BRIEF REPORT

Influence of Polyproline Region and Macro Domain Genetic Heterogeneity on HEV Persistence in Immunocompromised Patients

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Hepatitis E virus (HEV) can chronically infect immunocompromised patients. The polyproline region (PPR) and the macro domain of ORF1 protein may modulate virus production and/or the host immune response. We investigated the association between the genetic heterogeneity of HEV quasispecies in ORF1 and the outcome of infection in solid-organ transplant patients. Both sequence entropy and genetic distances during the hepatitis E acute phase were higher in patients whose infection became chronic than in those who cleared the virus. Hence, great quasispecies heterogeneity in the regions encoding the PPR and the macro domain may facilitate HEV persistence.

Keywords. hepatitis E; chronic infection; ORF1; polyproline region; macro domain.

Hepatitis E virus (HEV) is a major cause of enterically transmitted non-A, non-B hepatitis. It is also responsible for large outbreaks of waterborne acute hepatitis in tropical and subtropical countries as well as sporadic infections worldwide. Hepatitis E is a zoonotic disease in industrialized countries, with pigs, wild boar, and deer being the major animal reservoirs of HEV [1]. HEV, genus *Hepevirus*, family *Hepeviridae*, is an unenveloped, single-stranded, positive-sense RNA virus. Like all RNA viruses, HEV exists as a mixture of closely related variants defining a quasispecies. The approximately 7.2 kb genome of HEV has a coding region consisting of 3 open reading frames (ORFs). ORF2 encodes the capsid protein; ORF3 encodes a small protein implicated in virus egress [2], whereas ORF1 encodes a nonstructural protein that contains several putative functional domains. These include at least 4 enzyme activities: methyltransferase, cystein protease, helicase, and RNA-dependent RNA polymerase [2]. Homologies with other plant and animal positive strand RNA viruses have been used to identify other domains: the Y domain, the polyproline region (PPR), and the macro domain.

The PPR is genetically very diverse and could correspond to an intrinsically disordered region involved in virus adaptation [3]. In addition, the PPR does not seem to be required for HEV replication in vitro or in vivo [4]. Nonstructural virus genes that are not essential for replication are usually involved in modulating host immune responses [5]. The genomes of several virus families, including all members of *Coronaviridae*, rubella virus, and alphaviruses (*Togaviridae*), and HEV, have macro domains. The macro domain of the mouse hepatitis virus (MHV) influences the pathogenicity of the virus [6].

HEV can lead to chronic hepatitis in solid-organ transplant (SOT) patients [7]. But our knowledge of the factors associated with the development of chronic infection in SOT patients exposed to HEV is far from complete.

Our working hypothesis was that the genetic heterogeneity of the PPR or the macro domain play a role in the outcome of HEV infection in immunocompromised patients, as the PPR could modulate the host immune response and the macro domain could influence virus pathogenicity. We therefore analysed the characteristics of HEV quasispecies at the acute phase of hepatitis E in 2 groups of SOT patients, one whose infection became chronic and the other who cleared the virus.

MATERIALS AND METHODS

Patients and Samples

We studied 14 SOT patients who became acutely infected with hepatitis E between January 2004 and June 2009. The infection was diagnosed by the detecting HEV RNA using the real time polymerase chain reaction (PCR) and immunoglobulin M/immunoglobulin G anti-HEV antibodies by a commercial enzyme-linked immunosorbent assay [8]. Each patient was assigned to one of 2 groups according to the outcome of the infection. The first group (group I; 8 patients) had chronic infections,

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defined by persistently elevated liver enzyme activity and serum that was positive for HEV RNA for more than 6 months after diagnosis. The second group (group II; 6 patients) had resolving infections. Serum samples were collected from all patients at the acute phase of their infection and stored at −80°C.

Each patient underwent an exhaustive clinical and laboratory examination, including the immunosuppressive drugs used, the hepatic enzyme activities, and white blood cell count. The serum concentration of HEV RNA was measured by real-time PCR [9]. Virus genotype was determined by sequencing a 189-nucleotide fragment within the ORF2 gene. The sequences were compared to reference HEV strains (Genbank) as described elsewhere [10].

Amplification of an ORF1 Fragment Encompassing the PPR and the Macro Domain

Nucleic acids were extracted from serum samples and analysed by 1-step RT-PCR with the sense primer HEVORF1-S1 and anti-sense primer HEVORF1-A1 using the Super script III enzyme (Invitrogen, Cergy-Pontoise, France). The sequences of the primers are listed in Supplementary Table 1.

The sequence amplified included the PPR (nucleotide [nt] 2137–2340) and the macro domain (nt 2341–2829), using the genome 1b L08816 as a reference. The PCR products were purified using Qiaprep (Qiagen, Courtaboeuf, France) miniprep kits and stored at −20°C.

In total, 10 ng of the amplified sequence were directly ligated into 1 µL of PCR 4 vector (kit TOPO TA Cloning; Invitrogen) containing a gene conferring resistance to ampicilline. Recombinant plasmids were used to transform *Escherichia coli*-competent cells, and transformants were grown on ampicillin-coated plates at 37°C for 18 hours. The complementary DNA (cDNA) clones (20 from each patient sample) were analysed by PCR with the primers HEVORF1-S1 and HEVORF1-A1 (Supplementary Table 1).

