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 a partial homology with the HCV-encoded core protein sequence (17); both sequences show a high 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 human beings (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 (28,33) but none of these have previously investigated the presence of occult HCV. Up to date there are no data reporting on the detection of anti-GOR in patients with occult HCV infection.

The aims of this work have been to investigate whether anti-GOR can be detected in the sera of occult HCV-infected patients and to assess the diagnostic significance of GOR-antibody assay in 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, Gent, Belgium) and serum HCV RNA-negative (Amplicor HCV version 2.0; Roche Diagnostics, Branchburg, NJ; sensitivity of 50 IU/mL), and presented sustained abnormal liver function tests of unknown etiology for a minimum time of 12 months (tested every 3 months) prior to undergoing a liver biopsy for histological diagnosis (26) which demonstrated the presence of hepatic HCV RNA assayed by both PCR (110/110, 100%) and in situ hybridization (108/108 tested; 100%) as reported elsewhere (2). HCV RNA amplified from liver biopsies was genotyped by a standard method (Inno-LIPA HCV II, Innogenetics); all patients with occult HCV infection showed HCV1b (2). Other known causes of liver disease were excluded based on clinical, epidemiological and laboratory data: infection by HBV (hepatitis B surface antigen and serum HBV DNA negative), autoimmunity (negative for anti-nuclear and anti-mitochondrial antibodies, etc.), metabolic and genetic disorders, alcohol intake, drug toxicity, etc.; all subjects were negative for anti-HIV antibodies. There were no known risk factors for HCV infection; none of the patients referred clinical or biochemical history of acute hepatitis.

Control groups included: 110 patients with chronic hepatitis C (serum anti-HCV and HCV RNA-positive and abnormal transaminase values; all 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 non-viral 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 serum HBV DNA-positive: 15 hepatitis B e antigen-positive and 35 anti-HBe-positive). The study was approved by the ethics committee of the institution and was conducted according to the Declaration of Helsinki on human experimentation. Informed consent was obtained from the patients.

Enzyme immunoassay to detect IgG anti-GOR. 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 > 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 enzyme immunoassay. In brief, wells of a 96-well microtitre 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. Wells were washed with PBS pH 7.4 containing 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO) and non-specific sites blocked by incubating for 1 h at 37 °C with PBS containing 0.05% Tween 20 plus 10% heat-inactivated fetal bovine serum (Sera Laboratories International Ltd., West Sussex, UK). Serum samples were diluted 1:10 in blocking buffer and pre-incubated for 1 h at 37 °C with shaking; then, samples were allowed to react in duplicate with GOR-coated wells for 1h at 37 °C (100 µl/well) . Wells were washed five times as above and incubated (1 h at 37 °C) with horseradish peroxidase-conjugated rabbit polyclonal anti-human IgG (DakoCytomation A/S, Glostrup, Denmark) diluted 1:1000 in blocking buffer. After washing as above wells were reacted for 30 min at room temperature in the dark with 2,2'-azinobis-[3-ethylbenzthiazoline-6-sulfonic acid]-diammonium salt (ABTS; Pierce, Rockford, IL) and the absorbance value measured at 405 nm with a reference at 620 nm. A sample was considered reactive to IgG anti-GOR if the

absorbance value exceeded the mean absorbance values of 20 non-exposed, HCV-negative healthy volunteers plus five times the standard deviation. Typical cut-off values were below 0.11 absorbance units at 405/620 nm.

The specificity of the IgG anti-GOR antibody detection was assured by peptide inhibition assay as reported previously (21), in which serum samples were pre-incubated without or with the GOR peptide (10, 100 and 1000 μg/ml) in blocking buffer and then reacted in duplicate in the EIA as described above. A decrease of more than 50% in the absorbance values denoted inhibition of IgG anti-GOR detection. The reproducibility of the IgG anti-GOR assay was assessed in three separate runs, with the same samples from GOR IgG antibody-negative and -positive samples. The intra- and inter-assay coefficients of variation were 8.8% and 9.9%, respectively. Titration of IgG anti-GOR was done as described above in GOR IgG antibody-positive samples by serial two-fold serum dilutions (starting from 1:20) in blocking buffer and then reacted in duplicate in the EIA.

