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Serum Immunoglobulin G Antibodies to the GOR Autoepitope Are Present in Patients with Occult Hepatitis C Virus (HCV) Infection despite Lack of HCV-Specific Antibodies[▿]

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Antibody responses to the GOR autoepitope are frequently detected among anti-hepatitis C virus (anti-HCV)-positive patients with chronic hepatitis. Sera from 110 anti-HCV-negative patients with occult HCV infection, as diagnosed by detection of HCV RNA in hepatic tissue, were investigated for GOR antibody reactivity. A positive test for anti-GOR immunoglobulin G (IgG) was found for 22 (20%) of them. The frequency and titers of anti-GOR IgG were significantly lower than those in chronic hepatitis C patients (70/110, 63.6%; $P < 0.001$). Anti-GOR IgG was not detected in any of the 120 patients with HCV-unrelated liver disease. The anti-GOR IgG assay showed specificity and sensitivity values of 100% and 20%, respectively, among the sera from patients with occult HCV infection; the positive and negative predictive values were 100% and 44.3%, respectively. None of the clinical, laboratory, or histological characteristics of the patients with occult HCV infection were different according to GOR antibody status, except that the percentage of HCV RNA-positive hepatocytes was significantly greater ($P = 0.042$) in patients with occult HCV infection who tested positive for anti-GOR IgG. In conclusion, serum anti-GOR IgG is present in patients with occult HCV infection, despite a lack of detectable HCV-specific antibodies as determined by commercial tests. Testing for anti-GOR IgG in patients in whom HCV RNA is not detected in their sera may help with the identification of a subset of patients with occult HCV infection without the need to perform a liver biopsy.

Occult hepatitis C virus (HCV) infection has recently been described in patients with persistently abnormal liver function tests of unknown etiology (2). Occult HCV infection has been noted by other authors as well (4, 27). Because such patients are repeatedly negative by current assays for antibodies to HCV and HCV RNA in serum, occult HCV infection is identified by the detection of HCV RNA in hepatic tissue. Except for the serological profile, patients with occult HCV infection show characteristics similar to those observed in patients with chronic hepatitis C. Thus, HCV RNA has been detected in peripheral blood mononuclear cells of a high percentage of patients (2). Also, HCV replication has been demonstrated in peripheral blood mononuclear cells from patients with occult HCV infection (3), similar to the findings for patients with chronic hepatitis C. In addition, ultracentrifugation studies have revealed that the buoyant densities of HCV RNA from patients with occult HCV infection are comparable to those of particles found in the serum of patients with chronic hepatitis C (unpublished results). Furthermore, patients with occult HCV infection may potentially benefit from interferon-based therapies, as reported recently (18).

The GOR (GOR47-1) gene product is a host-derived antigen isolated from a cDNA library of host animals (16) which cross-reacts on immunoassays with the sera of HCV-positive patients. The human counterpart of the GOR gene product has recently been isolated (8); its sequence was highly conserved compared with the chimpanzee GOR gene sequence. Antibodies against another GOR epitope (termed GOR1-125), which is translated in humans, have been detected in some individuals but without an association with HCV infection (8). The detection of antibodies to the GOR47-1 autoepitope (anti-GOR) was first described in sera from non-A, non-B hepatitis cases (16). Since then, several studies have shown that the presence of anti-GOR is almost restricted to anti-HCV-positive individuals (14, 15). The sequence of the GOR (GOR47-1) epitope has partial homology with the HCV-encoded core protein sequence (17); both sequences show high degrees of conservation of residues essential for antibody binding (34). Antibodies against GOR are frequently detected among patients with overt HCV infection (6, 16, 21, 31). Thus, anti-GOR appears to be an antibody specifically related to HCV infection (15, 16).

On the other hand, there is little evidence of a relationship between autoimmunity and GOR in humans (13). However, because HCV infection may be associated with extrahepatic autoimmune disorders (20), such as cryoglobulinemia (5) and autoimmune hepatitis (15), the presence of serum factors associated with inflammatory conditions that could interfere with GOR antibody detection needs to be ruled out. Prior studies have found anti-GOR responses in a small percentage of individuals with chronic liver disease but without HCV RNA (28, 32), but none of these have investigated the patients for the presence of occult HCV. To date there have been no data on the detection of anti-GOR in patients with occult HCV infection.

