-γ, but is decreased in the presence of IL-10 (Alleva et al., 2002; Mahalingam & Lidbury, 2002; Matsuura et al., 2003; Sosroseno et al., 2002). In addition, the production of non-inflammatory cytokines is reported to be elevated in DHF/DSS patients, whilst the level of inflammatory cytokines is decreased. We therefore investigated the effect of infectious DENV–antibody complexes on the production of these cytokines. Results showed that IL-6 and IL-10 gene expression and the synthesis of these cytokines in supernatants were increased significantly (Fig. 5a–d). In contrast, the synthesis of IL-12 and IFN-γ was decreased significantly (Fig. 5e, f). The production of TNF-α was downregulated significantly during the early phase of infection, but rebounded to the same level as in DENV-infected cultures on day 3 (Fig. 5g). However, both ADE-DENV and DENV infection stimulated TNF-α production strongly in comparison with uninfected cells. These results suggested that DENV-ADE infection facilitated synthesis of anti-inflammatory cytokines, but blocked production of Th1 cell-promoting cytokines.

DISCUSSION

The majority of viral infections in both human and animals result in recovery and a state of resistance against reinfection. The resistant state is normally mediated by both memory T cell and B cells. However, in severe forms of secondary dengue infection, memory cells, as well as subneutralizing levels of antibodies, are reported to be mediators that regulate the severity of infection. T cells from previous DENV infections may participate in severe illness due to inefficiency in virus-infected cell elimination, as well as secretion of cytokines that are associated with disease severity (Bashyam et al., 2006; Mongkolsapaya et al., 2003). Subneutralizing levels of antibodies are hypothesized to increase the virulence of infectivity by promoting DENV infection via facilitating virus binding and entry into Fc-bearing cells, resulting in a large infected-cell mass (Kliks...
and in mouse models, where blocking of TNF-α reduces the mortality rate (Atrasheuskaya et al., 2003; Chen et al., 2006). Surprisingly, our system demonstrated a significant, but transient, suppression of TNF-α production during the early period of ADE infection in THP-1 cells. This transient suppression may benefit the initiation of DENV replication in THP-1 cells. In naturally DHF/DSS-affected infants, the level of TNF-α was higher than in healthy control subjects (Nguyen et al., 2004). Our data support this notion, in which both ADE infection and DENV infection induced TNF-α production significantly in comparison with uninfectected cells. Levels of IL-6 and IL-10 synthesis were elevated significantly in ADE infection, which is supported by studies of natural DHF/DSS. TNF-α, IL-6 and IL-10 may be involved in some biological responses that are typical characteristics of DHF/DSS. For example, in DHF/DSS infants, TNF-α may participate in increasing haematocrit, whilst IL-6 and IL-10 may mediate damage of the liver and coagulation systems, respectively (Nguyen et al., 2004).

DENV is extremely sensitive to the antiviral activity exerted by IFN-α/β (Shresta et al., 2004) and, in DENV infection, it has been shown that the NS4B protein is able to inhibit the IFN signalling pathway via STAT-1 and IFN-stimulated response element (ISRE) blocking (Ho et al., 2005; Munoz-Jordan et al., 2005), indicating that DENV may develop a defence mechanism in infected cells to fight against the antiviral effect of the IFN system. In our work, suppression of an innate anti-DENV molecule, the nitric oxide radical, is demonstrated through ADE infection. Nitric oxide is a diffusible radical that exerts an antimicrobial action against various pathogens, including viruses (Bogdan, 2001). For example, it inhibits the replication cycle of severe acute respiratory syndrome coronavirus and adenovirus in vitro via the inhibition of viral protein and RNA synthesis (Akaike & Maeda, 2000; Akerstrom et al., 2005; Cao et al., 2003). The recombinant virus variant coxsackievirus B3/IFN-γ activates nitric oxide production, which in turn reduces its own replication directly in vitro and in vivo (Jarasch et al., 2005). We demonstrated recently that exogenous nitric oxide inhibits NS5 activity, resulting in downregulation of the in vitro replication of clinical DENV isolates (Charnsilpa et al., 2005; Takhampunya et al., 2006). We investigated further and found that endogenous nitric oxide could suppress DENV replication in THP-1 cells. Moreover, an inverse correlation between plasma nitric oxide and circulating DENV RNA copies was demonstrated in secondary DHF, but not in flavivirus-non-immune DHF patients. These findings suggest that nitric oxide acts as one of the important immune mediators against DENV infection, and its level may be regulated by an ADE infection. Suppression of this mediator may contribute to the high viral load in secondary DHF patients. To investigate further the mechanism of ADE infection-suppressed nitric oxide production, expression of nitric oxide transcription activators was studied in ADE infection in THP-1 cells. Downregulation of IRF-1 and dephosphorylation of STAT-1 were found, accompanied by upregulation of IL-10. IL-10 is shown to suppress iNOS gene

