Viral Load, Antibody Titers and Recombinant Open Reading Frame 2 Protein-Induced Th1/Th2 Cytokines and Cellular Immune Responses in Self-Limiting and Fulminant Hepatitis E

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Key Words
Hepatitis E virus · Viral load · Cytokines · Antibody titers

Abstract
Objectives: Hepatitis E virus (HEV) is the predominant cause of water-borne epidemics, sporadic acute viral hepatitis (AVH) in adults and fulminant hepatic failure (FHF) among pregnant women and other adults in India. This preliminary study was designed to examine the association of viral load and certain host immune responses with uneventful recovery or progression to FHF. Methods: Viral load, anti-HEV antibody titers, rORF2p-induced Th1/Th2 cytokines levels and cellular immune responses were assessed in 47 patients with self-limiting hepatitis E and 14 FHF-E cases. The controls included 16 anti-HEV-IgM and IgG-negative healthy individuals. Results: In AVH category, the viral load was $2.4 \times 10^4 \pm 1.92 \times 10^4$ copies/ml while except for one, all FHF patients were negative for HEV RNA; anti-HEV-IgM and IgG titers were higher in the FHF group. The lymphocyte proliferative response to rORF2p was comparable in both groups. As compared to AVH, significantly higher levels of both Th1 (IFN-γ, IL-2 and TNF-α) and Th2 (IL-10) cytokines were recorded in FHF patients. Analysis of sequential samples differentiated FHF recovered and fatal patients with respect to IFN-γ and IL-12.

Conclusion: The results document increased Th1/Th2 responses and anti-HEV titers in FHF patients that warrant in-depth immunological studies.

Introduction

Hepatitis E virus (HEV) represents the major cause of water-borne epidemics in India and sporadic acute viral hepatitis among adults (AVH) [1, 2]. High mortality among pregnant women is the characteristic feature of hepatitis E epidemics [3, 4]. Among sporadic patients with fulminant hepatic failure (FHF), 37–62% of adult patients are related to HEV infection [5–7]. Exposure of susceptible individuals to HEV can lead to: (1) subclinical infection, (2) self-limiting clinical hepatitis, and (3) FHF. The basis of differential outcome of HEV infection is not well understood. FHF is one of the most challenging and life-threatening syndromes in clinical medicine and is characterized by massive hepatocellular necrosis and encephalopathy [8]. The etiology of FHF varies greatly worldwide. In developed countries like the United States and UK, the most common cause of FHF is drug-induced liver injury (most commonly acetaminophen toxicity) [9,
Both host and viral factors are associated with outcome of various infections. This preliminary study attempts to assess humoral and cellular responses as well as viral load in individuals with self-limiting hepatitis E and those progressing to FHF to help recognizing mechanisms associated with the development of FHF. The possibility of identifying prognostic markers for progression to FHF was another objective.

**Materials and Methods**

The study protocol was approved by the Human Ethical committee of the BJ Medical College and Sasoon General Hospital, Pune, Maharashtra.

**Study Population**

The patients were classified as AVH or FHF based on standard clinical and biochemical criteria [6, 11]. The diagnosis of hepatitis E was based on the presence of IgM antibodies to HEV (anti-HEV IgM). A study population of 78 (36 males, 12 females) was divided into three groups: (1) AVH group, consisting of 48 acute hepatitis E patients with uneventful recovery, (2) FHF group, comprising 14 hepatitis E patients progressing to fulminant hepatic failure, and (3) control group, including 16 apparently healthy adults negative for both IgM and IgG antibodies to HEV. Based on the outcome of the disease, the FHF patients were further classified as either fatal (n = 6) or recovered (n = 8). The patients as well as controls were enrolled during October 2003–September 2006 from the state of Maharashtra, Western India. FHF was defined when a patient with no history of liver disease developed encephalopathy (recovered group) (n = 14; mean POD: 9.93 ± 1.77 days). In the fatal group, the last sample was obtained within 24 h after the patient had recovered from hepatic encephalopathy (n = 14; mean POD: 9.93 ± 1.77 days).

Sixteen of the 48 AVH patients consented for follow-up. For the first sampling (n = 16), mean post-onset day of clinical symptoms (POD), i.e. number of days after the onset of clinical symptoms on which the sample was collected, was 13.75 ± 1.77 days. Subsequently, three sequential samples were collected at 14, 16, and 20 days’ interval (n = 16, 15 and 10, respectively). Of the 14 FHF patients admitted to the Medicine wards at Sasoon Hospital, Pune, 13 could be followed up daily or on alternate days. The first blood sample was collected within 48 h of onset of hepatic encephalopathy (n = 14; mean POD: 9.93 ± 1.57 days). The last sample was collected after the patient had recovered from hepatic encephalopathy (recovered group) (n = 7; mean POD: 13.57 ± 1.30 days). In the fatal group, the last sample was obtained within 24 h of the patient’s death (mean POD, 16.50 ± 1.42 days).

