RAPID COMMUNICATION

Serum Hepatitis C Virus Sequences in Posttransfusion Non-A, Non-B Hepatitis

By Motohiro Shibata, Tsuneo Morishima, Toyoichiro Kudo, Takako Maki, Shigeo Maki, and Yoshiyuki Nagai

We investigated 17 patients (12 males and 5 females, ages 2 to 57 years old) with posttransfusion non-A, non-B hepatitis to determine relationships between clinical courses and hepatitis C virus (HCV) markers. The patients were grouped according to time course of abnormal serum alanine aminotransferase (ALT) levels into three categories (chronic biochemical disease, biochemically resolved chronic disease, and acute disease). Latest serum samples (1.3 to 10.8 years after blood transfusion) were used to detect antibodies against C100-3 antigen (anti-HCV) by enzyme-linked immunosorbent assay and HCV sequences by polymerase chain reaction (PCR) assay. Of the 17 patients, 13 patients (76.5%) were anti-HCV positive and 8 patients (47.1%), including one anti-HCV negative case, were positive for HCV RNA. In total, 14 patients (82.4%) were positive for either HCV markers.

Recently, a cDNA from a small, positive-strand RNA virus, designated hepatitis C virus (HCV), was cloned, and diagnostic tests to detect antibodies against the recombinant C100-3 antigen (anti-HCV) have shown that anti-HCV was positive in about 80% of patients with posttransfusion non-A, non-B hepatitis (NANBH) and in 50% of patients with sporadic NANBH. In addition, recipients of anti-HCV positive blood products were 20 times more likely to have posttransfusion NANBH than recipients of anti-HCV negative blood. These results suggest that serum anti-HCV is one of the important markers for infectivity. Furthermore, the polymerase chain reaction (PCR) technique has enabled investigators to detect HCV sequences in liver tissues and sera from patients with NANBH as well as in sera from implicated blood donors. These results provide direct evidence that HCV is the major causative agent of NANBH.

A long-term observation of posttransfusion NANBH showed that more than one-half of patients develop chronic liver disease. Anti-HCV persisted for more than 6 years in patients with chronic liver disease, while it disappeared in a mean of 4 years in patients with acute liver disease that resolved within the first year. However, much less is known about the relation between clinical course and serum HCV sequences, a direct marker of viremia. In this study, we have measured HCV RNA and anti-HCV in sera from patients with different courses of posttransfusion NANBH and show a correlation between abnormal liver function and viremia.

With respect to clinical course, HCV RNA was detected in six of eight patients (75%) with chronic biochemical disease, and in two of five patients (40%) with biochemically resolved chronic disease. HCV RNA was not detectable in convalescent sera from four patients with acute disease. These results show that there is a relationship between clinical status and HCV viremia, but that normal liver function tests do not always represent the clearance of the virus. Viremia in two patients with normal ALT level suggests that hepatitis is not only caused by viral cytopathic effects, but also by immunologic reactions against virus-infected cells. Thus, PCR is useful in determining the persistence of HCV infection as well as to diagnose anti-HCV negative HCV infection.

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PCR. Two sets of oligonucleotide primers were synthesized for PCR. One pair of primers was to amplify a 458-bp sequence from the putative nonstructural region 3 of HCV RNA. 12 A sense primer, HCP10, was 5′-TGTCACTCAGGTCGATTTCA GGTCC-TAT (HCp20), and an antisense primer, HCP11, was 5′-ACTCTCACATTTTGATCCCACGAG-3′. The other pair of PCR primers were derived from a 5′-terminal noncoding region. An internal probe, HCpNS3, was 5′-GGAACTACTGTCTTCACGCAGA-3′, and an antisense primer, HCP14, was 5′-TGAGCTCATACCAAGCAGCAG-3′. The other pair of PCR primers was to amplify a 458-bp sequence from the putative nonstructural region 3 of HCV RNA cloned in Japan13 to amplify a 298-bp sequence. A sense primer, HCP14, was 5′-GGAACTACTGTCTTCACGCAGA-3′, and an antisense primer, HCP15, was 5′-TGTCACTCAGGTCGATTTCA GGTCC-TAT (HCp20), and an antisense primer, HCP11, was 5′-ACTCTCACATTTTGATCCCACGAG-3′. An internal probe, HCP20, was 5′-AAGCACCTAT CAGGAGATA-3′.