Chemokines such as TNF-α and MIF have been reported to be associated with the development of DHF/DSS in humans

Fig. 4. Kinetics of phosphorylation of STAT-1 and IRF-1 gene expression. THP-1 cells were infected with 0.1 p.f.u. per cell of DENV-2 16681 or the immune complex of DENV-2 16681 and ADE serum. Cells were harvested at 18, 24, 48 and 72 h post-infection. (a) Cell lysates were subjected to immunoblot for phosphorylated STAT-1 detection; (b) RNA from infected THP-1 cells was extracted and then used for quantification of IRF-1 gene expression using RT-PCR. Data are shown as means ± SD of three independent experiments. *Significantly different at P < 0.05.
Fig. 5. Detection of cytokine production. THP-1 cells were infected with DENV-2 16681 or with the immune complex of DENV-2 16681 and ADE serum. An m.o.i. of 0.1 was used. RNA was extracted from harvested cells at day 0–5 post-infection for detection of (a) IL-10 and (b) IL-6 gene expression. Supernatants were harvested for quantification of (c) IL-10, (d) IL-6, (e) IL-12, (f) IFN-γ and (g) TNF-α protein synthesis by ELISA. Three independent experiments were performed and results are expressed as means ± SD. *Significantly different at \( P < 0.05 \).
expression in lipopolysaccharide-stimulated macrophages via inhibition of STAT-1 and IRF-1 activity (Berlato et al., 2002). To integrate these data, the molecular process of DENV-ADE diminishing the anti-DENV response has been hypothesized as illustrated in Fig. 6. In the absence of an enhancing antibody, DENV enters into monocytes/macrophages via receptors such as heat-shock protein 70, 90 and CD14 (Chen et al., 1999; Reyes-Del Valle et al., 2005). Replication of virus in this situation turns on IL-12 and IFN-γ production, and signals elicited by the cytokine–receptor interaction then activate STAT-1 and IRF-1, resulting in activation of iNOS gene transcription and nitric oxide radical production, leading to a strong production of anti-DENV free radicals. However, in ADE infection, IL-10 was upregulated dramatically, whereas IL-12 and IFN-γ were suppressed significantly. IL-10 may act as an autocrine factor and bind to its specific receptor. Interaction of IL-10 and its receptor somehow inhibits STAT-1 and IRF-1 activation, thus inhibiting nitric oxide production.

In conclusion, the present investigation has demonstrated a relationship between DENV-ADE infection, innate immune response and cytokine-expression pattern in THP-1 cells. During primary DENV infections, replication of the virus in monocytes/macrophages is limited to a certain level due to effective intracellular anti-DENV activities. This results in a low viral burden. However, via infection in the presence of subneutralizing antibodies or secondary ADE infection, DENV is able to diminish the intracellular defence mechanisms, such as free radicals and antiviral cytokines, and to facilitate immune-suppressive cytokines, which may lead to increased viral production and disease severity. The present data demonstrate significant changes in response at 24 h post-infection. However, to understand further the impact of these two different entering mechanisms, molecular changes at the earlier time points should be investigated.

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REFERENCES