Determinations of rheumatoid factor and C-reactive protein were assayed in serum samples using latex agglutination tests (Biokit S.A., Barcelona, Spain). Presence of cryoglobulins was visually assessed by the appearance of a cryoprecipitate by blood coagulation at 37°C, centrifugation and incubation of serum at 4°C for 24 to 72 hours.

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

RESULTS

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

In patients with chronic hepatitis C, 70/110 (63.6%) had serum IgG anti-GOR. Thus, the frequency of GOR antibody detection was significantly higher in patients with chronic hepatitis C compared with individuals with occult HCV infection ($P < 0.001$). IgG anti-GOR was neither detected in 35 patients with cryptogenic liver disease nor in 35 others suffering from non-viral liver diseases, irrespective of the etiology of the disease; similarly, IgG anti-GOR was undetectable in fifty chronic hepatitis B patients.

To assess the analytical performance of the IgG anti-GOR 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" to evaluate the accuracy of the IgG anti-GOR test was the presence of hepatic HCV RNA that had allowed identifying occult HCV infection. Thus, the IgG anti-GOR assay showed values of specificity and sensitivity of 100% and 20%, respectively, among occult HCV-infected patient sera. Similarly, the predictive values (positive, PPV; negative, NPV) were 100% and 44.3%, respectively, considering 70 HCV RNA-negative patients with HCV-unrelated non-viral liver disease.

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

As regards the clinical, laboratory and histological characteristics, patients with occult HCV infection who tested positive to IgG anti-GOR did not differ from those who were GOR antibody-negative (table 1); the histological activity (average scores of necro-inflammation and fibrosis) tended to be greater, although not significantly, among IgG anti-GOR-positive patients with occult HCV infection (data not shown). On the other hand, the percentage of infected hepatocytes (that is, cells positive to genomic HCV RNA by in situ hybridization) resulted significantly greater ($P = 0.042$) in patients with occult HCV infection who tested positive to IgG anti-GOR (figure 3). However, the percentage of HCV-infected hepatocytes did not correlate significantly with IgG anti-GOR titres among the twenty-two GOR antibody-positive patients ($r_s = 0.311$, $P = 0.19$). In patients with overt chronic HCV infection the median percentage of infected hepatocytes observed by in situ hybridization was 8.0 (range 2.5 – 38.6), which resulted significantly higher ($P<0.001$) compared with occult HCV infection (median of 4.0, range 0.1 – 18.0), in agreement with a previous report (19).

With respect to rheumatoid factor, it was detected in the serum from 12 of the 110 (10.9%) patients with occult HCV infection, including one (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%) IgG anti-GOR-positive individuals. Finally, cryoglobulins were found in 14/110 (12.7%) patients with occult HCV infection; only one of them (4.5%) had IgG anti-GOR detectable.

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 IgG anti-GOR titres were found in most GOR antibody-positive individuals. Importantly, IgG anti-GOR was not detected in any of the patients without HCV irrespective of the etiology of the liver disease. Thus, despite repeated absence of serum anti-HCV antibodies by commercial immunoassays IgG anti-GOR can be found in patients with occult HCV infection. Most studies had only detected anti-GOR reactivity in HCV seropositive patients (6,9,14,15,21,31). However, a few reports identified anti-HCV-negative individuals who tested positive to anti-GOR (9,14) including blood donors (7,32); although, these 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, among occult HCV-infected patients HCV RNA is persistently negative in serum (2).

The frequency of IgG anti-GOR in occult HCV infection was significantly lower compared with a 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 chronic hepatitis C compared with occult HCV infection. We have reported recently that sera from some patients with occult HCV infection may demonstrate a positive reaction against HCV non-structural 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 antigen GOR is not merely due to sequence homology but to cross-reactivity

at the molecular level because of conservation of residues essential for antibody binding (34). Thus, de novo infection with HCV after liver transplantation produces an increase in IgG anti-GOR likely due to increased viral load and replication under immunosuppresion indicating that the immune response to GOR autoantibody is triggered by HCV (24).