RNA was prepared from 100 μL serum by treating in GIT buffer (4.0 mol/L guanidium thiocyanate, 0.1 mol/L Tris-HCl [pH 7.5], 1% 2-mercaptoethanol) and 0.5% sodium lauryl sarcosinate, followed by centrifugation through 5.7 mol/L CsCl/0.01 mol/L EDTA cushion at 140,000g for 16 hours in a SW 41 rotor (Beckman, Palo Alto, CA). The resulting RNA pellets were resuspended in H2O, and PCR was then performed according to the method described.12 Briefly, cDNA was synthesized with avian myeloblastosis virus (AMV) reverse transcriptase (BRL, Gaithersburg, MD) using an antisense primer. After adding a sense primer and Taq polymerase (2.5 U, Perkin Elmer Cetus, Norwalk, CT), 40 cycles of PCR were performed as follows: denaturation for 1 minute at 94°C, annealing for 2 minutes at 55°C (HCp10/11) or 50°C (HCp14/15), and extension for 3 minutes at 72°C. A 10-μL aliquot of PCR products was subjected to agarose gel electrophoresis, and visualized under UV fluorescence. The gels were then blotted onto Nytran (Schleicher & Schuell, Germany), and the blots were hybridized to an internal probe end-labeled with γ-32P-adenosine triphosphate (ATP) (New England Nuclear, Boston, MA) in 2X SET (1X SET = 0.15 mol/L NaCl, 1 mmol/L EDTA, 20 mmol/L Tris-HCl [pH 7.8]) containing 5X Denhardt’s solution, 100 μg/mL salmon sperm DNA, and 0.1% sodium dodecyl sulfate (SDS) at 45°C. The blots were washed in 2X SET and 0.1% SDS at 35°C, and autoradiographed.

The sensitivity of the PCR assay was determined by analyzing an infectious human serum with HCV titers of approximately 106 chimpanzee infectious doses (CID)/mL, kindly provided by the Association of Non-A, Non-B Hepatitis Research in Japan, Ministry of Welfare, Japan. Serial dilutions were prepared and assayed by PCR and Southern blot hybridization. Both primer sets (HCp10/11 and HCp14/15) could detect approximately 1 CID of the virus.

To avoid false positives with PCR, we applied the contamination prevention measures of Kwok and Higuchi.13 All PCR and Southern blots were run and analyzed in a blinded fashion.

**Anti-HCV assay.** Antibodies against C100-3 antigen were measured with the Ortho anti-C100-3 enzyme-linked immunosorbent assay (ELISA; Ortho, Raritan, NJ).

**Statistical analysis.** The differences between dichotomous variables were analyzed by Fisher’s exact test.

**RESULTS**

Serum was obtained from 17 patients with posttransfusion NANBH at latest biochemical examinations (1.3 to 10.8 years after blood transfusion) and examined for anti-HCV by ELISA and HCV RNA by PCR. Table 1

### Table 1. Summary of Patients Grouped According to Clinical Course

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Years After Blood Transfusion</th>
<th>ALT (IU/L)</th>
<th>HCV RNA</th>
<th>Anti-HCV</th>
<th>HCP10/11*</th>
<th>HCP14/15†</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Blood Transfusion</td>
<td>Peak</td>
<td>Latest</td>
<td></td>
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<tr>
<td>Group A (NANBH with chronic biochemical disease)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1. (13/M)</td>
<td>2.5</td>
<td>784</td>
<td>47</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2. (57/M)</td>
<td>1.2</td>
<td>76</td>
<td>40</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3. (10/F)</td>
<td>2.2</td>
<td>391</td>
<td>48</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4. (11/M)</td>
<td>3</td>
<td>1310</td>
<td>250</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>5. (6/M)</td>
<td>1.3</td>
<td>1626</td>
<td>54</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6. (11/M)</td>
<td>6.5</td>
<td>399</td>
<td>33</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7. (12/M)</td>
<td>2</td>
<td>1224</td>
<td>19</td>
<td>+</td>
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<td>8. (14/M)</td>
<td>1.5</td>
<td>1247</td>
<td>937</td>
<td>-</td>
<td>+</td>
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<td>9. (16/M)</td>
<td>10.8</td>
<td>130</td>
<td>9</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10. (56/F)</td>
<td>4.8</td>
<td>282</td>
<td>10</td>
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<td>+</td>
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<tr>
<td>11. (9/F)</td>
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<td>785</td>
<td>12</td>
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<tr>
<td>12. (14/M)</td>
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<td>13. (11/F)</td>
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<td>891</td>
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<td>Group C (NANBH with acute disease)</td>
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<td></td>
<td></td>
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<td>14. (7/M)</td>
<td>1.8</td>
<td>774</td>
<td>9</td>
<td>+</td>
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</tr>
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<td>15. (6/M)</td>
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<td>22</td>
<td>+</td>
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<td>-</td>
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<tr>
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<td>487</td>
<td>10</td>
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<td>-</td>
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<tr>
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<td>373</td>
<td>13</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</table>

*HCP10/11: PCR to amplify NS3 region.
†HCP14/15: PCR to amplify 5′-terminal noncoding region.

