Inflammatory Mediators in Dengue Virus Infection: Circulating Interleukin-12 and Interferon-γ

by


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Abstract

Interleukin (IL)-12 stimulates the production of interferon (IFN)-γ by T or NK cells, is a growth factor for preactivated T and NK cell, and enhances the cytotoxic activity of cytotoxic T cells and NK cells. To investigate the potential role of the IL-12/IFN-γ axis in dengue virus infection, we measured circulating levels of the p40-subunit of IL-12 and those of IFN-γ in 186 patients with this disease, and in 33 apparently healthy children as well as in 11 patients with bacterial infections as positive controls. Levels of IL-12-p40 were elevated in 90%, whereas those of IFN-γ were increased in 36.4% of the patients, respectively. Apparently healthy endemic children had similar levels of IL-12-p40, whereas they had significantly lower IFN-γ (p<0.01, WMW-test). In contrast, the patients with bacterial infections had similar levels of IL-12-p40 and IFN-γ as compared to the dengue patients. The levels of both cytokines were higher in the dengue patients without shock than in those with dengue shock syndrome (p<0.01). The IL-12-p40 and IFN-γ levels correlated with each other (r=0.2, p=0.00; two-tailed Spearman rank correlation). In addition, the IL-12 levels correlated with plasma protein levels, haematocrit value and the presence of ascites (r=0.15, p=0.04; r=-0.25, p=0.001; r=0.35, p=0.00, respectively). IFN-γ correlated with plasma protein levels, platelet counts and the presence of ascites (r=0.2, p=0.00; r=-0.20, p=0.004; r=0.23, p=0.002, respectively).

The results of this study indicate that there was no significant IL-12-p40 response in dengue patients as compared to controls, whereas IFN-γ levels were increased in a substantial number of patients with dengue virus infection. The findings do not allow for firm conclusions on the role of the IL-12/IFN-γ axis in the pathogenesis of dengue virus infection.

Keywords: Children, dengue virus infection, interferon-gamma, interleukin-12, shock.

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Introduction

Dengue is an acute infectious viral disease characterized by biphasic fever, headache, pain in various parts of the body, prostration, rash, lymphadenopathy, and leukopenia\(^1,2\). Dengue virus infection presents itself as two major clinical syndromes, dengue fever (DF) and dengue haemorrhagic fever (DHF)\(^1\). DF, the most common type of dengue illness, is a self-limited febrile disease. DHF is a more serious illness sometimes complicated by dengue shock syndrome (DSS). Based on data primarily collected in Thailand, the World Health Organization has proposed definitions for DHF and DSS, and recognizes four grades of severity\(^3\):

- **DHF Grade I**: Fever accompanied by non-specific symptoms with a positive tourniquet test as the haemorrhagic manifestation;
- **DHF Grade II**: Grade I accompanied by spontaneous haemorrhagic manifestation;
- **DHF Grade III**: Circulatory failure manifested by tachycardia with narrowing of the pulse pressure (<20 mmHg) or hypotension, and
- **DHF Grade IV**: Profound shock with undetectable blood pressure and pulse.

At present, the pathogenesis of dengue haemorrhagic fever is incompletely understood. Some studies suggest the involvement of T-cell responses in the pathogenesis of DHF and DSS: CD4\(^+\) cells from humans having suffered from dengue proliferate producing gamma-interferon (IFN-\(\gamma\)) in response to soluble dengue virus antigen\(^5\); circulating levels of sIL2R, sCD4 and sCD8 are significantly higher in patients with DHF than in healthy children\(^6\); serotype-cross-reactive C4\(^+\)CD8\(^-\) cytotoxic T-lymphocyte clones secreting interferon (IFN)-\(\gamma\) have been isolated from patients\(^7\); peripheral blood mononuclear cells from a dengue-4 immune donor have been shown to proliferate in response to a live dengue virus\(^8\); and, finally, serotype-cross-reactive, CD8\(^+\), class I-restricted, dengue virus-specific cytotoxic lymphocytes have been identified and it has been suggested that these cells may mediate viral clearance and contribute to shock by lysing dengue virus-infected cells in secondary infections\(^9\).

Interferon-gamma (IFN-\(\gamma\)) is a main cytokine released by CD8 effector T cells, capable of blocking viral replication\(^10\). IFN-\(\gamma\) is also a potent activator for phagocytic cells, increasing their bactericidal activity as well as their ability to produce cytokines. A major factor produced by infected phagocytes responsible for the induction of IFN-\(\gamma\) synthesis is interleukin-12 (IL-12). IL-12 consists of a heterodimer and is a potent inducer of cytokine production, particularly IFN-\(\gamma\), in T and NK cells. In addition, it is a growth factor for preactivated T and NK cells, and an enhancer of cytotoxic activity of both CD8\(^+\) T cells and NK cells\(^10,11,12,13\).