The aims of this work were to investigate whether anti-GOR can be detected in the sera of patients with occult HCV infec-

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tion and to assess the diagnostic significance of the GOR antibody assay with patients with occult HCV infection.

MATERIALS AND METHODS

Study subjects. One hundred ten patients with a diagnosis of occult HCV infection were enrolled in this study. They were serum anti-HCV negative (Innotest-HCV Ab IV; Innogenetics, Ghent, Belgium) and serum HCV RNA negative (Amplicor HCV, version 2.0; sensitivity, 50 IU/ml; Roche Diagnostics, Branchburg, NJ). They presented with sustained abnormal liver function tests of unknown etiology for a minimum of 12 months (with testing every 3 months) prior to undergoing a liver biopsy for histological diagnosis (26), which demonstrated the presence of hepatic HCV RNA, as assayed by both PCR (110/110; 100%) and in situ hybridization (108/108 patients tested; 100%), as reported elsewhere (2). The HCV RNA amplified from liver biopsy specimens was genotyped by a standard method (Inno-LIPA HCV II; Innogenetics); all patients with occult HCV infection were infected with HCV genotype 1b (2). Other known causes of liver disease were excluded on the basis of clinical, epidemiological, and laboratory data: infection with hepatitis B virus (HBV; hepatitis B surface antigen and serum HBV DNA negative), autoimmunity (negativity for antinuclear and antimitochondrial antibodies, etc.), metabolic and genetic disorders, alcohol intake, drug toxicity, etc. All subjects were negative for human immunodeficiency virus antibodies. There were no known risk factors for HCV infection; none of the patients had a clinical or biochemical history of acute hepatitis.

The control groups included 110 patients with chronic hepatitis C (serum anti-HCV and HCV RNA positive and abnormal transaminase values; all were infected with HCV genotype 1), 35 patients with cryptogenic liver disease (serum anti-HCV and HCV RNA negative and liver HCV RNA negative but abnormal transaminase values); 35 patients with nonviral liver disease (10 with autoimmune hepatitis, 10 with primary biliary cirrhosis, 5 with alcoholic hepatitis, and 10 with steatosis or steatohepatitis; all were liver HCV RNA negative); and 50 patients with chronic hepatitis B (all were serum HBV DNA positive, 15 were hepatitis B e antigen positive, and 35 were anti-hepatitis B e positive). The study was approved by the ethics committee of our institution and was conducted according to the Declaration of Helsinki on human experimentation. Informed consent was obtained from the patients.

Enzyme immunoassay (EIA) for detection of anti-GOR immunoglobulin G (IgG). A pentadecapeptide with the sequence GRRGQKAKSNPNRPL corresponding to the GOR (GOR47-1) epitope (16) was purchased from RayBiotech Inc. (Norcross, GA); the lyophilized peptide had a purity of >80%, as determined by high-performance liquid chromatography. The peptide was dissolved and diluted to a concentration of 1 mg/ml in deionized ultrapure sterile water.

Detection of IgG antibody to GOR was done by EIA. In brief, the wells of a 96-well microtiter EIA plate (Costar, Cambridge, MA) were coated with 10 μg/ml GOR peptide in 0.1 M sodium carbonate buffer (pH 9.6) for 18 h at 4°C. The wells were washed with phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO), and nonspecific sites were blocked by incubation for 1 h at 37°C with phosphate-buffered saline containing 0.05% Tween 20 plus 10% heat-inactivated fetal bovine serum (Sera Laboratories International Ltd., West Sussex, United Kingdom). The serum samples were diluted 1:10 in blocking buffer and were preincubated for 1 h at 37°C with shaking; then, the samples were allowed to react in duplicate with GOR-coated wells for 1 h at 37°C (100 μl/well). The wells were washed five times as described above and were incubated (1 h at 37°C) with horseradish peroxidase-conjugated rabbit polyclonal anti-human IgG (DakoCytomation A/S, Glostrup, Denmark) diluted 1:1,000 in blocking buffer. After the wells were washed as described above, they were reacted for 30 min at room temperature in the dark with 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)-diammonium salt (Pierce, Rockford, IL) and the absorbance value was measured at 405 nm with a reference at 620 nm. A sample was considered reactive to anti-GOR IgG if the absorbance value exceeded the mean absorbance values for 20 nonexposed, HCV-negative healthy volunteers plus five times the standard deviation. Typical cutoff values were below 0.11 absorbance units at 405/620 nm.