**Serology**

Blood samples were collected in K3 EDTA and plasma was separated within 4 h of collection. All samples were screened in ELISA for the presence of IgM antibodies against hepatitis A virus (anti-HAV IgM; Hepavase A-96, General Biologicals Corp., Taiwan), hepatitis B surface antigen (HBsAg; Surase B-96, General Biologicals), IgM antibodies to hepatitis B core antigen (anti-HBc IgM; Anticorase B-96, General Biologicals), antibodies to hepatitis C (anti-HCV; Ortho HCV 3.0, Ortho Clinical Diagnostics, USA), anti-HEV IgM and IgG employing recombinant ORF2 protein (rORF2p) [12] and serum alanine amino transferase levels (ALT; Span Diagnostics, India). Titers of anti-HEV IgM and IgG were determined by subjecting two-fold serum/plasma dilutions to ELISA protocols. All specimens from FHF cases were tested for HBV DNA [13] and HCV RNA [14].

**TagMan-Based One-Step Real-Time RT-PCR**

HEV RNA load (copies/ml) was determined by Taqman RT-PCR using primers and probe corresponding to HEV ORF1 (7300 Real Time PCR system, Applied Biosystems, Foster City, Calif., USA) according to the protocol described earlier [15]. Briefly, a 1,067-bp fragment of HEV genome (4,632–5,698 nt) was PCR amplified and cloned into pGEM-T Easy vector (Promega, Madison, Wisc., USA). Plasmid was linearised and in vitro transcription was done using T7 Riboprobe In Vitro Transcription System (Promega). Standard curve showed linear relationship (r² = 0.99) from 10 to 10¹⁰ RNA copies/reaction. The sensitivity of the assay was 100 copies/ml. HEV RNA was extracted from samples using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) and used. Every sample (plasma/serum) collected from AVH and FHF categories of patients were screened for the detection/quantitation of HEV RNA.

**Stimulation of PBMCs and Cytokine Assay**

Cytokine assay was carried out as previously described [16]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated, washed and resuspended in RPMI 1640 (Invitrogen, Carlsbad, Calif., USA) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen). PBMCs at a concentration of 2 × 10⁶ cells/well were set up in 6-well plate (Nunc, Denmark). Cells were stimulated with HPLC purified rORF2p from genotype-1 HEV [17]. Supernatants from cultured cells were harvested after 72 h of stimulation and stored at −70°C. Levels of IFN-γ, IL-2, IL-12, TNF-α, IL-10, and IL-4 were measured using ELISA ETM SETS, BD Pharmingen, San Diego, Calif., USA) in accordance with the manufacturer’s instructions. The sensitivity of ELISA SET used was 31.3 pg/ml for IL-12 and 4.7 pg/ml for IFN-γ and TNF-α and 7.8 pg/ml for IL-2, IL-10, and IL-4, respectively.

**Lymphocyte Proliferation Assay**

Lymphocyte proliferation assay (LPA) was carried out as previously described [17]. Briefly, 1 × 10⁵ cells/well PBMCs were cultured quadruplicately in 96-well flat bottom plate (Nunc, Denmark) at 37°C with 5% CO₂. Cells cultured with media and with PHA (5 µg/ml) (Sigma, USA) served as controls. 20 µg/ml of rORF2p was used as recall antigen. The optimum doses of PHA and rORF2p were decided based on previous dose response studies. On the 5th day of culture, the cells were pulsed with 1 µCi of tritiated thymidine (BARC, Mumbai, India) for 24 h. Cells were harvested onto GF/C filter (Whatman, UK) membrane and counts taken on a β-counter (LKB Pharmacia, Sweden) using standard protocol. Data for each sample was expressed as stimulation index (SI), calculated as the ratio between mean counts per minute (cpm) obtained in the presence and absence of the antigen. Samples showing SI values ≥3 were considered responders. The laboratory is approved as radioisotope laboratory by Atomic Energy Regulatory Board, Radiological Safety Division, Government of India.
**Statistical Analysis**

Inter- and intragroup comparisons were made by the Mann-Whitney and Wilcoxon signed-rank tests. The χ² test and Fisher’s exact test were used to compare frequencies. The statistical software SPSS 11.0 was used for statistical analyses. The values for POD of sample collection, age in years and cytokine levels (picogram/ml) are expressed as mean ± SE.