Considering a potential role for cytotoxic T lymphocytes in the pathogenesis of dengue, we hypothesized that the cytokines IL-12 and IFN-\(\gamma\) might be important in the pathogenesis of dengue virus infection. Hence, in this study we investigated the release of these cytokines in patients with dengue viral infection: plasma levels of the IL-12 p40 subunit as well as those of IFN-\(\gamma\) were measured in patients with dengue fever or dengue haemorrhagic...
fever in comparison to those in healthy children or patients with bacterial infections.

Materials and methods
The patients included in the study were admitted to the Department of Paediatrics of the Dr Sardjito General Hospital in Yogyakarta, Indonesia, between September 1995 and May 1996. They presented with fever lasting 2-7 days. A clinical diagnosis of DF or DHF was made according to the WHO criteria before the results of serological studies were known. The severity of illness was graded according to the WHO criteria for dengue haemorrhagic fever. A definitive diagnosis of dengue virus infection was made when patients had elevated levels of IgM antibodies with or without detectable IgG antibodies against a dengue virus according to the criteria described in the interpretation of MAC-ELISA results.

Two groups of patients served as controls. Patients with a bacterial infection (bacterial meningitis, sepsis or typhoid fever) served as "positive" controls. The diagnosis of these infections was based on clinical symptoms in combination with the results of cerebrospinal fluid culture and an elevated cell in the cerebrospinal fluid, blood culture, or a Widal serological test, respectively. Apparently healthy children in the outpatient department served as "negative" controls.

The clinical signs were registered in all patients at the time blood was collected for the present study. All patients were treated with supportive therapy, including infusion of crystalloid, plasma or whole blood when necessary, as a standard therapy. The protocol was approved by the Medical Ethics Committee of the Faculty of Medicine, Gadjah Mada University, Dr Sardjito Hospital. Informed consent was obtained from the parents of each patient included in the study.

Blood sampling
Blood was obtained from each patient within 1 day after admission and in a substantial number of patients also on subsequent days during hospital stay. Blood was collected in tubes containing soybean inhibitor (SBTI; Sigma Chem. CO., St. Louis, Mo), benzamidin and EDTA (100 ug per ml; 10mM and 10mM, final concentrations, respectively) to prevent activation of cells and of plasma cascade systems. The tubes were centrifuged for 10 minutes at 1,300 x g, and plasma was stored at -70°C in aliquots. The samples were transported on dry ice to Amsterdam, where they were stored at -70°C until tests were performed.

Laboratory investigations
IgM and IgG antibodies against dengue virus were measured in the Laboratory for Exotic Viral Infections, Institute of Virology, Erasmus Medical Centre, Rotterdam, the Netherlands. Briefly, an ELISA was used to detect DEN-2-specific IgG antibodies as described earlier. IgM antibodies against the recombinant E-protein were measured with a direct ELISA as described for the IgG antibody detection using an anti-IgM conjugate (Dakopatts, Glostrup, Denmark). IL-12-p40, IFN-γ, IL-6, and IL-8 were measured with ELISAs obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands, according to the manufacturer's instruction. Results were expressed as pg/ml. Levels of IL-12-p40...
exceeding 150 pg/ml and of IFN-γ exceeding 40 pg/ml were considered to be elevated. Leukocyte and platelet numbers were assessed according to standard techniques. Plasma protein was measured with a microhematocrite method. Heparinized blood was centrifuged for 10 minutes at 10,000-12,000 rpm\(^{15}\). The supernatant was analysed for protein content with a refractometer (Atago SPRN, Atago CO Ltd, Japan).

**Data analysis**

The difference between groups with respect to age was assessed by the Anova test. Differences in levels of IL-12-p40 and IFN-γ between cases and controls and between shock and normotensive patients were analysed by the Wilcoxon-Mann-Whitney (WMW) test. Differences in the proportion of elevated levels of IL-12 and IFN-γ between cases and controls, and the distribution of gender between DF and DHF were analysed by the Chi-square test. Correlations between IL-12-p40 and IFN-γ, respectively, and clinical and hemodynamic parameters were evaluated with the Spearman’s correlation test. A two-tailed p-value of less than 0.05 was considered to represent a significant difference. All statistical calculations were done in SPSS 6.0 for Windows 95.

