Expression and Purification of E2/NS1 Protein of Hepatitis C Virus and Detection of Anti-E2/NS1 Antibodies in Chronic Liver Disease Patients

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Key Words
Anti-E2/NS1 prevalence • Chronic hepatitis • E2/NS1 • Hepatitis C virus • Infection • Neutralizing antibodies

Abstract
Glycoproteins on the surface of viral particles present the main target of neutralizing antibodies. The structural proteins of most Flaviviruses are known to elicit neutralizing antibodies and, thus, to help in both the natural resolution of the infection and the protection from challenge with homologous hepatitis C virus (HCV). Because such antigens are associated with the viral clearance in both humans and chimpanzees, we aimed to express the E2/NS1 protein of HCV and to study the role of anti-E2/NS1 antibodies in the natural resolution of HCV infection. The prevalence of anti-E2/NS1 antibodies to recombinant E2/NS1 protein was seen by Western blot in chronic liver disease patients (15 chronic hepatitis and 12 cirrhosis patients), who were positive for anti-HCV and negative for HBV infection. The study also included 2 negative controls (positive for HBV infection and negative for anti-HCV antibodies) and 2 healthy controls (negative for both HBV and HCV infection). Anti-E2/NS1 was present in 20% of the chronic hepatitis and 16% of the cirrhosis patients. None of the controls were positive for anti-E2/NS1 antibodies. Serum samples positive for anti-E2/NS1 antibodies were also positive for HCV RNA by RT/PCR. Accordingly, the presence of anti-E2/NS1 may have very little or no role in the natural resolution of HCV infection.

Introduction
Hepatitis C virus (HCV), which has been identified as the principal agent of parenterally transmitted non-A, non-B hepatitis [5, 20], presents a serious health problem. Current estimates suggest that as many as 85% of the infected individuals remain persistently positive for HCV, and 5–20% of chronic HCV infections are associated with cirrhosis and hepatocellular carcinoma [1].

HCV is a member of the Flaviviridae. It consists of a 9.5-kb single-stranded RNA genome of positive polarity with a single large open reading frame coding for a 3011-amino-acid polyprotein [6, 15, 17, 35]. The polyprotein is then posttranslationally cleaved into various viral pep-
tides. The HCV gene order has been determined as 5' C-E1-E2/NS1-NS2-NS3-NS4A-NS4B-NS5A-NS5B 3' [2, 12, 22, 36]. The only available treatment for HCV infection is interferon-α. Despite extensive research, development of an effective therapeutic agent and a vaccine remains far from reality. The first and the most important step in designing a vaccine is the identification of host and viral components which are involved in the development of neutralizing immunity. The structural proteins of most Flaviviruses are known to elicit neutralizing antibodies and thus, help in the natural resolution of the infection. Earlier, antibodies to purified NS1 protein have been shown to protect immunized mice and rhesus monkeys against challenge with dengue virus [33] and yellow fever virus [32]. Various studies are available which support the hypothesis that anti-E2/NS1 can control HCV infection [4, 11, 19, 38]. Lee et al. [21] have identified a B-cell epitope located in a small domain of E2/NS1, spanning amino-acid residues 528–546. Therefore, it is predicted that anti-E2/NS1 antibodies may have some protective effect in HCV infection. Contrary to the above reports, there are studies which reveal that HCV infection persists despite the presence of a virus-specific cytotoxic T lymphocyte response and circulating antibodies to various HCV proteins [30, 39]. Thus, despite the fact that E2/NS1 is an efficient immunogen, the neutralizing capacity of these antibodies remains to be documented and induction of E2-specific cellular immune responses need to be studied extensively. So, this study was planned with the main aim to determine the prevalence of anti-E2/NS1 antibodies in patients with chronic liver disease due to HCV. To further assess the role of these antibodies in the resolution of HCV infection, RT-PCR of HCV was performed in anti-E2/NS1-positive samples.

**Materials and Methods**

**Source of C-E1-E2**

Plasmid pBRTM/HCV 1-3011 (a generous gift from Dr. C.M. Rice, Washington University; 1-3011 depicts the number of HCV amino acids encoded by the plasmid) with the whole 9.0-kb HCV genome inserted in it, was used as a source for the 2.0-kb fragment C-E1-E2 [12]. It is a pBR322-derived circular plasmid and contains tetracycline-resistant gene.

