CONCISE REPORTS

Anticardiolipin autoantibodies in serum samples and cryoglobulins of patients with chronic hepatitis C infection

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In memory of the late Professor David Geltner

Abstract

Objective—Chronic hepatitis C virus (HCV) has been linked to extrahepatic autoimmune phenomena. In addition, a variety of autoantibodies are found in patients with HCV. The prevalence, nature, and clinical significance of anticardiolipin (aCL) autoantibodies in serum samples of patients with HCV were therefore investigated.

Patients and methods—A prospective study of 48 consecutive patients with chronic HCV with no evidence of previous hepatitis B virus (HBV) infection or any other autoimmune disorder. Thirty patients with HBV and 50 healthy volunteers matched for age and sex served as control groups. Anticardiolipin antibodies in the serum samples and cryoprecipitates were measured by a sensitive enzyme linked immunosorbent assay (ELISA). The β_2 glycoprotein I (β_2 -GPI) dependency was determined by carrying out aCL assays in the presence or absence of fetal calf serum samples.

Results—High levels of IgG aCL antibodies were detected in serum samples of 21/48 (44%) patients with HCV. These autoantibodies showed no β_2 -GPI dependency. The control groups had much lower levels of aCL antibodies (20% in the patients with HBV and none in the normal volunteers). Cryoprecipitates from four patients with HCV (three aCL positive; one aCL negative) were further isolated. In two of the three aCL positive patients, specific cardiolipin reactivity was shown in the cryoprecipitates. The group of patients with HCV and aCL antibodies in their serum showed significantly higher total IgG levels, a higher incidence of antinuclear antibodies, and viraemia (HCV RNA) than the aCL negative patients. None of the patients with HCV and aCL antibodies showed any clinical manifestations related to those autoantibodies.

Conclusions—This study clearly shows a high prevalence of IgG aCL antibodies in the serum of patients with HCV and the

localisation of these antibodies in some cryoprecipitates. The role of these autoantibodies on the course of HCV infection and their clinical significance has not yet been determined.

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Hepatitis C virus is increasingly recognised as a significant cause of extrahepatic manifestations, such as Sjögren's syndrome, membranoproliferative glomerulonephritis, and leukocytoclastic vasculitis. In addition, several studies have shown a high incidence of cryoglobulins and autoantibodies, such as antinuclear antibodies (ANA), rheumatoid factor (RF), or antismooth muscle antibodies (ASTHMA) among patients with chronic HCV infection.¹

Anticardiolipin antibodies, which were initially described in patients with systemic lupus erythematosus (SLE), were also reported in association with several viral infections^{2 3} and in small cohorts of patients with HBV or alcoholic cirrhosis.⁴ The causes for the generation of aCL antibodies and their clinical significance in patients with liver disorders are not yet defined. The putative relation between chronic HCV infection and extrahepatic autoimmune phenomena led us to study the incidence of aCL antibodies and their clinical significance in a cohort of patients with chronic HCV.

Material and methods

PATIENT GROUP

Forty eight consecutive patients with HCV and at least six months of raised (three times above normal) liver enzymes were included in this study. All patients were evaluated at the beginning of the study for the presence of ANA, aCL, cryoglobulins, antimicrosomal, antimitochondrial (AMA), and ASTHMA antibodies. Thereafter, all patients were evaluated every three months for a mean (SD) follow up of 2 (0.4) years. Patients who had previous exposure to HBV or any autoimmune disease were excluded. None of the patients had a history of alcohol abuse. All patients were HCV seropositive by an Abbott Laboratories second genera-

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tion ELISA and a supplemental assay. HCV RNA was examined for by polymerase chain reaction (Hoffman-La Roche). HCV genotype was determined by the INNO-LIPA line probe assay. A liver biopsy was performed if appropriate. The histological activity was assessed by the Knodell score.⁵ Thirty patients with HBV and 50 HCV/HBV seronegative healthy volunteers matched for age and sex served as control groups.