### Results

#### Study Subjects

The patient group included only anti-HEV IgM positives. Thus, all the patients were negative for anti-HAV IgM, anti-HBc IgM and HBsAg. One HBsAg carrier with superimposed HEV infection was excluded from analysis (AVH category), the total number for analysis being 47.

The control group subjects were negative for hepatitis viral markers of recent infection as well as HBsAg, anti-HCV and anti-HEV IgG antibodies. Acute HEV infection was recorded more frequently among males than females (n = 35, 74.47% vs. n = 12, 25.53%, p = 0.000). The mean age was 27.09 ± 1.45 years. Of the 14 FHF patients, 5 were males and 9 were females; male to female ratio was 1:1.8. All the 14 FHF patients were negative for HBV DNA and HCV RNA.

Table 1 provides details of the FHF group. Overall case fatality rate (CFR) in this group was 42.86% (6/14). The mean age of FHF patients with fatal outcome (n = 6; 25.5 ± 0.94 years) was similar to that of surviving patients (n = 8; 26.4 ± 1.64 years). Of the 14 FHF cases, 7 were pregnant women (7/14, 50%). The mortality rate was higher among pregnant (4/7, 57.14%) than non-pregnant women (0/2). During the third trimester of pregnancy, very high (4/5, 80%) mortality was recorded. Importantly, 2/5 male FHF patients succumbed to the disease. The CFRs were 27.3% (3/11) and 100% (3/3), respectively, when the interval between the onset of jaundice and encephalopathy development was ≤2 and >2 weeks.

#### Viral Load

The first plasma sample collected from 47 AVH (mean POD, 12.84 ± 1.99 days) and 14 FHF patients (mean POD, 9.93 ± 1.57 days) were subjected to quantitation of HEV RNA by real-time RT-PCR. HEV RNA was detected in 22 of 47 (46.81%) AVH patients and the mean copy number was $2.4 \times 10^4 ± 1.92 \times 10^4$ copies/ml of plasma. Interestingly, HEV RNA could be detected only in one pregnant FHF patient with fatal outcome (1/14, 7.14%) ($4.17 \times 10^3$ copies/ml of plasma). No viral RNA could be detected in the follow-up samples of both AVH and FHF group patients.

#### Antibody Profiles

Anti-HEV IgM titer was observed earlier to be a function of time of sample collection after the onset of jaundice [1]. Therefore, for the comparison of titers, based on the post-onset week of collection of blood after the appearance of clinical symptoms, AVH and FHF patients were divided into two categories: <2 weeks and 2–4 weeks. Among FHF patients bled within two weeks, both anti-HEV IgM and IgG titers were significantly higher than the AVH group (p = 0.000 and p = 0.012, respectively) (fig. 1). Only IgM titers were significantly higher (p = 0.01) when samples obtained after >2 weeks were compared.

#### Th1/Th2 Cytokines

**AVH and Controls**

Only IL-12 was significantly elevated in the AVH group when compared with the controls (p = 0.011). Though statistically insignificant, IFN-γ levels in patients with AVH were 10.77 times higher than healthy controls ($58.46 \pm 19.55$ vs. $5.43 \pm 3.48$ pg/ml) (fig. 2). TNF-α and IL-2 levels were similar in AVH and control groups. No significant difference was found between AVH and control subjects for IL-10 (fig. 2) and IL-4 (AVH: $3.68 \pm 0.86$ pg/ml; control: undetectable).

**AVH and FHF**

Production of IFN-γ, IL-2 and TNF-α were significantly higher in patients with FHF than in those with AVH (p = 0.017, p = 0.002 and p = 0.000, respectively).

### Table 1. Clinical and demographic profile of FHF patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Recovered</th>
<th>Fatal</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (%)</td>
<td>8 (57.14)</td>
<td>6 (42.86)</td>
<td>14</td>
</tr>
<tr>
<td>Age</td>
<td>26.4 ± 1.64</td>
<td>25.5 ± 0.94</td>
<td>26 ± 1.39</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>19–45</td>
<td>23–35</td>
<td>19–45</td>
</tr>
<tr>
<td>Range</td>
<td>3:5</td>
<td>2:4</td>
<td>5:9</td>
</tr>
<tr>
<td>M:F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnancy:nonpregnancy</td>
<td>3:2</td>
<td>4:0</td>
<td>7:2</td>
</tr>
<tr>
<td>(female, n = 9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jaundice-encephalopathy interval, days</td>
<td>7.5 ± 1.1</td>
<td>13.17 ± 1.42</td>
<td>9.93 ± 1.57</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>3–13</td>
<td>7–20</td>
<td>3–20</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jaundice-encephalopathy interval, %</td>
<td>8 (72.7)</td>
<td>3 (27.3)</td>
<td>11 (78.6)</td>
</tr>
<tr>
<td>≤ 2 weeks</td>
<td>nil</td>
<td>3 (100)</td>
<td>3 (21.4)</td>
</tr>
<tr>
<td>&gt; 2 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

