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Toll like receptors in self-recovering hepatitis E patients with or without pregnancy

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ABSTRACT

Hepatitis E virus (HEV) causes high mortality among pregnant women. Pathogenesis of HEV, especially during pregnancy, is poorly understood. Our aim was to assess the role of Toll-like-receptors (TLRs) in hepatitis E patients with pregnancy (Antenatal care, ANC) or without pregnancy (non-ANC). The patient categories included acute-phase, non-ANC ($n = 46$) and ANC patients (2nd/3rd trimesters, $n = 13$) and non-ANC patients ($n = 31$) during convalescence. Controls included apparently healthy non-ANC ($n = 30$) and ANC subjects in the first ($n = 10$) and later (2nd/3rd, $n = 20$) trimesters. TLR2/TLR3/TLR4/TLR7/TLR8 levels were determined by flow-cytometry. Cytokine responses induced by TLR-specific-ligands-stimulated-PBMCs from ANC/non-ANC-patients and TLR-signaling-molecules (non-ANC-patients) were measured. PBMCs were used to assess gene expression levels by TaqMan-Low-Density-Array.

Compared to the temporal activation of TLR4/TLR7/TLR8 at protein and mRNA levels, the ANC-patients and controls exhibited reduced TLRs indicative of impaired TLR response. Stimulation of PBMCs with TLR-specific ligands led to the induction of type-I interferons, IFNβ by the non-ANC group and IFNα by the ANC category. Involvement of MyD88-independent (TLR3/TLR4) and MyD88-dependent (TLR4/TLR7/TLR8) pathways and association of TLR4/TLR7/TLR8 with recovery was documented in the non-ANC-patients. Except for robust type-I-interferon response, HEV infection could not modulate pregnancy-related diminished immune response. The results have implications in the understanding of HEV pathogenesis.

1. Introduction

Hepatitis E, a major public health concern in developing countries, is responsible for sporadic and epidemic acute viral hepatitis in adults. Hepatitis E virus (HEV) is predominantly transmitted via fecal-oral route, mainly through contaminated water. During epidemics, HEV is known to be responsible for high mortality among pregnant women (Antenatal care, ANC) especially in the third trimester [1–4]. More than 50% of the reported acute viral hepatitis cases from India are attributed to HEV in adult population [5,6]. In sporadic setting, men and non-pregnant women succumb to fulminant hepatitis E [6].

Though HEV infection is predominantly self-limiting, the fulminant outcome and severity during pregnancy necessitates understanding of the pathogenesis of HEV infection. Such studies are limited primarily because of the lack of small laboratory animal model. Though HEV infects rhesus monkeys, infection of pregnant rhesus monkeys with HEV in the third trimester did not lead to severe disease or mortality eliminating the use of primate models for such studies [7–9]. Pregnancy demands alterations in the immunologic system to accommodate the foetus and protect the mother against pathogens. This may lead to imbalance in the immune responses against invading pathogens.

In vertebrates, innate immune response is the first line of defence against invading microorganisms. The main players in innate immunity are phagocytes such as neutrophils, macrophages

Abbreviations: ANC, Antenatal care (pregnancy); HEV, hepatitis E virus; TLR, Toll like receptors; IRAK, interleukin-1 receptor-associated kinase; IkBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; TBK1, TANK-binding kinase 1; IRF, interferon regulatory transcription factor; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide; poly I:C, polyinosinic:polycytidylic acid; R848, TLR7/8-ligand.

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and dendritic cells. These cells can discriminate between pathogens and self by utilizing signals from the Toll like receptors (TLRs). Mammalian TLRs represent a family of pattern recognition receptors which detect conserved molecular components encoded by microorganisms [10]. Of the 13 mammalian TLRs known so far, TLR2, TLR3, TLR4, TLR7 and TLR8 have been shown to be involved in the immune responses against various viral infections [11]. Despite the identification of pregnant women as a high-risk group for HEV infection with high mortality and the added importance of the innate immune system in host defence during pregnancy, such data are not yet available. We report for the first time, association of TLRs in HEV infection with special reference to pregnancy.

2. Materials and methods

2.1. Ethical statement

National Institute of Virology, Pune, is the nodal organization in India for investigating suspected viral outbreaks and was invited by the state health authorities to investigate hepatitis outbreaks mentioned in the manuscript. Though, for the collection of blood samples from these patients, the institute does not need to obtain the consent, for the use of samples for research studies, written consent was obtained from all the study participants. The consent procedure was approved by the institutional "Ethical Committee for Research on Humans". The healthy pregnant women were bled on the request of the health authorities for the identification of IgM-anti-HEV positives so that they can be monitored for the symptoms and severity of the disease. Again, a written consent was taken for the use of the samples for this study.