The specificity of anti-GOR IgG antibody detection was ensured by a peptide inhibition assay, as reported previously (21), in which serum samples were preincubated without or with the GOR peptide (10, 100, and 1,000 μg/ml) in blocking buffer and were then reacted in duplicate in the EIA, as described above. A decrease in the absorbance values of more than 50% denoted the inhibition of anti-GOR IgG detection. The reproducibility of the anti-GOR IgG assay was assessed in three separate runs with samples from the same GOR IgG antibody-negative and -positive samples. The intra- and interassay coefficients of variation were 8.8% and 9.9%, respectively. Titration of anti-GOR IgG for GOR IgG antibody-positive samples was done as described above by serial twofold dilution (starting from 1:20) of serum in blocking buffer, and then the samples were reacted in duplicate in the EIA.

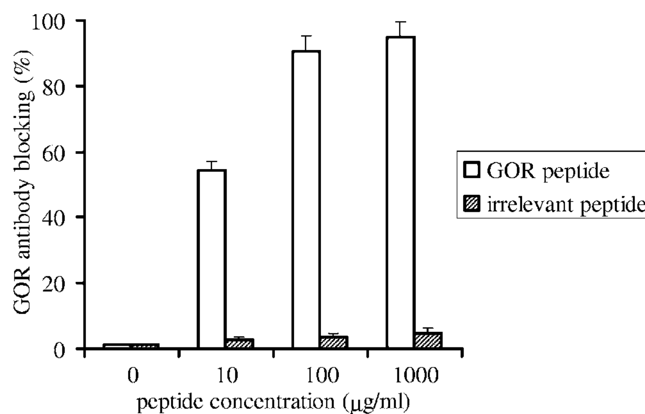

FIG. 1. Specificity of serum anti-GOR detection assay for patients with occult HCV infection. GOR antibody detection was blocked by preincubation with GOR peptide but not by an irrelevant peptide. The results shown are the mean percentages of anti-GOR blocking at each peptide concentration plus the standard error of the mean for five GOR antibody-positive individuals with occult HCV infection.

The serum samples were assayed for rheumatoid factor and C-reactive protein by latex agglutination tests (Biokit S.A., Barcelona, Spain). The presence of cryoglobulins was visually assessed by detection of a cryoprecipitate by blood coagulation at 37°C, centrifugation, and incubation of serum at 4°C for 24 to 72 h.

Statistical analysis. The results were analyzed by nonparametric tests by using the SPSS program (version 9.0; SPSS Inc., Chicago, IL). The chi-square test (or Fisher's exact test, when it was applicable) was used to compare frequencies. Correlations were done by using Spearman's rank correlation coefficient. All *P* values reported are two tailed.

RESULTS

Anti-GOR IgG was detectable in the serum of 22 of the 110 (20%) patients with occult HCV infection. The specificity of anti-GOR IgG antibody detection was demonstrated by the peptide inhibition assay, as shown in Fig. 1. Thus, preincubation of the serum samples with the GOR peptide resulted in a decrease of more than 50% in the absorbance values for anti-GOR IgG detection, whereas less than 5% blocking was noted following preincubation with an irrelevant peptide.

Among the patients with chronic hepatitis C, 70/110 (63.6%) had serum anti-GOR IgG. Thus, the frequency of GOR antibody detection was significantly higher in patients with chronic hepatitis C than in individuals with occult HCV infection ($P < 0.001$). Anti-GOR IgG was not detected in either the 35 patients with cryptogenic liver disease or the 35 other patients with nonviral liver diseases, irrespective of the etiology of the disease; similarly, anti-GOR IgG was undetectable in the 50 chronic hepatitis B patients.