IMMUNOLOGICAL EVALUATION

Antinuclear antibodies were determined at 1:40 dilution by immunofluorescence using methanol fixed Hep-2 cells as substrate. Antimitochondrial antibodies and ASTHMA were detected at 1:20 dilution using mouse kidney and stomach, respectively. Anti microsomal antibodies were detected at 1:20 dilution by the SERODIA-AMC (Fujirebio Inc, Tokyo, Japan) agglutination kit. Total IgG, IgM, and IgA in the serum samples was measured by nephelometry. Serum cryoglobulins were detected as described previously.⁶ Four cryoprecipitates were further incubated on ice for 12 hours at 4°C, washed three times with a small volume (0.5 ml) of ice cold phosphate buffered saline (PBS; pH 7.2) and then dissolved in warmed (37°C) PBS (1/10 vol/vol; Cryo/PBS) for two hours and tested for cardiolipin reactivity (at 37°C). Anticardiolipin antibodies were determined in triplicate at 1:100 dilution by a sensitive ELISA, as we described previously.⁷ Assays were developed with affinity purified alkaline phosphatase conjugated goat antihuman γ or μ chain specific antibodies (Sigma Chemical Co, St Louis, MO). Background values representing non-specific binding to "no antigen" wells coated with ethanol without cardiolipin were subtracted in all assays.⁷ To avoid false positive results originating from non-specific binding of high IgG levels, the mean (of the 50 healthy controls) +3SD was chosen as the upper level of normal cardiolipin reactivity. To determine the β_2 glycoprotein I (β_2 -GPI) dependency of the aCL antibodies we studied, concomitantly, cardiolipin reactivity in the presence or absence of 10% fetal calf serum (FCS) (as the source of β_2 -GPI) as described.⁸ For blocking and dilution, in the absence of FCS, we used 1% bovine serum albumin (BSA; Sigma Chemical Co).

STATISTICAL ANALYSIS

Values are presented as means (standard deviation). Statistical significance was confirmed by student's *t* test; *p* < 0.05 was considered significant.

Results

Forty eight consecutive patients with HCV (28 female, 20 male) and a mean age of 60 (14.9) years (range 43–77) were examined before starting any specific treatment. HCV RNA was found in 38 (80%) of the patients. Liver biopsy was performed in 22 patients. The mean (SD) Knodell score was 13.9 (2.78); cirrhosis was found in five patients (23%). Antinuclear antibodies (speckled) were detected in 12 (25%)

Figure 1 IgG aCL antibodies in 48 patients with hepatitis C virus (HCV), 30 with hepatitis B virus (HBV), and 50 normal volunteers matched for age and sex. Results represent OD units at 405 nm. Background values representing non-specific binding with "no-antigen" wells were subtracted in all assays. The upper level of normal (line) was defined as mean +3SD over normal controls (mean (SD) OD of controls 0.030 (0.019)).

and cryoglobulins in 19 (40%) of our patients. Low titres of AMA, antimicrosomal antibodies, and ASTHMA were detected in three (6%), two (4%), and one (2%) of these patients with HCV, respectively.

Figure 1 shows that high levels (>3SD than control) of IgG aCL antibodies were detected in 21 patients with HCV (44%). Much lower levels, 20% and 0%, were seen in the groups of 30 patients with HBV and the 50 healthy controls, respectively (*p* < 0.05). There was no difference in the age, sex, Knodell score or cirrhosis, the presence of cryoglobulins, and IgM or IgA levels between aCL positive and negative patients. However, cardiolipin reactivity was significantly associated with the presence of HCV viraemia (100% *v* 58.3%), hypergammaglobulinaemia (2395 (940) *v* 1664 (566) mg%), and high incidence of ANA (47.6% *v* 7.4%). The incidence of the HCV genotypes did not differ. Most of our patients, were type 1b which is the prevalent HCV type in Israel. No differences in the clinical course of HCV between the two groups were seen. In addition, during the follow up period, none of our patients with HCV and aCL antibodies showed any clinical manifestations related to those autoantibodies—namely, recurrent abortions, thrombosis, immune thrombocytopenia or haemolysis, vasculitis or livedo reticularis.

Figure 2 shows that aCL antibodies of patients with SLE required the presence of β_2 -GPI for cardiolipin reactivity (higher reactivity in FCS than in BSA assays). In contrast, the aCL antibodies of our HCV patients did not show any β_2 -GPI dependency since their reactivity with cardiolipin was even higher in the absence of β_2 -GPI (BSA assay).

To clarify the possible relation between aCL antibodies and the cryoglobulins, we tested the cardiolipin reactivity of four randomly selected cryoglobulins, obtained from three aCL positive and one aCL negative patient with HCV. As can be seen in table 1 cryoglobulins of two out of three aCL positive patients (patients 3,4) showed significant cardiolipin reactivity. As the concentration of the total IgG in the cryoglobulins was not measured, the quantitative ratio of aCL antibodies in the serum samples and the cryoglobulins could not be determined.

Figure 2 β_2 Glycoprotein 1 (β_2 -GPI) dependency of aCL antibodies in serum samples obtained from patients with hepatitis C virus (HCV) and systemic lupus erythematosus (SLE). Five serum samples obtained from patients with chronic HCV, two patients with SLE and two healthy volunteers were tested, concomitantly, for cardiolipin reactivity in the presence (10% fetal calf serum (FCS)) or absence (1% bovine serum albumin (BSA)) of β_2 -GPI. All serum samples were tested in triplicate at a dilution of 1:100. Results represent mean OD units at 405 nm. Background values representing non-specific binding to "no-antigen" wells (blocked with 10% FCS or 1% BSA, respectively) were subtracted in all assays. The standard deviation between triplicates was <10% in all assays.