**Results**

**Patients**

During September 1995 - May 1996, 235 patients, admitted to the hospital with fever lasting for 2 to 7 days suggestive of dengue infection, were included in the study. Only 186 patients fulfilled the serological criteria (IgM with or without IgG) for dengue virus infection. Seventy-one of these fulfilled the WHO criteria for dengue haemorrhagic fever (DHF) [22 cases for DHF-1 (11.8%), 20 cases for DHF2 (10.8%), 18 cases for DHF-3 (9.7%) and 11 cases for DHF-4 (5.9%)]. The other patients (115) with positive serology were considered to suffer from dengue fever (DF) (61.8%). The mean age (year) of patients with DF was: 8.15±3.15 years; with DHF-1: 9.73±3.15 years; with DHF-2 8.50±3.28 years; with DHF-3: 8.39±3.58 years; and with DHF-4: 7.40±2.32 years. The distribution of gender for DF was 51.8% male, 48.2% female; DHF-1: 68.2% male, 31.8% female; DHF-2: 40% male, 60% female; DHF-3: 33.3% male, 66.7% female; DHF-4: 54.5% male, 45.5% female. There was no statistically significant difference in age and gender distribution between groups (Anova: p=0.24; Chi-square: p=0.2).

**Levels of IL-12-p40**

Levels of IL-12-p40 in plasma samples obtained from 31 healthy children ranged from 139-1471 pg/ml, the majority of these children (96.8%) having elevated levels (Table 1, Figure 1). Plasma levels were elevated in 90% of the dengue virus infection patients with a range of <50-2550 pg/ml and a median of 322 pg/ml. The highest proportion of elevated IL-12-p40 levels was found in patients with DF (97.3%; range: 121-2550 pg/ml, median: 373 pg/ml), followed by DHF-1 (89.5%), DHF-2 (80%), DHF-3 (77.8%), and DHF-4 (54.5%). Thus, plasma levels of IL-12-p40 in the patients with DF or DHF were comparable with those in the apparently healthy children, as well as with those in the patients with bacterial infections (Table 1). IL-12-p40 levels did not fluctuate much during the course of the disease in the patients with DF or DHF (Table 2).
Table 1: Levels of IL-12-p40 and IFN-γ on admission in various groups of patients

<table>
<thead>
<tr>
<th>Group</th>
<th>Interleukin-12-p40 (pg/ml)</th>
<th>Interferon-γ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean no. of samples</td>
<td>Median (range)</td>
</tr>
<tr>
<td>Dengue virus infection</td>
<td>180</td>
<td>322(&lt;50-2550)*</td>
</tr>
<tr>
<td>DF</td>
<td>112</td>
<td>373(121-2550)*</td>
</tr>
<tr>
<td>DHF-I</td>
<td>19</td>
<td>235(128-1596)*</td>
</tr>
<tr>
<td>DHF-II</td>
<td>20</td>
<td>255(89-519)</td>
</tr>
<tr>
<td>DHF-III</td>
<td>18</td>
<td>189(57-504)</td>
</tr>
<tr>
<td>DHF-IV</td>
<td>11</td>
<td>209(&lt;50-294)</td>
</tr>
<tr>
<td>Healthy children</td>
<td>31</td>
<td>373(139-1471)</td>
</tr>
<tr>
<td>Bacterial infection</td>
<td>11</td>
<td>410(&lt;50-984)*</td>
</tr>
</tbody>
</table>

Wilcoxon-Mann Whitney test: *: levels of IL-12-p40 comparable with healthy children (p>0.05); +: levels of IFN-γ higher than those in healthy children (p<0.01).
**IFN-γ levels**

Levels of IFN-γ in plasma samples of healthy children ranged from <40-114 pg/ml with 16.1% having elevated levels (Table 1, Figure 2). In the patients with dengue virus infection IFN-γ levels were elevated in 36.4% (range: <40-3511 pg/ml, median: <40 pg/ml). The highest proportion of elevated IFNγ levels was found in DF (44.2%) with range <40-3511 pg/ml and median of <40 pg/ml, followed by DHF-1 (36.4%), DHF-2 (30%) and DHF-3 (16.7%). Remarkably, none of the patients with DHF-4 had elevated levels of IFN-γ.

When the dengue patient groups were considered separately, plasma levels of IFN-γ appeared to be significantly higher in the DF patients than in the healthy children but not in the DHF patients (Table 1). In addition, IFN-γ levels were not different between the dengue patient groups (with the exception of DHF-3 and DHF-4) and the patients with bacterial infections (Table 1). In the majority of patients with dengue virus infection the highest levels of IFN-γ occurred on the day of admission (Table 2).

