1 **ABSTRACT**

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3 Antibody responses to the GOR autoepitope are frequently detected among anti-hepatitis C
4 virus (anti-HCV)-positive patients with chronic hepatitis. GOR antibody reactivity has
5 been investigated in sera from 110 anti-HCV-negative patients with occult HCV infection
6 as diagnosed by detection of HCV RNA in hepatic tissue. A positive test to IgG anti-GOR
7 was found in 22 (20%) of them. The frequency and titres of IgG anti-GOR were
8 significantly lower compared with chronic hepatitis C patients (70/110, 63.6%; P<0.001).
9 IgG anti-GOR was not detected in any of the 120 patients with HCV-unrelated liver
10 disease. The IgG anti-GOR assay showed values of specificity and sensitivity of 100% and
11 20%, respectively, among occult HCV-infected patient sera; the predictive values
12 (positive, PPV; negative, NPV) were 100% and 44.3%. None of the clinical, laboratory and
13 histological characteristics of the patients with occult HCV infection were different
14 according to GOR antibody status except that the percentage of HCV RNA-positive
15 hepatocytes resulted significantly greater (P=0.042) in patients with occult HCV infection
16 who tested positive to IgG anti-GOR. In conclusion, serum IgG anti-GOR is present in
17 patients with occult HCV infection despite lack of detectable HCV-specific antibodies
18 using commercial tests. Testing for IgG anti-GOR in patients without HCV RNA detected
19 in serum may help in identifying a subset of occult HCV infection without performing a
20 liver biopsy.

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22 Key words: GOR antibody reactivity; anti-HCV-negative occult HCV infection; chronic
23 hepatitis C; host-derived antigen GOR; cross-reactivity.

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1 **INTRODUCTION**

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3 Occult hepatitis C virus (HCV) infection has been described recently in patients
4 with persistently abnormal liver function tests of unknown etiology (2). Occult HCV
5 infection has been noted by other authors as well (4,27). Because such patients are
6 repeatedly negative by current assays for antibodies to HCV and HCV RNA in serum,
7 occult HCV infection is identified by detection of HCV RNA in hepatic tissue. Except for
8 the serological profile, occult HCV infection show characteristics similar to those observed
9 in patients with chronic hepatitis C. Thus, HCV RNA has been detected in peripheral blood
10 mononuclear cells of a high percentage of patients (2). Also, HCV replication has been
11 demonstrated in peripheral blood mononuclear cells from occult HCV-infected patients
12 (3), in a similar way to patients with chronic hepatitis C. In addition, ultracentrifugation
13 studies have revealed that the buoyant densities of HCV RNA from occult HCV-infected
14 patients are comparable to those of particles found in the serum of patients with chronic
15 hepatitis C (unpublished results). Furthermore, patients with occult HCV infection may
16 potentially benefit from interferon-based therapies as reported recently (18).
17 The GOR (GOR47-1) gene product is a host-derived antigen isolated from a cDNA
18 library of host animals (16) which cross-reacts on immunoassays with sera of hepatitis C
19 virus-positive patients. The human counterpart of the GOR gene product has been isolated
20 recently (8); its sequence was highly conserved compared with that of the chimpanzee.
21 Antibodies against another GOR epitope (termed GOR1-125), which is translated in
22 humans, have been detected in some individuals without association with HCV infection
23 (8). The detection of antibodies to the GOR47-1 autoepitope (anti-GOR) was first
24 described in sera from non-A, non-B hepatitis cases (16). Since then, several studies have

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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.