Nucleotide Sequence Analysis

The PCR products of these amplifications were purified (QuickSpin columns; Qiagen) and sequenced using the fluorescent dye terminator method for Big DyeTerminator cycle sequencing (Applied Biosystems, Paris, France) with the primers HEVORF1-S1, HEVORF1-S2, HEVORF1-S3, HEVORF1-A1 and HEVORF1-A2 (Supplementary Table 1) on an Applied Biosystems ABI 3130 XL analyzer. Sequences were aligned using Clustal X and compared with those of HEV strains obtained from Genbank.

Calculation of Genetic Complexity and Diversity

We quantified the complexity of the HEV quasispecies by calculating the Shannon entropy: $S = -\Sigma_i\ (p_i \ln p_i)$, where *pi* is the frequency of each sequence in the viral quasispecies. The normalized entropies for both nucleotides and amino-acids, *Sn*, were calculated using $Sn = S/\ln N$, where N is the total number of sequences analysed.

We quantified diversity as the mean genetic distance calculated for all pairs of nucleotide sequences using Mega 4.0. The numbers of synonymous (*Ks*) substitutions per synonymous site and nonsynonymous (*Ka*) substitutions per nonsynonymous site were calculated with the Jukes-Cantor correction for multiple substitutions using MEGA 4.0. The Ka/Ks ratio is an indicator of the positive (>1) or negative (<1) selection pressure on a quasispecies [11].

Statistical Analysis

Proportions were compared by Fisher exact test. Quantitative variables were compared with the nonparametric Mann–Whitney test. Correlations between complexities or diversities of quasispecies were estimated by calculating Spearman rank correlation coefficient. A *P*-value below .05 was considered to be statistically significant.

Nucleotide Sequence Accession Numbers

The sequences have been deposited in the GenBank database under accession numbers KC911858 to KC912137.

RESULTS

Patient Characteristics

The clinical and biological characteristics of the patients are summarized in Supplementary Table 2. There was no significant difference between patients with chronic infections and those with resolving infections in terms of gender, age, or immunosuppressant treatment. The ALT activities of individuals with a chronic infection tended to be lower. Patients whose infection became chronic had lower total, CD3, CD4, and CD8 lymphocyte counts, but the differences were not significant. The serum HEV RNA concentrations at the acute phase of the 2 groups of patients were similar. They were all infected with HEV genotype 3f strains, except one whose infection was genotype 3c.

Association Between Quasispecies Heterogeneity and the Development of a Chronic Infection

We compared the sequence heterogeneity of 2 regions of ORF1 in patients with chronic HEV infection and those with resolving infections.

The mean nucleotide sequence entropy (nt complexity) of the PPR was higher in group I (0.63 [0.53–0.75]) than in group II (0.43 [0.38–0.52]; $P = .03$). Similar results were obtained for the macro domain (0.73 [0.60–0.91] in group I and 0.59 [0.50–0.54] in group II; $P = .005$) (Figure 1*A*).

The mean amino-acid (aa) sequence entropy (aa complexity) of the PPR was significantly higher in group I (0.51 [0.46–0.67]) than in group II (0.34 [0.20–0.37]; $P = .002$). Similar results were obtained for the macro domain (0.52 [0.43–0.75] in group I and 0.29 [0.24–0.32] in group II; $P = .03$; Figure 1*B*).

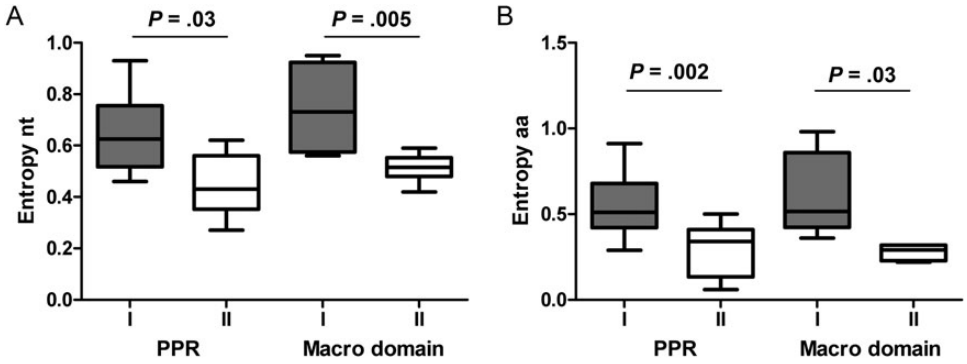

Figure 1. Box plot representation of quasispecies entropy for patients with chronic and resolving hepatitis E virus (HEV) infections. Gray boxes: chronic infection (I). White boxes: resolving infection (II). *A*, Mean values of nucleotide entropy (*B*) mean values of amino-acid entropy. The Mann–Whitney test was used to compare differences between the 2 groups.