**Relation of plasma levels of IL-12-p40 and IFN-γ to the presence of shock in dengue virus infection**

Twenty-nine of the 186 patients with dengue virus infection fulfilled the WHO criteria for shock. The plasma levels of IL-12-p40 and of IFN-γ were significantly higher in normotensively patients than those in patients with shock (Table 3).

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**Figure 2. Levels of IFN-γ in various groups of patients**

C: healthy children; C+: children with bacterial infections; DF: dengue fever, DHF I: dengue hemorrhagic fever I; DHF II: dengue hemorrhagic fever II; DHF III: dengue hemorrhagic fever III; DHF IV: dengue hemorrhagic fever IV.
**Relation of IL-12-p40 and IFN-γ to other variables**

A positive correlation between the plasma levels of IL-12-p40 and the plasma protein levels and a negative correlation with the haematocrit values was found. Plasma levels of IFN-γ correlated with the plasma protein levels. Also, a significant correlation between the levels of IL-12-p40 and of IFN-γ and the presence of ascites was found (Table 4). In contrast, the platelet counts correlated inversely with the plasma levels of IL-12 and of IFN-γ (Table 4).

**Table 2: Course of IL-12-p40 and IFN-γ in the dengue virus infection patients**

<table>
<thead>
<tr>
<th>Features</th>
<th>Interleukin-12 (pg/ml)</th>
<th>Interferon-γ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean no. of samples</td>
<td>Median (range)</td>
</tr>
<tr>
<td>Admissions</td>
<td>180</td>
<td>322(&lt;50-2550)</td>
</tr>
<tr>
<td>1 day after admission</td>
<td>81</td>
<td>285(&lt;50-3876)</td>
</tr>
<tr>
<td>2 days after admission</td>
<td>35</td>
<td>223(56-1745)</td>
</tr>
<tr>
<td>3, 4, 5, 6 days after admission</td>
<td>27</td>
<td>212(113-531)</td>
</tr>
<tr>
<td>Reconvalescence (7 days and later)</td>
<td>73</td>
<td>297(110-1933)</td>
</tr>
</tbody>
</table>

**Table 3: IL-12-p40 and IFN-γ in patients with shock versus normotensive patients**

<table>
<thead>
<tr>
<th>Group</th>
<th>Interleukin-12-p40 (pg/ml)</th>
<th>Interferon-γ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean no. of samples</td>
<td>median (range)</td>
</tr>
<tr>
<td>Shock patients</td>
<td>29</td>
<td>196(&lt;50-504)</td>
</tr>
<tr>
<td>Normotensive patients</td>
<td>151</td>
<td>345(89-2550)*</td>
</tr>
</tbody>
</table>

Wilcoxon-Mann Whitney test: *: levels of IL-12 in normotensive patients higher than those in patients with shock (p<0.01); +: levels of IFN-γ in normotensive patients higher than those in patients with shock (p<0.01).
Table 4: Relation levels of IL-12-p40 and IFN-γ to clinical, laboratory and haemodynamic variables

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Interleukin-12-p40 (pg/ml)</th>
<th></th>
<th></th>
<th>Interferon-γ (pg/ml)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean no. of samples</td>
<td>correlation coefficient</td>
<td>p-value</td>
<td>Mean no. of samples</td>
<td>correlation coefficient</td>
<td>p-value</td>
</tr>
<tr>
<td>Leukocyte count</td>
<td>178</td>
<td>0.04</td>
<td>0.3</td>
<td>182</td>
<td>0.08</td>
<td>0.6</td>
</tr>
<tr>
<td>Plasma protein level</td>
<td>178</td>
<td>0.15</td>
<td>0.04</td>
<td>182</td>
<td>0.2</td>
<td>0.00</td>
</tr>
<tr>
<td>Platelet count</td>
<td>174</td>
<td>-0.36</td>
<td>0.00</td>
<td>178</td>
<td>-0.2</td>
<td>0.004</td>
</tr>
<tr>
<td>Presence of ascites</td>
<td>168</td>
<td>0.35</td>
<td>0.00</td>
<td>170</td>
<td>0.23</td>
<td>0.002</td>
</tr>
<tr>
<td>Hematocrit value</td>
<td>177</td>
<td>-0.25</td>
<td>0.001</td>
<td>181</td>
<td>-0.12</td>
<td>0.08</td>
</tr>
<tr>
<td>Presence of bradycardia</td>
<td>179</td>
<td>0.13</td>
<td>0.07</td>
<td>183</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Discussion