To assess the analytical performance of the anti-GOR IgG assay, the sensitivity and specificity parameters were calculated with a threshold of detection set at 0.11 absorbance units, as described in Materials and Methods. The "gold standard" for evaluation of the accuracy of the anti-GOR IgG test was the presence of hepatic HCV RNA that had allowed the identification of occult HCV infection. Thus, the anti-GOR IgG assay showed values of specificity and sensitivity of 100% and 20%, respectively, among sera from patients with occult HCV infec-

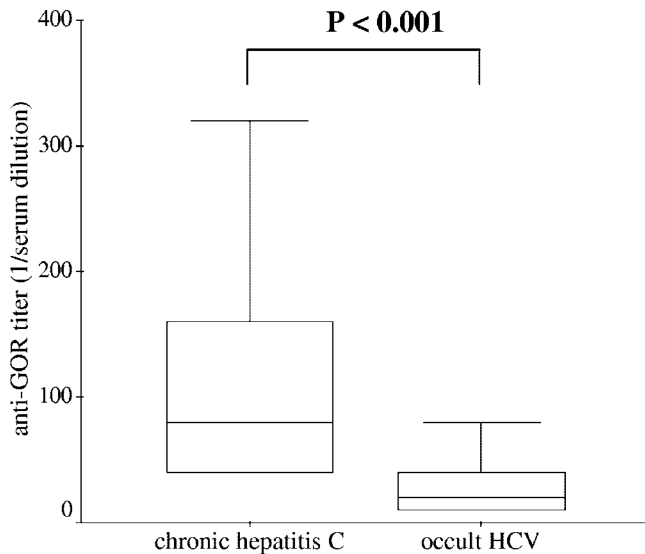

FIG. 2. Comparison of GOR antibody titers between patients with occult HCV infection and patients with chronic hepatitis C. The box plot representation shows the anti-GOR titers (expressed as the end-point serum dilution) measured in GOR antibody-positive patients with occult HCV infection (n = 22) and chronic hepatitis C (n = 70).

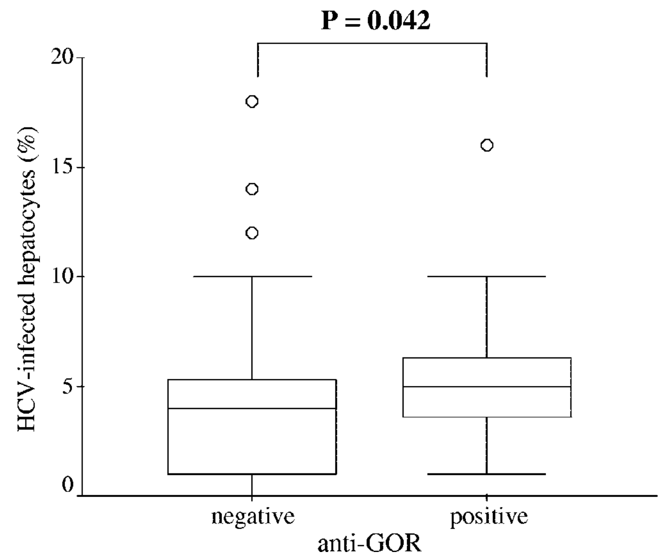

FIG. 3. Relationship between hepatic HCV infection and GOR antibody responses in patients with occult HCV infection. The box plot representation shows the percentages of HCV-infected hepatocytes in patients with occult HCV infection whose sera had negative (n = 87) and positive (n = 21) results by the anti-GOR detection test. Outliers are represented by single circles.

tion. Similarly, the positive and negative predictive values were 100% and 44.3%, respectively, when the results for the 70 HCV RNA-negative patients with HCV-unrelated nonviral liver disease were considered.

Titration of anti-GOR IgG showed a median value of 1:20 in patients with occult HCV infection, with the serum GOR antibody titers ranging from 1:10 to 1:80 (Fig. 2). In patients with chronic hepatitis C, the median anti-GOR IgG titer was 1:80, and the titers ranged from 1:40 to 1:320. Thus, GOR IgG antibody levels were significantly lower among individuals with occult HCV infection than among patients with chronic hepatitis C (P < 0.001) (Fig. 2). On the other hand, the analysis of anti-GOR IgG titers in sequential serum samples demonstrated minor changes in anti-GOR IgG levels among GOR antibody-positive patients with occult HCV infection. Similarly, there were no changes in the anti-GOR IgG titers among the GOR antibody-positive, untreated chronic hepatitis C patients within a 1-year period of the survey (data not shown).

