Reactivity to B Cell Epitopes within Hepatitis C Virus Core Protein and Hepatocellular Carcinoma

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ABSTRACT

Our aim was to investigate the existence of an association between B cell responsiveness to hepatitis C virus (HCV) core protein and progression of liver disease. In fact, the persistence of HCV infection is permitted by avoidance of viral clearance, despite chronic inflammation in the liver; this process ends with the development of hepatocellular carcinoma in many patients. On the basis of computerized prediction of antigenicity of the genomic sequence of HCV core protein, three 15-mer peptides (named Q15V, R15P, and G15V) were synthesized to be used as antigens in an enzyme immunoassay. Sera from 97 patients (65 males and 32 females) were tested: 43 patients had mild chronic liver disease (steatohepatitis, chronic persistent, or chronic active hepatitis) and 54 had cirrhosis, which was complicated by hepatocellular carcinoma (HCC) in 19. Seventy-six patients were positive to anti-HCV testing by second generation ELISA and 21 were negative. Rates of positivity for synthetic peptides in anti-HCV-positive versus anti-HCV-negative patients were as follows: 53 of 76 and 0 of 21 for anti-Q15V; 41 of 76 and 6 of 21 for anti-R15P; and 67 of 76 and 2 of 21 for anti-G15V. Rates of positivity to anti-Q15V and anti-G15V were similar among diagnostic groups (Pearson's $\chi^2$, 1.97, $P > 0.10$ and 0.45, $P > 0.10$), whereas anti-R15P antibodies were detected at a significantly lower rate in patients with HCC (2/13) in comparison to mild chronic liver disease (22/35) and cirrhosis (17/28) (Pearson's $\chi^2$, 9.42, $P < 0.01$). We conclude that anti-R15P antibodies are uncommon in anti-HCV-positive patients with HCC. During the course of chronic HCV infection, anti-R15P testing might help to identify a subgroup at higher risk to develop HCC.

INTRODUCTION

HCV is the agent responsible for the majority of cases of non-A, and non-B hepatitis (1). Its discovery was soon followed by the development of immunoenzymatic assays to detect in sera antibodies directed against NS (in first generation assays) (2) and both structural and NS (in second generation assays) (3) putative proteins of HCV. Most patients have antibodies directed against antigenic determinants of the putative HCV core (nucleocapsid) protein, which includes the first 190 amino acids of the polyprotein codified by the HCV genome (4). HCV core protein has a $M_r$ 22,000 and appears to be relatively well preserved among worldwide HCV isolates. Testing for antibodies to HCV core protein has significantly improved the serodiagnosis of non-A and non-B hepatitis. In fact, they appear earlier than antibodies to proteins derived from NS regions of HCV genome (5) and enhance by 31% the detection rate of HCV positivity in cryptogenic liver disease (6). Prevalence of antibodies to HCV core protein is closely related to chronic HCV infection, as indicated by HCV RNA detection (7), probably because of the high immunoreactivity of HCV core protein and of the high amino acid conservation of this protein among different HCV strains.

We recently analyzed the HCV core protein sequence searching for putative B cell epitopes (8). Antibodies directed against oligopeptides synthetized on the basis of computerized prediction of antigenicity of the HCV core protein were found in some healthy blood donors as well as in most patients with HCV-related chronic liver disease. We wondered whether, in chronic HCV infection, a distinct pattern of reactivity against these epitopes might be associated with progression of liver damage from chronic hepatitis to cirrhosis and eventually to HCC. In fact, a strong association has been demonstrated between development of HCC and exposure to HCV infection, marked by anti-HCV positivity (9–11). However, it is uncertain how HCV infection might be pathogenically related to hepatocellular carcinoma: HCV does not have a DNA intermediate and therefore it is not reputed to be able to integrate in the host genome. With the aim of investigating the existence of an association between B cell responsiveness to HCV core protein and progression of liver disease, the presence of antibodies to the three epitopes most immunoreactive was searched for in patients with chronic liver disease of a spectrum ranging from chronic hepatitis to HCC.