...
IL-12 levels in AVH and FHF patients were comparable (p > 0.05). IL-10 levels were significantly higher in FHF than AVH group (p = 0.008). IL-4 levels between FHF and AVH groups were comparable (1.0 ± 0.95 and 3.68 ± 0.86 pg/ml, respectively). Serum ALT levels were significantly higher in FHF patients (p = 0.000) (fig. 3).

Analysis of the sequential samples collected from AVH patients documented that IL-12 levels at the first sampling (n = 16, mean POD: 13.75 ± 1.77 days) were significantly higher compared to controls (p = 0.011) (fig. 4). At the time of the second sampling (n = 16, mean POD: 27.75 ± 1.77 days), IFN-γ levels rose to significant levels (p = 0.005) when compared to the first sample (fig. 4). At this time, the IL-12 levels returned to normal. IL-12 and IFN-γ levels were below the detection limits at the third (n = 15, mean POD: 42.93 ± 1.97 days) and fourth samplings (n = 10, mean POD: 64.3 ± 1.14 days) (<31.3 and <4.7 pg/ml, respectively).

Correlation between Cytokines and Outcome

At Admission. There was no significant difference in rORF2p-induced Th1/Th2 cytokines levels in FHF recovered (mean POD, 7.5 ± 1.1 days) and fatal groups (mean POD, 13.17 ± 1.42 days) (table 2).

At the Time of Death/Recovery. In contrast to the insignificant difference at the time of first sampling, higher IFN-γ and IL-12 levels were noted at last sampling in FHF recovered group as compared to fatal group (p = 0.042 and 0.011, respectively) (fig. 5). Due to the non-availability of samples, TNF-α level could not be determined for repeat specimens.

Lymphocyte Proliferative Response to rORF2p

Of the 47 AVH patients investigated, 31 could be subjected to the LPA. PBMCs of 7/31 (22.58%) of acute, 4/8 (50%) of FHF patients and 0/10 controls responded to
rORF2p. Among the responders, sequential samples were available for 3 FHF and 3 AVH patients and all responded till the last sample was collected 6–28 days after the initial sample. Lymphoproliferative response was recorded till the 48th POD for the AVH and the 12th POD for FHF, the last samples collected from these categories. The responders and nonresponders in both categories were compared with respect to IFN-γ and IL-12 cytokines levels (fig. 6). However, no association was recorded.
Discussion

This study confirms two of the earlier observations: high mortality in pregnant women, especially in the third trimester [3, 4], and HEV to be an important cause of FHF in non-pregnant women as well as men [18]. Overall mortality among FHF category was very high, i.e. 42.86%.

In 1993, during a common-source outbreak of hepatitis E in Karad, the ratio of clinical:subclinical HEV infections among pregnant women was estimated by us to be 1:13 and a substantial proportion of the subclinical infections were in the third trimester [19]. A crucial role of host immune response was envisaged. However, due to lack of a convenient cell-culture or animal model leading to large amounts of the virus of sufficient purity required for various immunological assays, such studies were hampered. Though rhesus monkey is an animal model for HEV, pregnant rhesus monkeys in the third para do not develop severe course of the disease [20]. Therefore, understanding the mechanism of the association of the pregnancy status with the severity of the disease cannot be studied in an animal model.

With the expression of the capsid protein (rORF2p) in the baculovirus expression system [17], a preliminary study was conducted during 2003–2006. Since all the FHF patients were admitted to a single unit of a medical college hospital, the treatment protocol was not responsible for differential outcome of FHF. An important observation was that when the patient developed FHF ≤2 weeks of the development of jaundice, proportion of deaths (3/11, 27.3%) was less than those developing after 2 weeks (3/3). Thus, early development of FHF may indicate a better prognosis.

An unexpected observation of absence of HEV RNA in all but one FHF cases suggests that the extensive liver damage may not be associated with excessive replication of the virus. It was not possible to obtain liver biopsies, and therefore actual replication in the liver cells could not be ascertained. These results are in accordance with Suzuki et al. [21] showing the absence of HEV RNA in 3 FHF patients. All HEV RNA positives investigated since 1981 belonged to genotype 1, and therefore the outcome was not related to infecting genotype (data not shown).