TABLE 1. Characteristics of the patients with occult HCV infection by GOR antibody status

Characteristic[a]	Result for: Anti-GOR-positive patients (n = 22)	Anti-GOR-negative patients (n = 88)	P value
Age (yr)[b]	45.5 (39.9–51.1)	45.1 (42.7–47.5)	0.86
Gender (no. of M/no. of F)	19/3	64/24	0.18
Duration of disease (yr)[b,c]	6.2 (3.4–9.0)	6.4 (3.6–9.2)	0.56
ALT (IU/liter)[b]	67 (51–84)	68 (59–79)	0.67
AST (IU/liter)[b]	38 (29–47)	41 (35–46)	0.86
GGTP (IU/liter)[b]	111 (73–148)	94 (76–112)	0.32
Necroinflammation[d]	11 (50)	31 (35)	0.20
Fibrosis[d]	5 (23)	17 (19)	0.72
Cirrhosis[d]	1 (4)	2 (2)	0.88
Steatosis[d]	4 (18)	14 (16)	0.79

[a] M, male; F, female; ALT, alanine aminotransferase; AST, apartate aminotransferase; GGTP, γ-glutamyltransferase.
[b] Expressed as the mean (95% confidence interval of the mean).
[c] The estimated duration of abnormal liver function tests since the first alteration was detected.
[d] Expressed as the number (percent) of cases.

The clinical, laboratory, and histological characteristics of the patients with occult HCV infection who tested positive for anti-GOR IgG did not differ from those of the individuals who were GOR antibody negative (Table 1); the histological activity (average scores for necroinflammation and fibrosis) tended to be greater, although not significantly, among the anti-GOR IgG-positive patients with occult HCV infection (data not shown). On the other hand, the percentage of infected hepatocytes (that is, cells positive for genomic HCV RNA by in situ hybridization) were significantly greater (P = 0.042) in patients with occult HCV infection who tested positive for anti-GOR IgG (Fig. 3). However, the percentage of HCV-infected hepatocytes did not correlate significantly with the anti-GOR IgG titers among the 22 GOR antibody-positive patients (Spearman's rank correlation coefficient = 0.311, P = 0.19). Among the patients with overt chronic HCV infection, the median proportion of infected hepatocytes observed by in situ hybridization was 8.0% (range, 2.5% to 38.6%), which was significantly higher (P < 0.001) than that among patients with occult HCV infection (median, 4.0%, range, 0.1 to 18.0%), in agreement with the findings presented in a previous report (19).

Rheumatoid factor was detected in the serum from 12 of the 110 (10.9%) patients with occult HCV infection, including 1 (4.5%) of the 22 GOR antibody-positive individuals. Similarly, C-reactive protein was detectable in 15/110 (13.6%) patients with occult HCV infection, including 1/22 (4.5%) anti-GOR IgG-positive individuals. Finally, cryoglobulins were found in 14/110 (12.7%) patients with occult HCV infection; anti-GOR IgG was detectable in only 1 of them (4.5%).

DISCUSSION

In this study, we have observed a 20% frequency of IgG antibody reactivity to the GOR autoepitope in the serum of anti-HCV-negative patients with occult HCV infection. Low anti-GOR IgG titers were found in most GOR antibody-positive individuals. Importantly, anti-GOR IgG was not detected in any of the patients without HCV infection, irrespective of the etiology of the liver disease. Thus, despite the repeated absence of serum anti-HCV antibodies as determined by commercial immunoassays, anti-GOR IgG could be found in patients with occult HCV infection. Most studies have detected anti-GOR reactivity only in HCV-seropositive patients (6, 9, 14, 15, 21, 31). However, a few reports identified anti-HCV-negative individuals who tested positive for anti-GOR (9, 14), including blood donors (7, 33), although those studies did not exclude the presence of occult HCV infection. In addition, it has been reported that detection of anti-GOR without anti-HCV is not associated with hepatitis C viremia (1). In this way, the sera of patients with occult HCV infection is persistently negative for HCV RNA (2).