The low level of IgG anti-GOR antibodies detected in 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 occult HCV-infected patients 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 resulted significantly greater in patients with occult HCV infection who tested positive to IgG anti-GOR. The mechanism(s) that regulate humoral immune responses during occult HCV infection are not well known. In humans the GOR (GOR47-1) gene product cannot be translated into a protein (8) and so antibody responses to GOR and HCV may be independently regulated as suggested in 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 individuals with occult HCV infection, the subset of GOR IgG antibody-positive patients did not show a different clinical background compared with their IgG anti-GOR-negative counterparts (9). However, a greater number of IgG anti-GOR-positive patients had signs of necro-inflammation, which is similar to 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 the hepatitis C virus (19); although, liver cirrhosis is present in around 4% of these patients.

Finally, rheumatoid factor, C-reactive protein and/or cryoglobulins were detected in the serum of 10-14% of occult HCV-infected patients. Frequencies of such factors were lower than those commonly found in chronic hepatitis (25), suggesting that this may reflect differences in the host response to HCV between occult HCV and chronic hepatitis C patients. 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 already reported in 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 react with the GOR autoepitope on enzyme immunoassays; although, this frequency is lower compared to GOR reactivity in patients with chronic hepatitis C. Because IgG anti-GOR is not detected in patients with HCV-unrelated liver disease detection of IgG antibodies to the GOR seems to reflect cross-recognition with viral sequences during occult HCV infection, even in the absence of detectable HCV-specific antibodies using commercial tests. Testing for IgG anti-GOR might be used to screen HCV RNA-negative patients and thus help in identifying at least a subset of occult HCV infection without performing a liver biopsy. Nevertheless, even after implementation of IgG anti-GOR testing the majority of patients would still need a liver biopsy for accurate diagnosis of occult HCV infection.

1 **ACKNOWLEDGMENTS**

2

3 The authors have no conflicting financial interests. This work has been supported in part

4 by a grant from "Fundación de Investigaciones Biomédicas", Madrid (Spain).

5

REFERENCES

1. **Baath, L., and A. Widell.** 1994. Anti-GOR without anti-HCV core is not associated with hepatitis C viremia. Vox Sang. **66**:151.
2. **Castillo, I., M. Pardo, J. Bartolomé, N. Ortiz-Movilla, E. Rodríguez-Iñigo, S. de Lucas, C. Salas, J.A. Jiménez-Hefferman, A. Pérez-Mota, J. Graus, J.M. López-Alcorocho, and V. Carreño.** 2004. Occult hepatitis C virus infection in patients in whom the etiology of persistently abnormal results of liver-function tests is unknown. J. Infect. Dis. **189**:7-14.
3. **Castillo, I., E. Rodríguez-Iñigo, J. Bartolomé, S. de Lucas, N. Ortíz-Movilla, J.M. López-Alcorocho, M. Pardo, and V. Carreño.** 2005. Hepatitis C virus replicates in peripheral blood mononuclear cells of patients with occult hepatitis C virus infection. Gut **54**:682-685.
4. **Dries, V., J. von Both,M. Muller, G. Gerken, P. Schirmacher, M. Odenthal, R. Bartenschlager, U. Drebber, K.H. Meyer zum Buschenfeld, and H.P. Dienes.** 1999. Detection of hepatitis C virus in paraffin-embedded liver biopsies of patients negative for viral RNA in serum. Hepatology **29**:223-229.
5. **Ferri, C., A.L. Zignego, and S.A. Pileri.** 2002. Cryoglobulins. J. Clin. Pathol. **55**:4-13.
6. **Hayashi, J., K. Nakashima, Y. Kishihara, M. Ohmiya, E. Yoshimura, M. Hirata, and S. Kashiwagi.** 1993 Improved detection of antibodies to hepatitis C virus by the second-generation assay in patients with chronic non-A, non-B liver disease. J. Infect. **26**:287-294.