Detection of hepatitis C virus antibodies with new recombinant antigens: assessment in chronic liver diseases

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A new serological assay to detect antibodies against hepatitis C, based on a recombinant protein (BHClO) which incorporates structural and non-structural viral antigens, was tested in 67 healthy subjects and 409 patients with various forms of liver disease. Results were compared with the current assay based on the recombinant non-structural viral antigen c100 and with the recently introduced second-generation assay, Ortho2. None of the healthy subjects was positive by any of the assays. In patients with chronic non-A, non-B hepatitis the prevalence of anti-BHClO was 96.8%, higher than anti-c100 (83.3%, p < 0.001) and similar to Ortho2 (94.3%). False-positive results were less frequently found when BHClO was used. These findings show that assays incorporating structural and non-structural antigens provide higher sensitivity to detect hepatitis C virus infection and they define an almost exclusive role of hepatitis C virus in the genesis of chronic non-A, non-B hepatitis.

Key words: HCV antibody; Polymerase chain reaction; Chronic hepatitis C; Chronic hepatitis B; Alcoholic liver disease; Primary biliary cirrhosis; Hepatocellular carcinoma

Since 1989 with the discovery of part of the RNA genome of the non-A, non-B hepatitis (NANBH) agent, now known as 'hepatitis C virus' (HCV), an assay for circulating viral antibodies has been available. In this assay a recombinant non-structural protein (c100) from a chimpanzee HCV isolate (1) is used. Anti-c100 antibodies have been found in 58–84% of patients with chronic NANBH (1–5). However, HCV sequences have been detected in serum of cases negative for anti-c100 indicating that a higher proportion of NANBH can be HCV-related (6). On the other hand, false-positive results in patients with autoimmune chronic active hepatitis (7), in blood donors (8,9) and in patients with other diseases (10) have been reported. Thus, a second-generation assay is required.

We describe results from a serological test to detect anti-HCV antibodies based on a recombinant antigen (BHClO) which incorporates structural and non-structural viral proteins, where the non-structural moiety does not contain the c100 region. This novel test showed enhanced sensitivity and specificity in the identification of HCV infection. The results obtained with new-generation assays indicate an almost exclusive role of HCV in the genesis of chronic NANBH and will probably allow a more efficient prevention of post-transfusion hepatitis.

Materials and Methods

Subjects

Serum samples, stored at -40 C, from 409 patients with liver disease and 67 healthy blood donors were analyzed. Twelve blood donors had been followed over a number of years and had never been implicated in any case of post-transfusion hepatitis. They were considered as accredited donors. Patients were divided into four groups:

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Chronic NANBH

This group included 156 patients (55 female, mean age 45.6 years, range 12-72) and fulfilled the following criteria: liver biopsy showing chronic persistent or chronic active hepatitis with or without cirrhosis, the presence of raised serum alanine aminotransferase levels for more than 6 months, negative for HBsAg, alcohol consumption less than 50 g/day, antinuclear antibodies negative or at titre lower than 1/100, absence of potential hepatotoxic drugs, normal levels of ceruloplasmin and $a_1$-antitrypsin. None of these patients was receiving or had received antiviral or immunosuppressive therapy. In 54.4% there was previous exposure to blood products.

Chronic hepatitis B

Serum samples were obtained from 148 HBsAg-positive patients (33 female, mean age 39.2 years, range 5-78) with biopsy-proven chronic hepatitis (6.1% chronic persistent, 68.9% chronic active and 25.0% cirrhosis); 38.5% of these patients with hepatitis B virus (HBV) infection showed positive serum HBV-DNA [determined by molecular hybridization using a 32P-labelled HBV-DNA full-length probe (pBR322-HBV) on serum spots blotted on nitrocellulose filters (11)].

Hepatocellular carcinoma

This group was formed by 27 patients (six female, mean age 65.2 years, range 47-76) five of whom were HBsAg-positive and the other eight showed positive anti-HBs or anti-HBc.

Other liver diseases

Seventy-eight serum samples from patients with other forms of liver disease (34 female, mean age 48.2 years, range 9-73) were tested. This group included: 33 patients with alcoholic liver disease (9 steatosis, 11 alcoholic hepatitis and 13 cirrhosis), 26 primary biliary cirrhosis, 10 non-alcoholic steatosis, 4 inactive cryptogenic cirrhosis, 2 idiopathic cholestasis, 2 liver disease associated with ulcerative colitis and 1 congenital hepatic fibrosis. Liver biopsy was performed in all cases. None of them was receiving immunosuppressive therapy.