PATIENTS AND METHODS

We studied sera collected from 97 patients (65 males and 32 females; mean age, 53.8 ± 15.4 years, range, 21–81) referred to our institute to undergo a complete diagnostic work-up. Seventy-six patients (46 males and 30 females; mean age, 54.1 ± 15.2 years, range, 21–81) were positive and 21 were negative (19 males, and 2 females; mean age, 52.8 ± 6.5 years, range, 24–86) to an enzyme immunoassay for anti-HCV antibodies. Forty-three patients had mild chronic liver disease: in detail, 8 had liver steatohepatitis, 17 had chronic persistent hepatitis, and 18 had chronic active hepatitis. Fifty-four patients had cirrhosis, which was complicated by hepatocellular carcinoma in 19. Diagnoses of chronic persistent and chronic active hepatitis and liver steatohepatitis were all established by histopathological evaluation of liver samples obtained by percutaneous liver biopsy. Cirrhosis was diagnosed clinically on the basis of evidence of portal hypertension, ascites, hypoalbuminemia, and hypergammaglobulinemia and was confirmed histologically in the majority of patients. Hepatocellular carcinoma was diagnosed in the presence of raised (≥400 µg/liter) serum levels of α-fetoprotein and/or suggestive radiological imaging; it was always confirmed histologically or at necropsy. None of the patients had received immunosuppressive or antiviral therapy in the 12 months preceding their evaluation.

HCV Serology. Serum samples were tested for anti-HCV antibodies by means of a second generation ELISA (Ortho Diagnostics Inc., Raritan, NJ). Besides, all sera were tested by means of an enzyme immunoassay for the presence of serum antibodies to epitopes identified in the putative HCV core protein, as previously described by our group (8). Briefly, according to computer-generated prediction of antigenicity of the putative HCV core protein, three peptides (Q15V, QKKNKRNTNRRPQDV; R15P, RKTSSRQPRRQGP; and G15V, GGVYLLPRRGPRLGV) were synthetized in a semisynthetic peptide synthesizer (model NSP 4000; NeoSystem, Strasbourg, France) to be used as antigens in a conventional enzyme immunoassay. Both positive and negative samples were included in each run; absorbance was read in a spectrophotometer equipped with a 450- to 620-nm filter. Samples were considered positive for absorbancies 3 SD above the mean absorbance of...
negative controls. Sixty-three sera with positive results at conventional enzyme immunoassay were available for Matrix HCV supplemental assay, which allows recognition of specific antibodies to four different recombinant proteins. One of these is c100-3, a fusion protein expressed in yeast; it is presumed to contain primarily NS4 sequences. Three recombinant proteins are expressed in *Escherichia coli*: HC-34, presumed to contain core structural protein sequences; HC-29, presumed to contain NS3 sequences; and HC-23, presumed to contain NS4 sequences (entirely contained within the c100-3 protein).

**HCV RNA Determination.** Serum samples from 55 of 76 patients HCV positive at the screening test were suitable for HCV RNA determination, which was carried out by PCR amplification. For PCR, nested primers derived by the highly conserved 5' untranslated region of the HCV genome were used essentially as described previously (12, 13). Briefly, serum samples were immediately frozen after collection and kept at ~20°C until use. After thawing, aliquots of 200 µl were diluted on ice with 1 ml RNA extraction buffer (MBS, Italy) and thoroughly mixed. Then, serum proteins were removed by phenol-chloroform extraction. RNA was precipitated by the addition of one volume of ice-cold isopropanol and permanence on ice for 10 min. After pelleting by centrifugation in a microcentrifuge (15 min, 12,000 rpm, 4°C), RNA was washed once with 75% ethanol, vacuum dried and resuspended in 50 µl diethyl pyrocarbonate ultrapure H₂O. The RNA suspension, 15 µl diluted with 5 µl diethyl pyrocarbonate ultrapure H₂O, was heated at 65°C for 2 min to linearize RNA. Then, it was maintained at 55°C for 10 min in the presence of 50 pm of outer antisense primer (GGTGCACGGTGCTACGAGACCT').