The mean pair-wise within-sample genetic distance (diversity) of the PPR was higher in group I (0.0058 [0.0039–0.010]) than in group II (0.0030 [0.0025–0.0032]; $P = .006$). The macro domain diversity was also greater in group I (0.0050 [0.0038–0.011]) than in group II (0.0023 [0.0019–0.0025]; $P = .002$; Figure 2).

The nt complexity and diversity of the PPR were positively correlated with those of the macro domain ($r = 0.69$ $P = .009$ and $r = 0.83$ $P = .0005$, respectively).

Association Between the Ka/Ks Ratio and the Development of a Chronic Infection

The ratios of nonsynonymous substitutions per nonsynonymous site (Ka) and synonymous substitutions per synonymous site (Ks) for the PPRs seemed to be higher in group I (0.51 [0.38–0.70]) than in group II (0.93 [0.61–1.75]), but the difference was not statistically significant ($P = .30$). The Ka/Ks ratios for the macro domains in group I (0.54 [0.17–0.93]) and group II (0.37 [0.27–0.38]; $P = .90$) were similar.

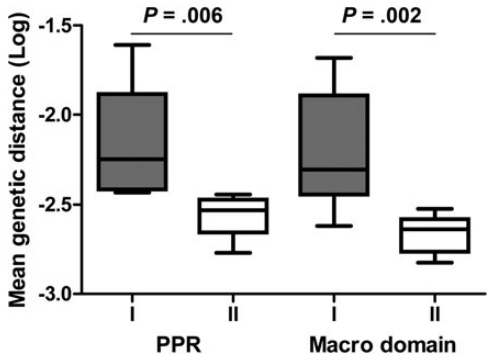

Figure 2. Box plot representation of quasispecies mean genetic distances for patients with chronic and resolving hepatitis E virus (HEV) infections. Gray boxes: chronic infection (I). White boxes: resolving infection (II). The Mann–Whitney test was used to compare differences between the 2 groups.

DISCUSSION

It was shown recently that an HEV infection can evolve to a chronic infection in SOT recipients [7]. The viral determinants associated with the persistence of such an infection are poorly documented. We therefore investigated the influence of the genetic heterogeneity of HEV quasispecies on the outcome of infection, focusing on the PPR and the macro domain.

Both the complexity and diversity of the PPR and the macro domain were higher in viral population of the patients whose infection became chronic than in those who cleared the virus. It has been shown that the quasispecies heterogeneity in the ORF2 region encoding the capsid protein during the acute phase of infection is associated with the development of a chronic HEV infection [12]. Our data support the finding that great genetic heterogeneity of the quasispecies in patients whose infection become chronic seems to favor the appearance of variants that can persist, as reported for HCV infections [13]. Although the diversity of the region preceding the PPR was higher in patients with chronic infection, nt and aa complexities were not different in the 2 groups (data not shown), suggesting that the higher heterogeneity in chronic patients was not a general effect seen across the entire region studied. This great genetic heterogeneity could also reflect an inadequate control of the viral replication, but no correlation was found between quasispecies heterogeneity and viral concentrations. PPR appears to be dispensable for in vitro HEV replication, but it is required for in vivo infection, suggesting that it is involved in infecting cells with innate immunity [4]. Although the role of the macro domain in the replication of HEV is unknown, it does not seem to be essential for the replication of coronavirus in cell culture [14]. It was recently suggested that genes that are

dispensable for virus replication are involved in modulating the host immune response, like down-regulating interleukin 1β (IL-1β) or tumor necrosis factor α (TNF- α) secretion [5]. The macro domain is also involved in the inflammation caused by the mouse hepatitis virus (MHV), and the substitution of a strictly conserved amino-acid residue is responsible for reducing the secretion of inflammatory cytokines [6]. The great quasispecies heterogeneity in patients whose infection became chronic may include some variants that reduce inflammatory cytokine production, which could facilitate HEV persistence. This could explain the lower serum concentrations of interleukin 1 (IL-1) receptor antagonist and TNF-α found in patients whose infection became chronic [12].

We find that the complexities and diversities of the PPR and the macro domain are correlated. These 2 regions may well have evolved together as the protein encoded by ORF1 does not seem to be cleaved [15]. We also studied the correlations between the ORF1 and ORF2 as this study and our previous one on ORF2 diversity [12] were carried out on the same patients (except for 3). We also find that the complexity and diversity of the regions in ORF1 and ORF2 that were studied are correlated (data not shown), suggesting that they, too, have evolved together.

Finally, we find no differences in the Ka/Ks ratios of the studied regions, even though the Ka/Ks ratios of the PPR seemed to be higher in patients who cleared the virus. Alternatively, the Ka/Ks ratio may not allow us to infer differences in selection pressure. In both regions, Ka/Ks ratios indicate negative selection, probably due to structural or functional constraints. A limitation of our study is the small number of patients in each group and an implication of immunological factors in the evolution toward chronicity cannot be excluded.

We conclude that the high genetic heterogeneity of the PPR and the macro domain at the acute phase of an HEV infection is associated with persistence of the virus. This association may be due to the appearance of mutants able to modulate the host immune response. Further investigation is now needed to confirm this association.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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