Serological studies

The Wellcome NANBH assay is an enzyme-linked immunosorbent assay (ELISA) based on a recombinant viral antigen polypeptide named BHC10 encompassing polypeptides from both the core region of HCV (c22) and also the greater part of the NS5 region (putative replicase) of the virus (12). ELISA plates (Nunc, Denmark) coated with BHC10 antigen were incubated at 37°C for 45 min with 200 µl/well of a 1/20 serum dilution. Two positive and three negative sera were used as controls. In each plate after washing 6 times with Tween-saline (0.05% NaCl and 0.05% Tween-20), 100 µl of 1/12 000 anti-human horseradish peroxidase conjugate was added and incubated at 37°C for 30 min. After 6 washings with Tween-saline, the plates were developed at room temperature for 20 min with 100 µl of a solution of 0.2 mg/ml of 3,3',5,5'-tetramethylbenzidine in 0.05 M citrate buffer (pH 5) containing 0.04% hydrogen peroxide. The reaction was stopped by adding 50 µl/well of 2 M sulphuric acid. The plates were read at 450 nm using a Titertek Multiscan MK II. The cut-off was calculated with the accredited blood donor samples. In each assay the cut-off was 0.15 optical density units added to the average reading of the three negative control sera.

Antibodies to c100 were assayed using the Ortho-HCV enzyme-linked immunoassay (Ortho Diagnostics Systems, Raritan, NJ). Positive results were confirmed in all cases by re-testing in duplicate. The second-generation assay from Ortho Diagnostics Systems, which includes the c100-c33 antigens (non-structural) and the c22 antigen (structural) of HCV, was used to test 150 serum samples from NANBH and 98 samples from chronic hepatitis B.

Serological markers of HBV infection were investigated by radioimmunoassay or ELISA with commercial reagents (Ausria II, Ausab, Corab, IMx HBsAg, Ausab EIA, IMx core; Abbott Laboratories, Chicago, IL).

Detection of HCV sequences in serum

Following reverse transcription of NANBH, viral sequences to cDNA amplification using the polymerase chain reaction (PCR) was performed using a tested primer technique. This assay was originally developed with primers from the NS5 region (12) and subsequently modified to improve sensitivity by the use of primers from the 5'-non-coding region of the virus (13). The 5'-non-coding region primers were used in all experiments reported in this paper.

Statistics

The chi-square test and the ratio difference test were used to compare frequencies between groups. For comparison of means, Student's t-test was employed.

Results

All the 67 healthy blood donors studied were negative for both anti-BHC10 and anti-c100. In chronic NANBH,
antibodies to HCV antigens were found more frequently with BHC10 as a substrate than with c100 (96.8% vs. 83.3%; p < 0.001) (Fig. 1). In contrast, in chronic hepatitis B, the prevalence of anti-BHC10 was significantly lower than anti-c100 (18.9% vs 29.7%; p < 0.001). Similarly in the group of miscellaneous liver diseases anti-BHC10 was detected in only 4% of patients (one case of primary biliary cirrhosis who had received blood transfusions and two cases of alcoholic cirrhosis), a proportion which was significantly lower than the prevalence of anti-c100 in this group (11.5%; p < 0.001). In patients with hepatocellular carcinoma the frequency of positive cases for anti-BHC10 was similar to that of anti-c100 (55.5% vs 59.3%; p = n.s.). In this group of patients with liver cell cancer (n = 27) anti-HCV antibodies were present in 18 cases, of whom 10 were negative for all serological markers of HBV infection.

Among the 156 patients with chronic NANBH studied, 130 cases were positive for both anti-c100 and anti-BHC10. None of the chronic NANBH patients was positive for anti-c100 in the absence of anti-BHC10. Twenty-six patients with chronic NANBH were negative for anti-c100; 21 of the anti-c100 negative patients were positive for anti-BHC10. In these 21 cases we used the PCR technique to investigate the presence of HCV sequences in serum and we found that HCV-RNA could be detected in 19 (Table 1). PCR was also performed in 10 of the chronic NANBH patients who were positive for both BHC10 and c100 and was positive in 8 (Table 1). As mentioned, 5 cases with chronic NANBH were negative in both c100 and BHC10 assays. Of these patients one had high titres of smooth muscle antibodies and improved with steroids (suggesting autoimmunity), three were positive for both anti-HBs and anti-HBc (suggesting covert HBV infection), and only one had all serological markers negative. Interestingly, only the latter was PCR-positive.

To see whether there was some clinical or pathological peculiarity in chronic NANBH patients with isolated positivity for anti-BHC10, we compared this subgroup (n = 21) with the larger population of chronic NANBH patients showing positivity for both anti BHC10 and anti-c100 (n = 130). No differences between these subgroups were found in age, sex, liver histology, biochemistry or autoantibodies (Table 2). The only significant difference was a predominance of sporadic NANBH cases in the subgroup of patients with isolated positivity for anti-BHC10 (Table 2).

In the patients with chronic hepatitis B, 24 subjects were positive for both anti-c100 and anti-BHC10. Twenty additional patients were positive for anti-c100 but negative for anti-BHC10. Of these, 12 were tested by PCR for the presence of HCV-RNA in serum and all were negative. Only four patients with chronic hepatitis B were positive for anti-BHC10 and negative for anti-c100. All of these were also negative for HCV-RNA in serum (Table 1). However, PCR was positive in three out of ten cases with chronic hepatitis B that were positive for both BHC10 and c100 (Table 1).