Table 1 Cardiolipin reactivity in the serum and cryoprecipitates of patients with chronic HCV

Patient	Cardiolipin reactivity*	
	Serum†	Cryoprecipitates†
1	0.012	0.006
2	0.330	0.023
3	0.430	0.180
4	0.260	0.095

*Cardiolipin reactivity presented as mean OD units at 405 nm.
 †Serum samples were diluted (in phosphate buffered saline (PBS)) at 1:100 and the cryoprecipitates were diluted (in PBS) at 1:10. Background values representing non-specific binding to non-antigen wells were subtracted in all assays. Standard deviation between triplicates did not exceed 10% in all assays.

Discussion

Our study clearly shows a high incidence (44%) of IgG aCL antibodies in serum samples of patients with chronic HCV hepatitis, which is significantly higher than seen in our two control groups. In addition, we have shown the presence of cardiolipin reactivity in some cryoglobulins obtained from aCL positive HCV patients.

The high prevalence of IgG aCL antibodies (fig 1) is not an artefact originating from a non-specific binding of polyclonal hyperglobulinaemia. Firstly, cardiolipin reactivity was defined only when the result was 3SD above that of a control group matched for age and sex. Furthermore, the use of "no-antigen" wells in all cardiolipin assays eliminates the background originating from non-specific binding. Secondly, although the group of patients with IgG aCL antibodies had higher IgG levels, some patients had high IgG levels but no aCL antibodies, and vice versa. Moreover, there was no close relation between the IgG aCL antibody titre and the total IgG levels. Lastly, a cohort of patients with rheumatoid arthritis who had comparable levels of total IgG had a very low incidence of aCL antibodies in our assay (Stoeger *et al*, unpublished data).

There are few reports demonstrating the presence of antiphospholipid antibodies (determined by aCL or lupus anticoagulant assays) in patients with HCV. Matsuda *et al*,⁹ Levy *et al*,¹⁰ Prieto *et al*,¹¹ Biron *et al*,¹² and Cacoub *et al*¹³ reported that 13%, 14%, 22%, 33%, and 20%, respectively, of their HCV patients had significant titres of aCL antibodies. In agreement with our study, most of those reports did not find any clinical significance for the presence of aCL antibodies.⁹⁻¹⁰ Nevertheless, Prieto *et al* showed a high prevalence of portal hypertension, thrombotic events, and thrombocytopenia,¹¹ and Biron *et al* found more liver fibrosis in patients with HCV and aCL antibodies.¹² Differences in demographic and genetic background, HCV subtypes, and lengths of follow up periods may explain the differences between the various studies.

The fact that none of our patients had any aCL related clinical manifestations may reflect the nature and fine specificity of these auto-antibodies. As was shown for Q fever, HIV, and syphilis,^{2,3} and in agreement with Cacoub *et al*,¹³ the aCL antibodies of our HCV patients, did not require the presence of β_2 -GPI (fig 2). Thus reactivity with the natural anticoagulant β_2 -GPI ("pathogenic" aCL antibodies in SLE) may cause specific clinical manifestations that do not occur in association with "reactive" aCL antibodies, like those of patients with HCV.

Cryoglobulins were detected in 39.6% of our HCV patients, which is similar to other observations.^{6,13-15} As reported by Cacoub *et al*,¹³ we found no correlation between the presence of aCL antibodies and cryoglobulins. Nevertheless, we were able to show, for the first time, specific cardiolipin reactivity in two cryoprecipitates isolated from aCL positive HCV patients (table 1). Studies of cryoglobulins obtained from large number of aCL positive and negative patients are needed to elucidate the role of aCL antibodies in HCV related cryoglobulins.

The patients with HCV who showed high titres of aCL antibodies had a higher incidence of HCV viraemia together with hypergammaglobulinaemia and ANA, suggesting the existence of a chronic inflammatory state. Persistent HCV infection leads to endothelial and hepatic damage that can cause alteration of the expression of cell surface phospholipids and induction of proinflammatory cytokines, which together may promote the generation of aCL antibodies. The presence of aCL antibodies in the cryoprecipitates (table 1), may suggest reactivity of these autoantibodies with HCV epitopes which can be found in the cryoprecipitates.¹⁵ The close association between the presence of aCL antibodies and the persistent HCV viraemia may support the latter mechanism. Further studies of a large cohort of patients with HCV, currently under progress, are needed to determine the exact role and clinical significance of aCL antibodies in patients with HCV.

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