2.2. Study subjects

Table 1 provides details of the study population. The diagnosis of hepatitis E was based on the presence of anti-HEV-IgM antibodies in ELISA [5] and only IgM-anti-HEV positives were included in the study. The patient categories included (1) non-ANC hepatitis E patients during the acute ($n = 46$, non-ANC-acute) and convalescent ($n = 31$, non-ANC-Convalescent) phases of the disease (2) pregnant women in the 2nd and 3rd trimester of pregnancy suffering with acute hepatitis E ($n = 13$, ANC-acute). These patients were identified during epidemics of hepatitis E in the state of Maharashtra, India (2009–2011). Two types of apparently healthy, anti-HEV antibodies negative control groups included (1) Non-pregnant subjects ($n = 30$) and (2) pregnant women ($n = 30$, 10/trimester). All the study subjects were screened for both IgG and IgM-anti-HEV antibodies (5), IgM-anti-HAV antibodies, HBsAg, IgM-anti-HBc, anti-HCV and anti-HIV antibodies (ELISA, Abbott, USA). The patients were negative for the serological markers for hepatitis A/B/C/HIV, while the controls were negative for all the markers examined.

A detailed clinical examination was done for all the AVH cases. All AVH-E patients had typical symptoms of acute viral hepatitis, such as sudden onset of fever, nausea, vomiting, weakness, jaundice, and elevated serum ALT levels. The number of individuals in different categories varied for different parameters evaluated.

2.3. Isolation, culture, and stimulation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from controls/patients by density gradient centrifugation using Ficoll-Hypaque (Sigma–Aldrich, USA). Cell pellets were stored in 500 μl *RNA* Later solution (Life technologies, USA) at −80 °C for gene expression analysis. PBMCs (1×10^6 cells) were cultured with RPMI-1640 with 10% fetal bovine serum (FBS), stimulated separately with 10 μg/ml each of TLR ligands poly I:C, LPS or R848 (Invivogen, USA) for 6 h at 37 °C with 5% CO_2, supernatants were stored at −80 °C till tested.

2.4. Flow cytometry analysis

Anti-human antibodies for TLR2-FITC, TLR3-PE, TLR4-PE, TLR7-FITC and TLR8-FITC (Imgenex, USA) were used for cell surface/intracellular staining as describe previously [12]. Stained cells were resuspended in 500 μl PBS with 1% Paraformaldehyde for flow cytometry analysis. Intracellular levels of IRAK4-PE, IkBα-PE, NFkB-PE, TBK1-PE and IRF7-PE were measured by using the BD-PhosFlow kit (BD Biosciences, USA) according to the manufacturer's instructions. Briefly, 100 μl of whole blood was stimulated with TLR ligands (poly I:C, LPS and R848) and incubated at 37 °C with different antibodies, 15 min for IRKA4, 45 min for IkBα and NFkβ and 30 min for TBK1 and IRF7. Cells were fixed with BD-Phos-Flow lysed/Fix Buffer at 37 °C for 15 min, pelleted, washed with PBS, permeabilized by Perm Buffer II/III for 30 min on ice, washed with stain-buffer incubated with fluorochrome-conjugated antibodies of IRAK4, IkBα, NFkB (pS529), TBK1 and IRF7 in the dark at room temperature for 30 min, washed twice with stain-Buffer, and resuspended in same Buffer, for FACS analysis. On the basis of dot plots lymphocytes and monocytes were gated and analyzed using BD-FACS Diva software. TLR levels and other markers were calculated as median fluorescence intensity (MFI).

2.5. Cytokine measurements

Cytokines/interferons released by the PBMCs were determined by using a custom premixed Milliplex Map Kit (Millipore, USA) for 8 cytokines/interferons i.e. IL1α, IL1β, IL6, IL12p40, IL12p70, TNFα and IFNα on Bio-Plex Protein Array System (Bio-Rad, CA); IFNβ levels were measured by IFNβ ELISA kit (PBL interferon source, NZ), as per the manufacturers' instructions. For statistical analysis, a value of 0.2 pg/ml was used for samples showing undetectable concentrations.

Table 1
Characteristic features of study groups.

Categories	No.	Age (mean ± se)	Male:Female	Anti-HEV IgM/IgG status	POD (mean ± se)	ALT (IU/ml; mean ± se)
Non-ANC controls	30	24.8 ± 0.6	16:14	−/−	NA	28.8 ± 1.6
1st trimester ANC controls	10	21.9 ± 1.1	00:10	−/−	NA	26.2 ± 1.1
2nd trimester ANC controls	10	20.8 ± 0.7	00:10	−/−	NA	28.9 ± 1.2
3rd trimester ANC controls	10	21.1 ± 0.8	00:10	−/−	NA	21.1 ± 1.0
Non-ANC acute patients	46	37.6 ± 2.5	30:16	+/+	6.9 ± 1.1	224.6 ± 53.1
Non-ANC convalescent	31	26.3 ± 1.2	19:12	+/+	38.2 ± 2.3	24.8 ± 2.9
2nd + 3rd trimester ANC acute patients	13	22.3 ± 1.1	00:13	+/+	13.5 ± 1.9	109 ± 32.7

NA, not applicable.
POD, post onset disease.