The cDNA synthesis was carried out in a final volume of 25 µl at 37°C for 60 min in the presence of 2.5 mM of each deoxyribonucleotide, 40 units RNase inhibitor, and 400 units Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL) in 50 mM Tris-HCl buffer (pH 8.2) containing 70 mM KCl, 10 mM MgCl₂, and 5 mM dithiotreitol. The generated cDNAs (25 µl) were amplified in the presence of both sense (AACTACTGGTCCTACGCAGGA) and antisense outer primer in a final volume of 100 µl. Two sequential cycles of amplification were performed under the conditions recommended by the manufacturer (Perkin-Elmer/Cetus). These included: initial 5-min denaturation at 95°C, 35 cycles of 95°C for 1.5 min, 55°C for 2 min, 72°C for 3 min, and then 7-min extension at 72°C. Ten µl of the first amplification were added to 90 µl reaction mixture for the second PCR using a set of inner primers (sense, ATGGGTTAAGTATGAGTT; antisense, GCCGACCAACTCAGCGCT). Negative samples were included in each run to control for contamination. Amplification products were separated on 2.0% agarose gels stained with ethidium bromide and visualized under UV light to be identified as bands of 188 base pairs by direct comparison with molecular weight markers.

**Statistical Analysis.** Pearson’s χ² test was applied to detect differences among categorical variables. Spearman’s rank correlation was used to correlate the S/CO ratios of antibodies directed to HCV core protein as measured by a conventional assay (Matrix HCV) with those measured by means of immunoassays using synthetic peptides. All statistical tests were performed by means of the BMDF statistical software package, release 7.0 (14).

**RESULTS**

Table 1 reports the detection rates of anti-Q15V, anti-R15P, and anti-G15V antibodies in the studied population, grouped according to the diagnosis. Anti-R15P antibodies were detected at a significantly lower rate in patients with HCC in comparison to patients with mild chronic liver disease and with cirrhosis not complicated by HCC (Pearson’s χ², 9.42; P < 0.01). In contrast, anti-HC-34, anti-G15V, and anti-Q15V did not differ among the diagnostic categories. Finally, Table 4 presents the result of serum HCV RNA testing. No significant difference was detected in the rate of positivity of serum HCV RNA in the patients grouped according to diagnosis.

**DISCUSSION**

The prevalence of HCV seropositivity in HCC is substantial and virtually comparable to HBV, which is also associated with the development of HCC (15, 16). Both HCV and HBV may function independently in the pathogenesis of HCC (11). Late in its natural history, HBV infection ends with integration of the viral genome into the host, a process which is suspected to be implicated in its oncogenicity (17, 18). How HCV infection might be responsible for the development of neoplasia remains largely speculative at present. Currently, one of the most favored hypotheses is that HCV might be carcinogenic indirectly via chronic inflammation. In fact, chronic HCV-related liver disease is characterized by a long natural history, progressing slowly toward advanced forms of liver damage through decades of generally mild clinical disease (19). The strategy adopted by HCV to escape viral clearance by the host for such a long time includes viral diversity, i.e., the ability to generate viral variants with different antigenic properties. It is conceivable that, under selection pressure on protective B or T cell epitopes, HCV variants might emerge which are difficult to recognize for the immune system and might thus be associated with ongoing viral replication, cellular damage, and inflammation (20). Patients with cirrhosis of the liver, which represents often the final outcome of this process, are now recognized as one of the most common categories of acquired immunodeficiency (21). Notably, at the time these patients develop HCC, they have defective T cell immune function (22): therefore, neoplastic clones...
with HCC is that the lack of ability to elicit an antibody response to anti-HCV testing (second generation enzyme immunoassay) grouped according to their diagnosis. Statistical analysis (Pearson’s $\chi^2$) is also given.

Table 3 Rates of positivity to anti-HC-34 antibodies and to synthetic 15-mers Q15V, R15P, and G15V in the patients positive to commercial anti-HCV testing (second generation enzyme immunoassay) grouped according to their diagnosis