The frequency of anti-GOR IgG in patients with occult HCV infection was significantly lower than the 63.6% GOR IgG antibody reactivity found in patients with chronic hepatitis C, which is similar to the frequency reported by several authors in patients with overt HCV infection (10, 12, 14, 16, 21). Also, anti-GOR levels were greater in patients with chronic hepatitis C than in patients with occult HCV infection. We have recently reported that sera from some patients with occult HCV infection may demonstrate a positive reaction against HCV nonstructural proteins on immunoblot assays, suggesting a very low level of specific antibody production (23). In chronic hepatitis C, the presence of antibodies reactive to the host-derived GOR antigen is not merely due to sequence homology but is also due to cross-reactivity at the molecular level because of the conservation of residues essential for antibody binding (34). Thus, de novo infection with HCV after liver transplantation produces an increase in anti-GOR IgG levels, likely due to the increased viral load and replication under conditions of immunosuppression, indicating that the immune response to GOR autoantibody is triggered by HCV (24).

The low level of anti-GOR IgG antibodies detected in patients with occult HCV infection may reflect not only exposure to HCV (22) but also an ongoing productive HCV infection within the liver (2). Indeed, HCV replication has been demonstrated in peripheral blood mononuclear cells from patients with occult HCV infection as well (3). This may result in discrete amounts of antigen production and then presentation to antibody-producing cells. Interestingly, the percentage of infected hepatocytes was significantly greater in patients with occult HCV infection who tested positive for anti-GOR IgG. The mechanism(s) that regulates the humoral immune responses during occult HCV infection is not well known. In humans the GOR (GOR47-1) gene product cannot be translated into a protein (8), and so the antibody responses to GOR and HCV may be independently regulated, as has been suggested for patients with chronic hepatitis C (11). In patients with chronic hepatitis C, anti-HCV antibodies usually persist for decades, although these may eventually disappear after recovery from HCV infection (29, 30).

Among the individuals with occult HCV infection, the subset of GOR IgG antibody-positive patients did not show a clinical background different from that of their anti-GOR IgG-negative counterparts (9). However, a greater number of anti-GOR IgG-positive patients had signs of necroinflammation, which is similar to the finding in patients with chronic hepatitis C, in whom reactivity to GOR had been correlated with liver disease activity (21). Nevertheless, compared with chronic hepatitis C, occult HCV infection seems to be a less aggressive form of the disease caused by HCV (19), although liver cirrhosis is present in about 4% of these patients.

Finally, rheumatoid factor, C-reactive protein, and/or cryoglobulins were detected in the sera of 10 to 14% of the patients with occult HCV infection. The frequencies of these factors were lower than those commonly found in patients with chronic hepatitis (25), suggesting that this may reflect differences in the host response to HCV between patients with occult HCV infection and patients with chronic hepatitis C. In addition, the presence of these factors was not associated with the GOR IgG antibody status. These data are in line with the notion that the significance of GOR is little during triggering of autoimmune phenomena by HCV, and thus, GOR is unlikely a marker of induced autoimmunity, as has already been reported for chronic HCV infection (13). Indeed, histological features of autoimmune disease were absent in all patients.

In conclusion, we have found that sera from 20% of the patients with occult HCV infection react with the GOR autoepitope in EIAs, although this frequency is lower than the GOR reactivity of patients with chronic hepatitis C. Because anti-GOR IgG is not detected in patients with HCV-unrelated liver disease, the detection of IgG antibodies to GOR seems to reflect cross-recognition with viral sequences during occult HCV infection, even in the absence of detection of HCV-specific antibodies by commercial tests. Testing for anti-GOR IgG might be used to screen HCV RNA-negative patients and thus help to identify at least a subset of occult HCV infections without performing a liver biopsy. Nevertheless, even after implementation of anti-GOR IgG testing, the majority of patients would still need a liver biopsy for the accurate diagnosis of occult HCV infection.

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