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1 **MATERIALS AND METHODS**

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3 **Study subjects.** One hundred ten patients with a diagnosis of occult HCV infection
4 were enrolled in this study. They were serum anti-HCV-negative (Innotest-HCV Ab IV,
5 Innogenetics, Gent, Belgium) and serum HCV RNA-negative (Amplicor HCV version 2.0;
6 Roche Diagnostics, Branchburg, NJ; sensitivity of 50 IU/mL), and presented sustained
7 abnormal liver function tests of unknown etiology for a minimum time of 12 months
8 (tested every 3 months) prior to undergoing a liver biopsy for histological diagnosis (26)
9 which demonstrated the presence of hepatic HCV RNA assayed by both PCR (110/110,
10 100%) and in situ hybridization (108/108 tested; 100%) as reported elsewhere (2). HCV
11 RNA amplified from liver biopsies was genotyped by a standard method (Inno-LIPA HCV
12 II, Innogenetics); all patients with occult HCV infection showed HCV1b (2). Other known
13 causes of liver disease were excluded based on clinical, epidemiological and laboratory
14 data: infection by HBV (hepatitis B surface antigen and serum HBV DNA negative),
15 autoimmunity (negative for anti-nuclear and anti-mitochondrial antibodies, etc.), metabolic
16 and genetic disorders, alcohol intake, drug toxicity, etc.; all subjects were negative for anti-
17 HIV antibodies. There were no known risk factors for HCV infection; none of the patients
18 referred clinical or biochemical history of acute hepatitis.

19 Control groups included: 110 patients with chronic hepatitis C (serum anti-HCV
20 and HCV RNA-positive and abnormal transaminase values; all with HCV genotype 1); 35
21 patients with cryptogenic liver disease (serum anti-HCV and HCV RNA-negative and liver
22 HCV RNA-negative but abnormal transaminase values); 35 patients with non-viral liver
23 disease: 10 with autoimmune hepatitis, 10 with primary biliary cirrhosis, 5 with alcoholic
24 hepatitis and 10 with steatosis or steatohepatitis (all were liver HCV RNA-negative); and

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1 50 patients with chronic hepatitis B (all serum HBV DNA-positive: 15 hepatitis B e
2 antigen-positive and 35 anti-HBe-positive). The study was approved by the ethics
3 committee of the institution and was conducted according to the Declaration of Helsinki on
4 human experimentation. Informed consent was obtained from the patients.

5 **Enzyme immunoassay to detect IgG anti-GOR.** A pentadecapeptide with the
6 sequence GRRGQKAKSNPNRPL corresponding to the GOR (GOR47-1) epitope (16) was
7 purchased from RayBiotech Inc. (Norcross, GA); the lyophilized peptide had a purity >
8 80% as determined by high-performance liquid chromatography. The peptide was
9 dissolved and diluted to a concentration of 1 mg/ml in deionized ultrapure sterile water.

10 Detection of IgG antibody to GOR was done by enzyme immunoassay. In brief, wells
11 of a 96-well microtitre EIA plate (Costar, Cambridge, MA) were coated with 10 μg/ml
12 GOR peptide in 0.1 M sodium carbonate buffer pH 9.6 for 18 h at 4 °C. Wells were washed
13 with PBS pH 7.4 containing 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO) and
14 non-specific sites blocked by incubating for 1 h at 37 °C with PBS containing 0.05%
15 Tween 20 plus 10% heat-inactivated fetal bovine serum (Sera Laboratories International
16 Ltd., West Sussex, UK). Serum samples were diluted 1:10 in blocking buffer and pre-
17 incubated for 1 h at 37 °C with shaking; then, samples were allowed to react in duplicate
18 with GOR-coated wells for 1h at 37 °C (100 μl/well). Wells were washed five times as
19 above and incubated (1 h at 37 °C) with horseradish peroxidase-conjugated rabbit
20 polyclonal anti-human IgG (DakoCytomation A/S, Glostrup, Denmark) diluted 1:1000 in
21 blocking buffer. After washing as above wells were reacted for 30 min at room
22 temperature in the dark with 2,2'-azinobis-[3-ethylbenzthiazoline-6-sulfonic acid]-
23 diammonium salt (ABTS; Pierce, Rockford, IL) and the absorbance value measured at 405
24 nm with a reference at 620 nm. A sample was considered reactive to IgG anti-GOR if the

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

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

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

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

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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.

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