<table>
<thead>
<tr>
<th></th>
<th>Mild chronic liver disease (N = 35)</th>
<th>Liver cirrhosis (N = 28)</th>
<th>Hepatocellular carcinoma (N = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Q15V</td>
<td>Positive 25 (71.4)</td>
<td>21 (75.0)</td>
<td>7 (53.8)</td>
</tr>
<tr>
<td></td>
<td>Negative 10 (28.6)</td>
<td>7 (25.0)</td>
<td>6 (46.2)</td>
</tr>
<tr>
<td>Anti-R15P</td>
<td>Positive 22 (62.9)</td>
<td>17 (60.7)</td>
<td>2 (15.4)</td>
</tr>
<tr>
<td></td>
<td>Negative 13 (37.1)</td>
<td>11 (39.3)</td>
<td>11 (84.6)</td>
</tr>
<tr>
<td>Anti-G15V</td>
<td>Positive 30 (85.7)</td>
<td>25 (89.3)</td>
<td>12 (92.3)</td>
</tr>
<tr>
<td></td>
<td>Negative 5 (14.3)</td>
<td>3 (10.7)</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>Anti-HC-34b</td>
<td>Positive 29 (83.6)</td>
<td>21 (75.0)</td>
<td>9 (90.0)</td>
</tr>
<tr>
<td></td>
<td>Negative 2 (6.5)</td>
<td>1 (4.5)</td>
<td>1 (10.0)</td>
</tr>
</tbody>
</table>

$^a$ Results are expressed both as absolute numbers and as percentage (numbers in parentheses). Statistical analysis (Pearson’s $\chi^2$) is also given.

might emerge as a result of chronic inflammation and escape recognition and killing because of defective immune response.

In the present study, a strict correlation was found between the presence of antibodies to the entire HCV core protein and the presence of antibodies to the three epitopes identified within its predicted amino acid sequence. In fact, serum immunoreactivity against HCV core protein and against core epitopes were virtually superimposable both qualitatively and quantitatively. Qualitatively, the concordance of these tests was complete. Quantitatively, seroreological response to each of the three epitopes, measured as S/CO ratios, was related the degree of serological response to HCV core protein in toto, measured by S/CO of anti-HC-34 antibodies. Moreover, one of the two patients negative to the screening test for HCV but found positive for antibodies to at least one of the synthetic peptides was also HCV RNA positive. Thus, we believe that the analysis of the immunoreactivity against these three synthetic peptides truly represents a dissection of the immunoreactivity against the HCV core protein. Antibodies to the synthetic peptide R15P were detected at a significantly lower rate in patients with HCC in comparison to benign chronic liver disease. It might be that humoral immune response against R15P tends to disappear in time, explaining why it is often undetectable in patients with HCV-related HCC, an event which occurs late in the natural history of HCV infection. On the other hand, in patients with benign chronic liver disease both rates of positivity for anti-R15P antibodies and their S/CO ratios did not differ among the diagnostic categories (chronic persistent or active hepatitis and cirrhosis) (data not shown). Moreover, no difference was detected in the frequency of positivity for both serum HCV RNA and anti-HC-34 antibodies among benign and malignant HCV-related chronic liver disease, confirming previous reports showing that in chronic HCV infection viremia (and presumably the antigenic stimuli) persists indefinitely even in the most advanced forms of liver disease (23, 24). An alternative hypothesis to explain the low rate of immunoreactivity against R15P in patients with HCC is that the lack of ability to elicit an antibody response to this epitope might mark viral mutants better suited to escape immune surveillance by the host. In fact, even one point mutation within the genomic sequence coding for R15P might determine a change in the tertiary structure of the peptide, resulting in a modification of its immunogenic properties. An association between relative lack of response to interferon with frequent progression to cirrhosis and infection by specific HCV subtypes has already been described (25–27). It is conceivable that some HCV strain might possess greater oncogenic potential as a result of a more efficient strategy of viral persistence, maintaining longer a condition of chronic inflammation in the liver parenchyma. However, for each of the diagnostic categories anti-R15P antibodies were not frequently found, a finding not consistent with this epitope having a crucial role in immune recognition by the host. Finally, reactivity to HCV antigens is diminished in immunosuppressed in comparison with immunocompetent patients and immunosuppressive treatment after bone marrow transplantation is associated with loss of immunoreactivity to HCV antigens (28). Thus, reactivity to R15P might be lost as a result of the immunodeficiency associated with emerging of HCC.

Whatever the reason for this might be, anti-R15P antibodies are uncommon in patients with HCC. To date, aggressive screening strategies for early detection of HCC in patients with cirrhosis have not been proved to result in better therapeutic results and longer survival (29). If our findings will be confirmed in larger and prospective studies, anti-R15P antibodies could help to identify within HCV-positive patients a subgroup at higher risk to develop HCC.

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