**Construction of Recombinant Plasmid**

Structural protein encoding fragment C-E1-E2 was PCR amplified from pBRTM/HCV 1-3011. Primers used were sense primer 5' ACC TGG GAA TTC CCC GGG TAC CCT TGG C 3' (position: nt 225–250 of HCV genome) and antisense primer, 5' TAT TAC GGA ATT CTC CAA AGC CGC CTC 3' (position: nt 2235–2259 of HCV genome). PCR was performed under the following conditions: 94°C: 5 min (1 cycle); 94°C: 45 s; 59°C: 2 min; 72°C: 4 min (repeated for 30 cycles), and 72°C: 7 min (1 cycle; Robocycler, Stratagene; Germany). After digestion with EcoRI, the amplified product was eluted from the low-melting-point agarose gel by Supelco Spin columns (Supelco, Bellefote, Pa., USA) and ligated into the EcoRI site of the plasmid pTrc99 A (Pharmacia Biotech). White colonies, which appeared after transformation on the LB-agar/amp/Isopropylthio β-D-galactoside/Xgal plates, were screened for the presence of recombinant plasmid by restriction digestion, SDS-PAGE analysis and Western blot, using rabbit anti-E2/NS1 antisera, WU105 (a generous gift from Dr. C.M. Rice).

**Protein Purification**

Once confirmed, E2/NS1 was purified from 1 liter of culture according to the method described by Maniatis et al. [23] and Martinson et al. [24]. A large proportion of the aggregates was released from the Escherichia coli cells by the use of lysozyme. The proteins contaminating the inclusion bodies were removed by washing them with Triton X-100 and EDTA, and, as expected, most of the protein of our interest was present in the pellet fraction. The denaturant used for solubilizing recombinant E2/NS1 in our study was 10 M urea at pH >9.0. 30 ml of alkaline solution containing 50 mM KH2PO4 (pH 10.7), 1 mM EDTA (pH 8.0) and 50 mM NaCl was added and the solution was stored at room temperature for about 10 min. pH was maintained between 10.7 and 13.0 with KOH. After 30 min, pH was neutralized to 8.0 with HCl, and the solution was further stored at room temperature for 1 h [10]. It was then centrifuged at 12,000 rpm for 15 min at 4°C, and the supernatant was analyzed by SDS-PAGE to determine the extent of solubilization. WU105-specific 35-kd E2/NS1 protein was eluted from the polyacrylamide gel using an Amicon Centrulutor micro-electroeluter (Millipore, Bedford, Mass., USA) according to the manufacturer’s instructions. Polyclonal antibodies were raised in rabbits against this purified protein.

**Prevalence of Anti-E2/NS1 Antibodies in Patient Sera**

27 chronic liver disease patients (15 with chronic hepatitis and 12 with cirrhosis) who were positive for anti-HCV antibodies (Ortho 3.0 ELISA, Germany) and negative for HBsAg (Monolisa; Sanofi, France) and HBV DNA (PCR was performed with primers designed from the surface region of the HBV genome) were included in this study. Two healthy controls (negative for both anti-HCV and HBV DNA) and 2 negative controls (positive for HBV DNA and negative for anti-HCV) were also included in the study. The following primers were used for PCR of HBV DNA: antisense: 5' TTC AAG CCT CCA AGC TGT GCC TTG G 3' (nt 2274–2294), and sense: 5' TCT GCG ACG CGG CGA TTG AGA 3' (nt 2235–2259). To see the prevalence of anti-E2/NS1 in the patient sera, Western blot was performed as described by Towbin et al. [37]. Both solubilized and purified proteins (E2/NS1) were transferred separately onto the nitrocellulose paper and were reacted with anti-HCV-positive serum or normal healthy/negative control, rabbit anti-E2/NS1 antisera, and color was developed by adding the substrate. RT/PCR was also performed for the serum, which was positive for the anti-E2/NS1 antibodies, and the primers for RT/PCR were designed from the 5' non-translated region.