Irrespective of the outcome of the disease, the FHF patients bled within 2 weeks of onset of jaundice had significantly higher anti-HEV IgM and IgG titers. Subsequently, only IgM titers were higher. The role of overproduction of antibodies in the pathogenesis of FHF needs further investigations. A lack of correlation between the proliferative responses to HEV capsid protein and the presence of HEV-specific IgG in plasma was observed. Lymphoproliferative response to rORF2p was not different in AVH or FHF cases; T cell epitopes on rORF2p were recognized by both patient categories.

Comparison of Th1 and Th2 cytokine levels led to some interesting findings. Earlier, we had used plasma samples for the estimation of cytokine levels during different stages of acute-resolved hepatitis E. However, in plasma the levels remain below the detection limits of the assay. This was in sharp contrast to the observations with

Table 2. Production of cytokines from PBMCs of FHF recovered (POD, 7.5 ± 1.1 days) and fatal patients (POD, 13.17 ± 1.42 days) at the time of admission in response to rORF2p antigen

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>FHF recovered (n = 8)</th>
<th>FHF fatal (n = 6)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>209.26 ± 74.82</td>
<td>100.22 ± 91.35</td>
<td>NS</td>
</tr>
<tr>
<td>IL-2</td>
<td>35.19 ± 15.45</td>
<td>177.57 ± 80.01</td>
<td>NS</td>
</tr>
<tr>
<td>IL-12</td>
<td>170.11 ± 120.90</td>
<td>444.19 ± 144.19</td>
<td>NS</td>
</tr>
<tr>
<td>TNF-α</td>
<td>364.24 ± 19.84</td>
<td>280.22 ± 19.10</td>
<td>NS</td>
</tr>
<tr>
<td>IL-4</td>
<td>1.31 ± 1.31</td>
<td>ND</td>
<td>NS</td>
</tr>
<tr>
<td>IL-10</td>
<td>148.88 ± 96.66</td>
<td>330.28 ± 156.53</td>
<td>NS</td>
</tr>
</tbody>
</table>

ND = Not detected; NS = FHF recovered vs. fatal, not significant (p > 0.05).

Fig. 6. Comparison of IFN-γ and IL-12 levels among AVH and FHF patients showing lymphoproliferative response/no response. Values indicate mean ± SE pg/ml. NS = Nonsignificant.
hepatitis A [22]. As a result, we measured the cytokine levels from the supernatants of PBMCs stimulated with HEV-specific recombinant antigen. In AVH, only IL-12 (Th1) levels were elevated when compared to controls. In contrast, significantly higher levels of both Th1 (IFN-γ, IL-2 and TNF-α) and Th2 (IL-10) cytokines were present in FHF patients when compared with the AVH category (fig. 3). The concomitant significant rise in serum ALT levels in FHF patients indicating degree of liver damage suggests probable role of these cytokines in pathogenesis of HEV infection. A substantial increase in IL-10 production along with the higher levels of Th1 cytokines in FHF patients indicates involvement of anti-inflammatory response. It is likely that during FHF, IL-10 was released to limit inflammatory response probably induced by IFN-γ, IL-2 and TNF-α. Thus, both inflammatory and anti-inflammatory reactions were present in FHF patients.

To understand the association of these cytokines with the outcome of FHF, fatal and recovered groups were compared. At the time of admission, no significant difference was recorded. However, during the later stages of the disease, IFN-γ and IL-12 levels were significantly higher in recovered than fatal patients strongly suggesting an important role of these cytokines in modulating severe course of the disease. We need to investigate these cytokines/related mechanisms in FHF patients during subsequent studies.

We further compared IFN-γ and IL-12 cytokine levels between both categories of the patients with respect to lymphoproliferative response. No significant correlation was recorded suggesting that T cell activation, as assessed by LPA, may not be playing a key role.

An observation of IL-12 administration in pulmonary model of Cryptococcus leading to increased IFN-γ levels deserves mention here [23]. In hepatitis E, an initial rise in IL-12 levels resulting in increased IFN-γ levels in the later samples may be a distinct possibility. Analyses of sequential samples in AVH patients (fig. 4) documented the involvement of IL-12 in inducing IFN-γ at a later stage probably leading to recovery. It seems that patients responding with an increased IL-12 production will promote Th1 development and stimulate the production of IFN-γ. Absence of IL-12 in FHF fatal cases strongly indicates that this cytokine may be instrumental in recovery. A detailed analysis of the roles of IL-12 and IFN-γ in the pathogenesis of hepatitis E must be taken up on priority in countries with high disease endemicity.

In conclusion, the results demonstrate the role of cytokines in self-limiting as well as fulminant hepatitis E and emphasize the need for in-depth immunological studies.

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