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shows the summary of the results of ELISA and PCR assays together with clinical data. The 17 patients were grouped according to their clinical courses into three categories as defined in Materials and Methods. Anti-HCV was positive in most patients of group A and B (Table 1), and a total of 13 patients (76.5%) had anti-HCV (Table 2). HCV RNA was amplified with two sets of primers from NS3 region (HCp10/11) or 5'-terminal sequences (HCp14/15). Representative results of PCR are shown in Fig 1. The DNA products had an expected size of 458 bp (HCp10/11) or 298 bp (HCp14/15), and hybridized with the internal probes. The two primer sets worked equally in detecting the viral genome with one exception (case 6), and a total of eight patients (47.1%) were positive for HCV RNA. With respect to clinical course, HCV RNA was detected in six of eight (75%) patients with chronic biochemical disease (group A), and two of five (40%) patients with biochemically resolved chronic disease (group B). It has to be noted that a single case (no. 8) was positive for HCV RNA even though anti-HCV was negative. In contrast, all four patients with acute disease (group C) were negative for HCV RNA in the convalescent sera.

In total, 14 patients (82.4%) were positive for either HCV markers. Six patients who received blood transfusions but did not develop hepatitis and 16 healthy individuals were negative for both HCV RNA and anti-HCV (Table 2).

DISCUSSION

In this study, we examined sera for HCV RNA and anti-HCV, and found that 82.4% of patients with posttransfusion NANBH were positive for either HCV markers. These results confirm that HCV is the major causative agent for posttransfusion NANBH.1

HCV has considerable variations in nucleotide sequences.10,11 These variations could cause false-negative results by PCR. To minimize false-negative results, we used two different sets of primers for PCR. A single case (no. 6), which is positive only by one of the two primer sets, appears to reflect such sequence variability. However, the fact that the two primer sets gave the same results in the other cases suggests that the regions corresponding to the primer sequences could be conservative.

Van der Poel et al7 reported that several cofactors in anti-HCV positive blood products indicate a high risk of HCV infectivity. A raised serum ALT was a highly significant cofactor for recipient HCV infection, compared with a normal serum ALT (86% v 11%). Therefore, in this study, patients with NANBH were grouped by time course of abnormal serum ALT levels into three categories. Serum HCV RNA was detected in 75% of the group A patients with chronic biochemical disease, 40% of the group B patients with biochemically resolved chronic disease, and none of the group C patients with acute disease. These results show that there is a relation between abnormal liver function and HCV viremia. However, it should be noted that HCV RNA was positive in two patients with biochemically resolved chronic disease. Especially noteworthy, patient no. 9 was HCV RNA positive despite normal biochemical examinations for more than 8 years. This case should be carefully followed-up for exacerbation of liver function. Alternatively, there might be asymptomatic HCV carriers, as suggested by the presence of normal blood donors who transmit HCV hepatitis to recipients.

There is a long delay (mean, 22 weeks) between infection and anti-HCV seroconversion,4 and, therefore, PCR is particularly useful for diagnosing HCV infection during the acute phase.14 In some patients with chronic HCV infection the delay of anti-HCV seroconversion seems to be longer, or anti-HCV may disappear soon.8 Weiner et al4 reported HCV RNA could be detected in liver from anti-HCV negative patients with chronic NANBH. In this study, we also found an anti-HCV negative case who was positive for serum HCV genome. Thus, the absence of anti-HCV does not always exclude HCV infections in chronic NANBH, and PCR is useful for diagnosing such cases.

In conclusion, analysis of patients with posttransfusion NANBH for their clinical course and serum HCV RNA showed that present or recent elevation of serum ALT is likely to be associated with viremia. However, normal liver function does not always represent the clearance of the virus because HCV RNA was also detected in some patients with biochemically resolved chronic disease. These results suggest that hepatitis is not only caused through injury of hepatic cells by viral cytopathic effects, but also by immunologic reactions against virus-infected cells, as in the case of hepatitis B virus infection.15

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