In this study we found elevated plasma levels of IL-12-p40 in the majority of patients and elevated plasma levels of IFN-γ in a substantial number of patients with dengue virus infection when using cut-off values for normal Dutch adults. In vivo, IL-12 may occur in at least 2 different forms: as so-called p40 and p70 forms. The latter form consists of a 35 kD chain linked to a 40 kD polypeptide chain, and is bioactive. The p40 form consists of the 40 kD chain only, and, except maybe for an inhibiting effect on the IL-12 receptor, is not bioactive. Because IL-12-p40 circulates at much higher levels than the p70 form, its measurement is used as an indirect parameter for the production of bioactive IL-12. We also measured IL-12-p70 in some of our patients and found levels to be detectable in only a small part of them (data not shown). An important biological effect of IL-12 is the induction of IFN-γ synthesis. IFN-γ levels significantly correlated with IL-12-p40 levels, supporting the notion that IL-12-p40 in our patients indeed reflected the production of bioactive IL-12.

IL-12 is produced by monocytes or macrophages stimulated by bacterial, parasitic or virus infections. In animal models, IL-12 is indeed released into circulation following a challenge with endotoxin. Consistent herewith, the levels of IL-12-p40 are increased in the majority of children with meningococcal sepsis. During some virus infections, such as those with lymphocyte choriomeningitis virus, IL-12 may impair cytotoxic T lymphocyte generation. Our data do not allow for conclusions regarding the cellular source of IL-12, but one scenario is that it originated from monocytes or macrophages infected with dengue virus.

Surprisingly, the levels of IL-12-p40 were also elevated in most of the apparently healthy children. The relatively comparable levels of IL-12-p40 in patients with dengue.
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virus infection and the apparently healthy children in the outpatients department could be due to the fact that these children were not really healthy. They may have suffered from chronic conditions such as parasitic infestations, which is very common in the area where the study was done. If this is the case, there is no reason to presume that in the dengue patients this was otherwise and therefore there does not seem to be an important IL-12-p40 response in dengue infection. On the other hand, IFN-γ levels were significantly higher in dengue patients than in the apparently healthy children. In another study also increased levels of IFN-γ have been found in children with dengue infections as compared with healthy controls(20). In a recent study IFN-γ concentrations were measured in children with fever caused by dengue infection of less than 72 hours duration(21). The duration of fever in dengue is typically 3-5 days and plasma leakage tends to occur at or around the time of defervescence. Therefore, this study design allowed for the assessment of plasma levels of this cytokine in a time period preceding the period of maximal plasma leakage(22), in contrast to our study in which children were admitted to the hospital not before the third day of the disease and often later. In the above-mentioned study(21) the mean plasma IFN-γ levels were significantly higher in children with dengue than those with nondengue febrile illness, but not different between patients with DHF and those with DF, but few patients with DHF 3 and none with DHF 4 were included. The day of peak IFN-γ production for all patients occurred before or on the day of defervescence. Therefore, in our study, we may have missed peak levels, particularly since IFN-γ has a short plasma half-life. Nevertheless, IFN-γ levels in our study correlated with the presence of ascites which is considered a marker of plasma leakage. In vitro, IFN-γ can increase permeability in endothelial cell monolayers(23) and upregulates expression of TNF receptors on myeloid and epithelial cells, thereby rendering these cells more sensitive to the effects of TNF-α, which can also increase the permeability of endothelial cell monolayers(23,24).

However, in our study, IFN-γ concentrations (and also of IL-12-p40) were significantly lower in patients with dengue shock syndrome than in dengue patients without shock. This finding is not easy to explain, since plasma leakage is considered an important mechanism inducing shock. Although a positive correlation between IFN-γ levels and protein levels and an inverse correlation between IFN-γ and the haematocrit levels also does not support a role for IFN-γ in inducing plasma leakage, this is in contrast with the finding that patients who developed ascites had higher levels of IFN-γ. In summary, in our patients with dengue who were admitted to the hospital relatively late in the course of the disease (by which we may have missed early responses) we did not find an important IL-12-p40 response when compared to apparently healthy children, whereas IFN-γ levels were significantly higher than in these negative controls. The study provided conflicting results as to the potential pathogenetic role of IFN-γ in the induction of plasma leakage. Therefore, the data do not allow for firm conclusions on the role of both cytokines in the pathogenesis of dengue virus infection.
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