The BHC10 assay was also compared with the second-generation assay from Ortho (Ortho2). In chronic NANBH 94.7% of cases were positive by both tests. However, when chronic hepatitis B was studied, more cases were positive with Ortho2 (32.6%) than with BHC10 (15.3%; p < 0.001).

Discussion

Chronic hepatitis C is a common viral disease that often progresses to cirrhosis (14). The first-generation assay currently used to detect anti-HCV antibodies is
TABLE 2
Comparison of clinical, histological, biochemical and serological features in two groups of patients with chronic NANBH with different anti-HCV patterns

<table>
<thead>
<tr>
<th>Subgroups of chronic NANBH</th>
<th>Anti-BHC10 (+)</th>
<th>Anti-BHC10 (+)</th>
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<tbody>
<tr>
<td>Anti-c100 (+)</td>
<td>Anti-c100 (+)</td>
<td></td>
</tr>
<tr>
<td>(n = 130)</td>
<td>(n = 21)</td>
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| Female | 36.1% | 23.8% |
| Age (years) | 46.2 ± 1.2 | 46.3 ± 3.6 |
| No parental exposure | 40.5% | 85.7% |

Liver histology
- CAH: 67% 48%
- CPH: 10% 48%
- Cirrhosis: 23% 33%

Biochemistry
- AST (IU/l): 97.7 ± 8.6 70.1 ± 10.9
- ALT (IU/l): 156.9 ± 11.2 111.6 ± 14.1
- Alkaline phosphatase (IU/l): 150.0 ± 6.00 151.4 ± 8.90
- Albumin (g/dl): 4.2 ± 0.05 4.3 ± 0.12
- r-Globulin (g/dl): 1.5 ± 0.05 1.3 ± 0.09

HBV markers
- Anti-HBs: 21.4% 35.0%
- Anti-HBe: 29.2% 52.0%

Autoantibodies
- ANA: 9.7% 4.8%
- AMA: 1.6% 0.0%
- SMA: 19.5% 9.5%

* Mean ± standard error.

Based on the recombinant protein c100 derived from the non-structural portion of the HCV genome. Although the use of this assay represents an important step in the history of NANBH, recent reports have shown that it may fail to identify HCV infection in some patients (6).

In our group of patients with NANBH the prevalence of anti-c100 was 83.3%. This rate is similar to other European series (2,3,5) which also include post-transfusional and sporadic cases. Perhaps one of the most relevant points of the present study is the finding that the test based on BHC10, a recombinant antigen encompassing both structural and non-structural viral proteins [the core region of HCV (c22) and the greater part of the NS5 region], has a significantly higher sensitivity than the assay that includes only non-structural antigens. A sensitivity similar to BHC10 was observed with the second-generation assay from Ortho, which also includes structural and non-structural antigens.

The prevalence of anti-HCV in chronic NANBH rose with the BHC10 assay to nearly 97% (151 out of 156 cases were positive for anti-BHC10). In the group of patients with chronic NANBH we observed that while all subjects positive for anti-c100 were also positive for anti-BHC10, there were 21 additional cases which were positive only for anti-BHC10. The presence of HCV infection in these cases was demonstrated by the detection of HCV-RNA in 19 of them. Interestingly in the subgroup with isolated anti-BHC10 there was a significant increase in the relative number of patients without known exposure to blood products, suggesting that some sporadic cases with chronic hepatitis C may have a different pattern of humoral response against viral antigens.

In a recent report from Esteban et al. (16) 30% of drug addicts were negative for anti-c100 and half of these showed sustained elevation of transaminases in the absence of active HBV infection. These authors postulated the existence of chronic seronegative HCV infection or the participation of other viruses in the genesis of the liver lesion. Remarkably in our study, only five patients with chronic NANBH were seronegative for anti-BHC10, and, as mentioned, three of them were suspected of having covert hepatitis B infection and one had probably autoimmune chronic hepatitis. The remaining unique seronegative patient was found to have HCV sequences in serum. These findings have several implications. Firstly, they indicate that HCV is probably the sole agent responsible for chronic NANBH, when care is taken to exclude HBV infection. Secondly, the improved sensitivity of new-generation assays will allow better identification of HCV carriers and more efficient prevention of post-transfusional hepatitis.

One of the problems with the first-generation assay is the reported existence of false-positive results (7,8). In the present study there is a contrast between the significantly higher frequency of anti-BHC10 in chronic NANBH and the significantly lower prevalence of this antibody in chronic hepatitis B and in the miscellaneous group of liver diseases. In chronic hepatitis B all cases positive for anti-c100 but negative for anti-BHC10 tested by PCR were negative for HCV-RNA, suggesting that these cases might be in fact false-positive results. Since the prevalence of positive cases in chronic hepatitis B is comparable using c100 or Ortho2 and both assays share ~100, our results suggest that there may be cross-reactivity between c100 and HBV antigens.

Finally, as reported in previous studies (16), this paper shows a high prevalence of HCV markers in hepatocellular carcinoma. The finding of anti-HCV antibodies in patients with primary liver tumors who are seronegative for HBV points to a significant role of HCV in hepatocarcinogenesis.

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References